Use of Face-Mask Sampling as a Means of Characterising the Microbiota Exhaled from Human Respiratory Tract in Health and Disease

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Abstract

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Introduction: Facemasks carrying a gelatine sampling matrix have been used to sample exhalations from TB patients. The aim here was to further develop this approach by evaluating molecular detection, quantitation and capture of signals in healthy volunteers and in HIV-uninfected patients with suspected *Pneumocystis jirovecii* pneumonia (PJP).

Methods: DNA isolation was optimised with NaOH or collagenase to dissolve gelatine followed by bead beating. Assays for selected marker and degradative proteins were established as were six *16S*rDNA-directed qPCRs with different phylogenetic specificities. Mask samples were taken from healthy volunteers over 15, 30 and 60 minutes with four different respiratory efforts. Pathogen detection was tested in patients with suspected PJP (*MtLSU* target) and a subset of samples were subjected to microbiome profiling.

Results: In healthy volunteers, capture of exhaled bacteria appeared maximal at 15 minutes while accumulation continued up to 60 for proteins. Reading-out loud produced most SP-A, albumin and α-amylase. *P. jirovecii* was detected in 7/20 patients diagnosed and 3/19 patients suspected but not diagnosed with PJP. The median captured signal was 8.59×10^4 (IQR= 3.01×10^5 – 1.81×10^4) *MtLSU* copies/mask. Blood β-D-Glucan results correlated with the mask results (Spearman r=0.65; p<0.0001) while other relevant clinical indices did not. Microbiome results provided further evidence for different microbial outputs between individuals and with different respiratory efforts and samples. However, the sampling system provided a high background *16S* signal.

Conclusion: The mask approach has been significantly improved towards sampling material exhaled from the upper and lower respiratory tracts with evidence that different breathing activities produce different yields. Sampling in suspected PJP adds diagnostic likelihood in cases with positive β-D-Glucan results and reinforces evidence that *P. jirovecii* is an airborne infection. Microbiome studies revealed several differences, but conclusions were limited by high background signals. Mask sampling is promising non-invasive investigative and diagnostic tool.

A hyperlinked soft copy is available on the enclosed CD

Acknowledgement

When it comes to this section, it comes to a not less challenging one than others.

I have a long list in mind and if I mention some, I might forget many…

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To end up, while PhD projects must involve an original contribution to knowledge as my project does, the most important message of my work is that being disabled must not exclude us from making that contribution whatever the difficulties we face.

Dedicated to whom is interested in my writing

xxx

Papers and presentations from this study

Co-author:

1. Face-mask sampling reveals antimicrobial resistance genes in exhaled aerosols from patients with chronic obstructive pulmonary disease and healthy volunteers (BMJ Open Respir Res 5(1):e000321)

2. Exhaled *Mycobacterium tuberculosis* output and detection of subclinical disease by Face-Mask-Sampling in prospective observational studies (Accepted at Lancet Infect Dis)

Author of selected presentations at:

1. Annual Conference 2016 of Microbiology Society, Liverpool

2. First Annual Midlands Academy of Medical Sciences Research Festival 2016, Leicester (This project was selected among the top ten from the University of Leicester)

3. 16th Annual Joint Respiratory Research Day 2016, Leicester

4. British Association of Lung Research Summer Meeting 2016, Sheffield

5. M4 Midland Molecular Microbiology Meeting 2016, Leicester

6. EMBO (Excellence in the Life Sciences) Conference Tuberculosis 2016, Paris

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List of abbreviations, symbols & units of measurements

A	Adenosine
A230	Absorbance at 230nm
A260	Absorbance at 260nm
ADC	Albumin-dextrose-catalase
AEC-I	Alveolar epithelial cells type-I
AFB	Acid-fast bacilli
AHC	Agglomerative Hierarchical Clustering
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guérin
BDG	(1,3)-Beta-D-glucan
BHI	Brain heart infusion
BOS	Bronchiolitis obliterans syndrome
Bp	Base pair
C	Cytosine
CASS	Cough aerosol sampling system
CC10	Clara cell 10 kDa protein
CC16	Clara Cell Secretory Protein 16
CD4+	Cluster of differentiation 4

CDC	The Centers for Disease Control and Prevention
CF	Cystic Fibrosis
CFU	Colony Forming Unit
cm	Centimetre
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-reactive protein
CT	Computerized tomography
C_t	Cycle threshold
CXR	Chest x-ray
DHPS	Dihydropteroate synthetase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
dsDNA	Double-Stranded DNA
EBC	Exhaled Breath Condensate
ECIL	The European Conference on Infections in Leukaemia
EDTA	Ethylenediaminetetraacetic acid
E_H	Shannon equitability index
ELISA	Enzyme-linked immunosorbent assay
EMP	The Earth Microbiome Project

EORTC/MSG	The European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group
ETA	Endotracheal aspiration
ETS	Endotracheal secretions
F:P	Proteobacteria to Firmicutes ratio
FITC	Fluorescein isothiocyanate
FN	False negative
FP	False positive
FRC	Functional Residual Capacity
g	Gram
g	Gravity (centrifugation speed; preceded by x)
G	Guanine
H	Shannon–Wiener index
HARRT	Highly active antiretroviral treatment
HβG	Human β-globin
HEPA	High-efficiency particulate air
HIV	Human Immunodeficiency Virus
hr(s)	Hour
IB	Instructed breathing
IC	Intermittent coughing

IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	Interquartile range
IS	Insertion sequence
J	Joule
K	Kilo
KDa	KiloDalton
KGy	KiloGray
KR	Kilorads
L	Litre
LDH	Lactate dehydrogenase
LLD	Lower limit of detection
LOB	Limit of blank
LOD	Limit of detection
LOQ	Limit of quantification
LRT	Lower respiratory tract
m	Metre
M	Molar
MASS	Mask aerosol sampling system
Mb	Megabase
MDR	Multi-drug-resistant TB

MDS	Multidimensional scaling
MGB	Minor groove binder
min	Minute
mL	Millilitre
mM	Milli Molar
mm	Millimetre
MOUDI	Micro-orifice uniform deposit impactor
mRNA	Messenger RNA
MSG	Major surface glycoprotein
Mtb	*Mycobacterium tuberculosis*
MTC	*Mycobacterium tuberculosis* complex
MtLSU	Mitochondrial large subunit
MW	Molecular weight
n	Number or sample size
NB	Normal breathing
NC	Negative control
NCBI	The National Center for Biotechnology Information
ng	Nanogram
NHS	The National Health Service (UK)
nm	Nanometre
nmole	Nanomole
NP40	Nonidet P40

NPV	Negative Predictive Value
NTM	Nontuberculous Mycobacteria
nU	Nano unit
^{o}C	Degree Celsius
OD	Optical density
OTU	Operational Taxonomic Unit
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PExA	Particles of exhaled air
pg	Pico gram
PJP	*Pneumocystis jirovecii* pneumonia
PMA	Propidium monoazide
PPD	Purified-protein-derivative
PPV	Positive predictive value
psi	Pounds per square inch
PVA	Polyvinyl alcohol
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Real-time quantitative PCR
r	Correlation factor
R^2	Coefficient of determination
RASC	Respiratory Aerosol Sampling Chamber

rDNA	Ribosomal DNA
RDP	Ribosomal Database Project
RL	Reading-out loud
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RTLF	Respiratory Tract Lining Fluid
RV	Residual Volume
SCGB1A1	Clara Cell Phospholipid-Binding Protein
S_{Chao1}	Chao1 index
SD	Standard deviation
SE	Standard error
sec	Second
S_J	Jaccard coefficient
SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
S_S	Sørensen–Dice coefficient
T	Thymine
TAE	Tris-acetate-EDTA
TB	Tuberculosis
TLC	Total Lung Capacity

TN	True negative
TP	True positive
U	Unit
URT	Upper respiratory tract
UV	Ultraviolet radiation
v/v	Volume per volume
VC	Vital Capacity
w/v	Weight per volume
WBC	White Blood Cells
μ	Micro
μg	Microgram
μJ	Micro Joule
μL	Microlitre
μm	Micrometre
1-D	Simpson index
2D	Two Dimensional
3D	Three Dimensional
α	Alpha
β	Beta
γ	Gamma
μM	Micromolar

Asterisks used to denote the degree of statistical significance

*	$p \leq 0.05$
**	$p \leq 0.01$
***	$p \leq 0.001$
****	$p \leq 0.0001$

Chapter One: General introduction

1.1. Introduction

Clinical face-masks have been widely used to protect the wearer from airborne infection hazards and, conversely, to reduce infectivity from individuals with respiratory infection. Work in this laboratory has developed use of face-masks to sample exhaled bacteria from patients with pulmonary tuberculosis (TB) (Cheah, 2010; Williams *et al*., 2014). The present study started from this point.

As with almost any other tools, there are several variables that may affect the overall performance.

We aimed through this project first to evaluate and develop mask processing methods, then to investigate the effect of different instructions to wearers on sample content reflecting the lower respiratory tract (LRT) in health and to explore applicability of the developed tool in disease, taking *Pneumocystis jirovecii* pneumonia (PJP) as a challenging paradigm. A further analysis of our samples, presented in Chapter Five, enabled a first exploration of the mask utility in studying the exhaled microbiome in health and disease.

1.2. *Mycobacterium tuberculosis* (Mtb) as a model of airborne infection

Respiratory infections claim millions of lives annually. Among the examples are TB and acute LRT infection, two of "*the big five*" commonest causes of morbidity and mortality worldwide per the estimates of the World Health Organization 2015 and the Global Burden of Disease Study 2016 (WHO, 2015; GBD, 2016).

Although the airborne route is well linked to respiratory infections, understanding the physiology and pathophysiology of aerosolization, the mechanism(s) of transmission and the biology of the aerosolized particles are far from satisfactory and there remain fundamental gaps in defining these processes. Any tool developed to address these gaps should ideally be non-invasive, cost-effective and clinically timely.

The earliest study on the role of aerosol-like material in infectiousness was probably by Koch who demonstrated animals developing TB-like disease following inhalation of sprayed bacilli (Koch, 1883 cited in Wells *et al*., 1948).

Chapin (1910) raised the concept of airborne infection when he discussed "*the number of living tubercle bacilli in the air of consumptives' apartments*" and when he related school attendance with the spread of scarlet fever and diphtheria. Chapin, however, was interested in dispersal of large droplets over short distances and recommended preventing "*exchange of saliva and other secretions*".

Wells (1934) invented "*airborne contagion*" idiom and been known the author of "*droplet-nuclei*" term which he coined to describe the status of exhaled droplets after losing their moisture and evaporate.

Several differences have been known between aerosols and droplets, possibly the most important is the size. The shape of the particle, initial velocity, humidity, composition, site of origin, site of deposit and time remaining

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suspended in air are other aspects of the difference. 5µm has traditionally been the cut-off size between aerosols (<5 µm) and droplets (>5µm). Particles of less than 10µm in diameter have been classified as respirable, while those larger than 10µm up to 100µm have been classified as inspirable (Nicas and Sun, 2006; Atkinson and Wein, 2008; Stilianakis and Drossinos, 2010). In terms of size, droplet-nuclei are comparable to aerosols (Cole and Cook, 1998). Exhaled particles from a generator, that are directly or indirectly inhaled by a receiver, can also be classified according to the site of deposit to extra-corpus including indoor and outdoor environment and intra-corpus including nasopharyngeal, tracheobronchial and alveolar (Roy and Milton, 2004). The smaller the particle diameter, the more distant the extra-corpus deposition site and the deeper the intra-corpus one.

In fact, the conventional view in clinical practice in relying on acid-fast bacilli (AFB)-smear positivity to determine the infectiousness burden of pulmonary (and laryngeal) TB patients and whether they are dischargeable from isolation facilities has been potentially challenged by sufficient evidence of TB transmissibility from AFB-negative smear cases (Blahd *et al*., 1946; Catanzaro, 1982; Di Perri *et al*., 1989; Behr *et al*., 1999). In addition, the occurrence of nosocomial dissemination after discharging respiratory-isolated patients to general medical wards has been documented (Beck-Sague *et al*., 1992).

While it is self-evident that expectorated sputa themselves do not intermediate airborne dissemination that is fundamentally based on aerosolized droplet nuclei as affirmed further by Dharmadhikari *et al*. (2011)'s studies, this leaves many questions on the relationship(s) between sputum and infectious aerosols unanswered.

Several epidemiological and analytical investigators like Hernandez-Garduno *et al*. (2004) and Jones-Lopez *et al*. (2013) highlighted a dissociation between

sputum positivity and transmission risk. In this context, recent transcriptomic and gene expression studies performed on specimens collected from TB patients provided evidence that aerosols exhaled by TB patients are not fine particles of their expectoratable sputum since each holds a distinguishable biosignature (Nardell *et al*., 2016).

1.3. Tools used to collect exhaled Mtb

Probably the earliest study on collection of aerosol-like material in respiratory infections was by Boston (1901) who collected exhaled output from TB patients on microscope slides stained to detect Mtb (Figure 1.1) (Boston, 1901 cited in Williams *et al*., 2014).
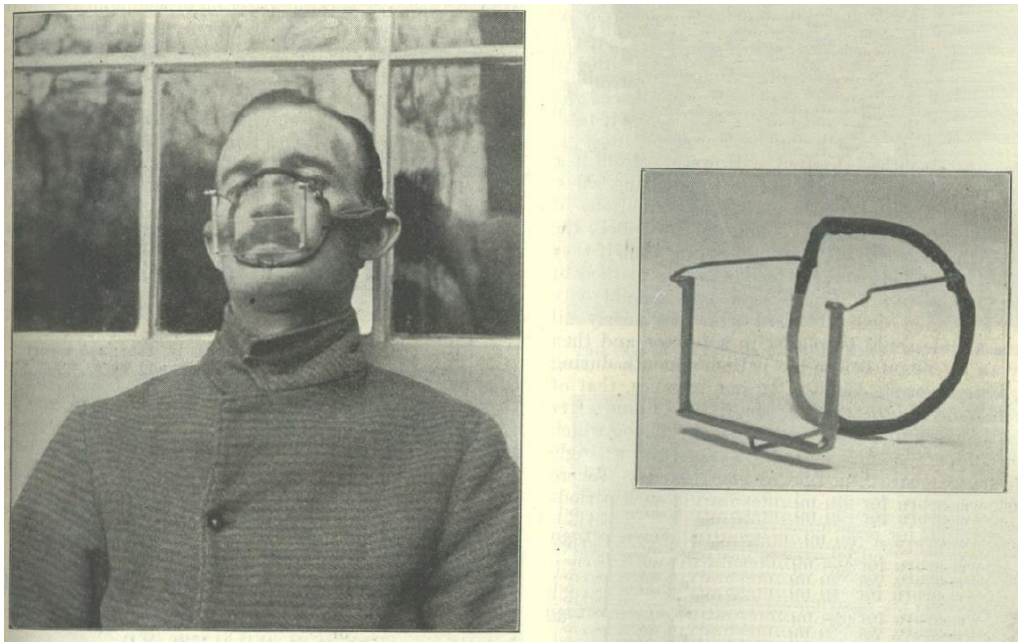
Figure 1.1 Boston mask to detect exhaled Mtb
This was reproduced from Boston (1901) with permission of the American Medical Association

Next attempts to collect microbe-carrying aerosols have involved a variety of methods, including impaction, impingers, and gelatine or nucleopore air filters incorporating air pumps.

Andersen (1958) designed a cascade impactor as a mechanical air sampler applied for determining the particle size of viable aerosols on agar plates placed on six size-differentiating stages (having jet size diameters ranging from 0.01–0.046µm). This tool was re-applied by Wendt's group in 1980 and Falkinham's group in 1990 for collecting nontuberculous mycobacteria (NTM) aerosolised from natural water sources.

Micro-orifice uniform deposit impactor (MOUDI) was a similar sampler developed by Marple's team in 1991 by integrating the impactors of Khulmey's and Marple's teams (1981 cited in Marple *et al*., 1991) to aerodynamically size particles of 0.056–18µm diameters. Schafer *et al*. (1998) validated the MOUDI on aerosolized suspensions from *Mycobacterium tuberculosis* complex (MTC), comparing its performance to that of disposable plastic filter containers, and applying it in 2003 for sampling NTM aerosolized from communal swimming pools.

Several animal models using Guinea pigs (characterised by their susceptibility), rabbits and other species were devised as optimal living air samplers to study TB infectiousness (Lurie and Wells, 1941; Wells *et al*., 1948; Ratcliffe and Palladino, 1953; Riley *et al*., 1959; Ratcliffe, 1960; Riley *et al*., 1962; Loudon *et al*., 1969; Escombe *et al*., 2007 and 2009; Dharmadhikari *et al*., 2011). However, these models were encountered by limitations of costs, needs for specially constructed infrastructure, results interpretation challenges and susceptibility differences between animal and human hosts.

Fennelly and colleagues (2004) devised the Anderson samplers to develop the CASS for direct quantification of culturable Mtb exhaled by TB patients during deliberate coughing (Figure 1.2). This tool detected 25% of 16 AFB-positive smear patients whose CASS-based estimates widely ranged from 18 to 3,798 exhaled particles per hr. Fennelly and colleagues noted a correlation trend between number of coughs and colony forming units (CFU) counts of exhaled aerosols, alongside a positive correlation between the latter and AFB score of paired sputa. They also reported rapid reduction in collected Mtb aerosols during initial weeks of commencing effective anti-TB, postulating an association between infectious aerosol production and ineffective management of TB.
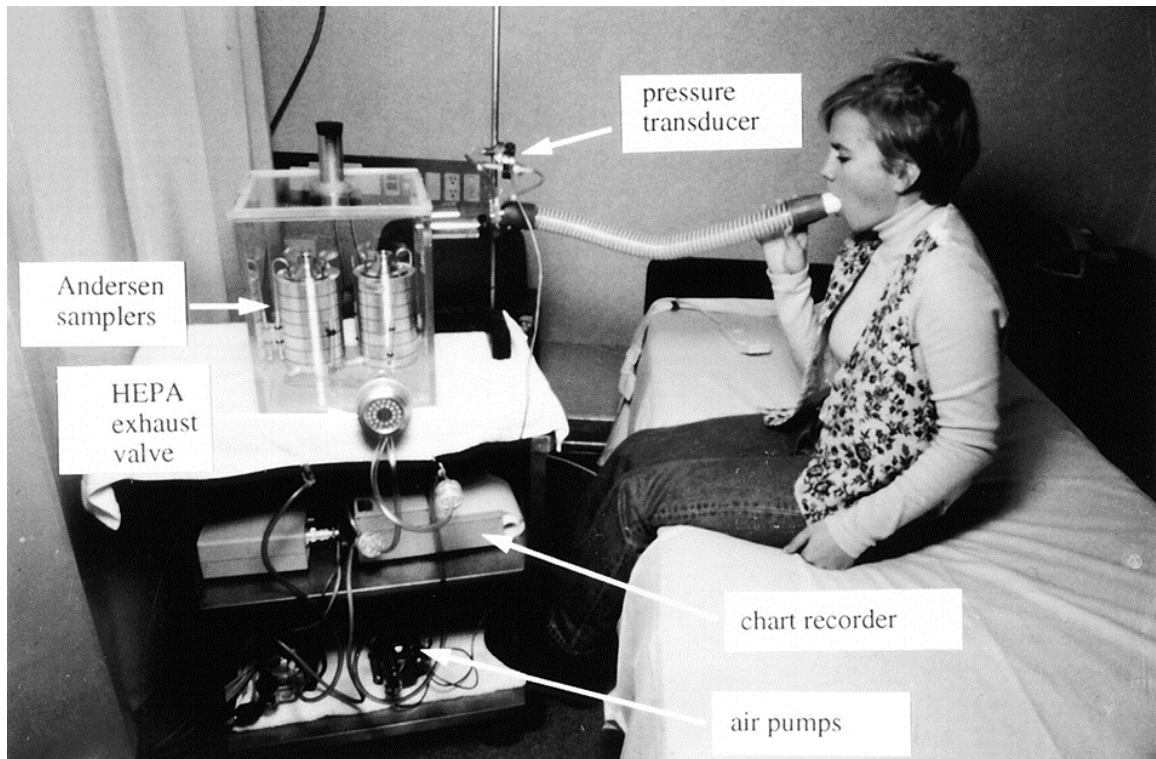
Figure 1.2 Cough aerosol sampling system (CASS) at the bedside
This was reproduced with permission of the American Thoracic Society. Copyright © 2019 American Thoracic Society. Fennelly *et al*. 2004. Cough-generated aerosols of Mycobacterium tuberculosis: a new method to study infectiousness. Am J Respir Crit Care Med, 169, 604-9. *The American Journal of Respiratory and Critical Care Medicine* is an official journal of the American Thoracic Society.

However, the CASS faced several limitations including time to obtain culturing results, cultivability requirement per se, specific infrastructure requirements, fungal contamination and desiccation of media and bacilli due to prolonged sampling. The Fennelly patients were also sampled during sputum induction and their majority were with multi-drug-resistant (MDR) TB, limiting further data representativity of natural aerosolization and applicability for drug-susceptible cases.

Fennelly *et al*. (2012) associated the production of cultivable aerosols of culture-positive TB patients with their subjectively-assessed force of coughing, but they excluded smear-negative cases from their screening and culture-negative cases from their final analysis and applied a deliberate rather than normal pattern of coughing.

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Liu and Novoselac (2014) developed a cough box to study the spread behaviour of three particle sizes (0.77, 2.5, and 7µm) generated from coughing. They found that the larger particles had a lower concentration in the vicinity of a receiver occupant positioned in close proximity to the box. However, Liu and Novoselac used very simplified manikins compared to natural aerosolization and studied this model at a fixed separation distance (1.2m).

More recently, Wood *et al*. (2016) developed the Respiratory Aerosol Sampling Chamber (RASC) to enable sampling of exhaled particles and organic matter. The RASC comprised an array of particulate impingers, impactors and filters, incorporated for aerodynamic particle size detection, viable and non-viable sampling for molecular- or culture-based downstream analyses, real-time CO_2 monitoring and cough sound-recording (Figure 1.3).

Patterson *et al*. (2017) used the RASC to detect 1-hr Mtb exhalations from 35 untreated TB patients presented with GeneXpert sputum-positivity. ~93% of collected samples were positive by droplet Digital Polymerase Chain Reaction (PCR) and ~43% did so by culturing on solid media. While they reported a correlation between culturability and cough rate, the former did not correlate with sputum smear positivity nor with radiographic findings, in particular pulmonary cavitation. Based on the culture positivity, they estimated the bacillary load in exhaled air as 0.09 CFU/litre and at 4.5×10^7 CFU/mL for exhaled particulate aerosols which CFU concentrations were around 1−2 log folds higher than that of sputum. Among the limitations for the culture approach was the reliance on solid media and associated fungal contamination, and for both approaches was the required infrastructure and thus their limited applicability in different clinical settings.
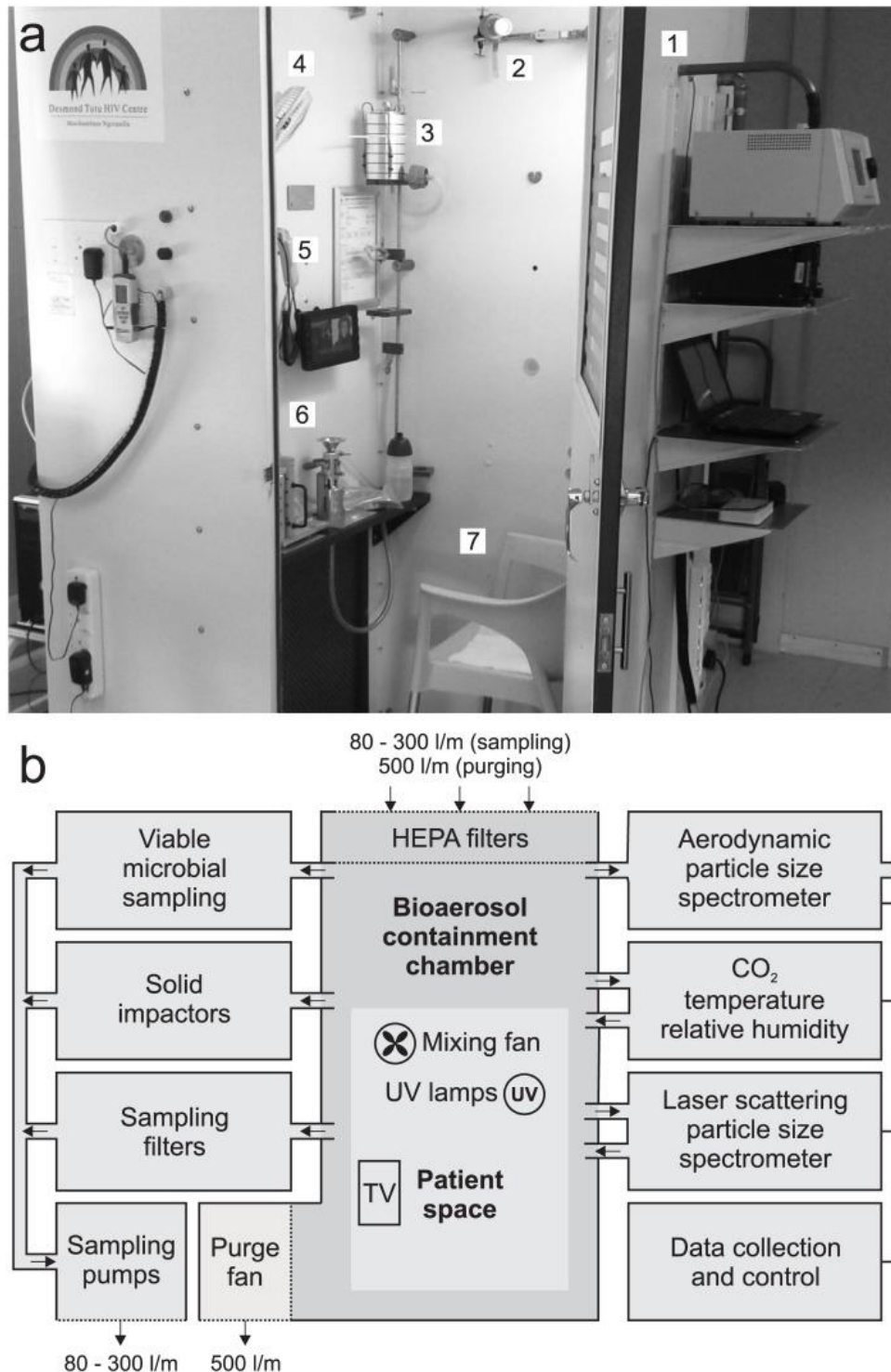
Figure 1.3 The Respiratory Aerosol Sampling Chamber (RASC)
(A) Photograph of the RASC (with the door open) on site in a community TB clinic (1) aerodynamic particle sizer (2) Filter samplers (3) Andersen impactor (4) Mixing fan (5) CO_2, temperature and RH (6) PM10 impactor (7) Chair for participant. (B) Block diagram depicting the fluidic and electronic configuration of the RASC. Thick connecting lines indicate airflow and aerosol paths; thin lines indicate electronic connections. All air leaving the RASC is HEPA filtered. This was reproduced from Wood *et al.*, 2016 (open-access article permitting unrestricted use).

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1.4. Use of facemasks in medical literature

Historically, facemasks have been implied and evaluated in the medical field for different purposes. The goals included protections of wearers and, conversely, their surroundings from airborne infection hazards (Nardell, 1991; Fennelly, 1998; Fennelly and Nardell 1998; Fennelly *et al*., 2005), investigations of the mask as a potentially-infectious source for the wearer (Heimbuch *et al*., 2014, Tseng *et al*., 2016), and sampling and studying exhaled material from the wearer (Huynh *et al*., 2008; Cheah, 2010; Williams *et al*., 2013 and 2014).

1.4.1. Protection of both wearers and their surroundings

With regards to airborne infections, the mask protection rule has remained argumentative since its first recommended use in the last decade of the 19th century in the USA to prevent occupational TB for health care workers. This was mainly because of that minimal or even no epidemiological data were available to demonstrate effectiveness in protection against nosocomial TB (Fennelly, 1998; Fennelly *et al*., 2005).

Fennelly and Nardell (1998) evaluated the relative efficacy of personal respirators in terms of protection with increased concentrations of infectious aerosols or decreased rates of room ventilation. They found the infection risk significantly decreased with increasing room ventilation or personal respiratory protection. Thus, the relative efficacy of the personal respirators in terms of protection decreased with increasing room ventilation rates or decreasing the concentrations of infectious aerosols, suggesting that the risk of occupational TB can be lowered by using personal respirators combined with modest room ventilation rates, particularly for newly diagnosed cases.

Similarly, Basu (2007)'s and Lee (2016)'s studies carried-out in low-resource and high-resource settings, respectively, agreed on that the implementation

of particulate respirators combined with other control measures can offer additional personal protection against nosocomial TB if properly used, particularly when a supposed protection of administrative and environmental controls fails to offer so.

In addition to that, Mogridge *et al*. (2016) developed a miniature, low-weight sampler and evaluated its performance in terms of diminishing the mask protection efficiency after being incorporated with FFP masks, following their assessment of the sampler in terms of particles and mass collection efficiency. Applying the requirements of the European Committee for Standardization (CEN, 2009) for respiratory protective devices, Mogridge and colleagues concluded no significant effect of that incorporation on the protection offered by FFP3s.

On the other hand, Nardell (1991) reviewed a number of strategies applied for interrupting TB transmission and found that traditional surgical masks provided the wearer with minimal or no protection.

After the CDC proposed use of respirators with high-efficiency particulate air filters (HEPA respirators) in response to outbreaks of MDR TB, alongside proposed application of other precautions measures against nosocomial TB, Adal *et al*. (1994) conducted a cost-effectiveness analysis in a high-resource area using local data of those exposed to occupational TB and their positivity rates of purified-protein-derivative (PPD) skin test. The results suggested that more than one year of using HEPA respirators was required to prevent a single conversion of the PPD test and thus the hospital would require >40 years to prevent one case of occupational TB with a considerable cost. Adal's group concluded that the use of HEPA respirators would offer negligible and cost-ineffective protection. Unsurprisingly, Harries *et al*. (1997)'s review on measures against nosocomial TB using data from low-resource settings with

high co-incidence rates of TB and HIV also concluded that applying such measures including HEPA respirators was unaffordable.

Roth (2005) monitored the use of N95 respirators as an occupational TB control measure by 145 workers in a 50-bed hospital in a resource-limited setting. Roth found that these respirators were infrequently used, particularly with high-risk procedures and in high-risk areas. In addition, this study noted a failure in wearing the mask tightly as directed. Roth concluded that this measure was neither adequate nor cost-effective in resource-limited settings, highlighting the need for alternative measures, particularly when the risk cannot be controlled by administrative and engineering measures.

The masks have also been used outside airborne infections. For example, for protection of wearers from volatile chemicals and other agents.

1.4.2. The mask as a potential infectious source for the wearer

Other groups studied the face-mask from another perspective. Tseng *et al*. (2016) investigated surgical face masks as an infective fomite rather a protective means. Tseng and colleagues coated the mask surfaces with an antimicrobial surfactant and demonstrated an inhibitory activity of this coating material after exposing the surface to aerosolised bacteria.

Similarly, other studies assessed decontamination methods of different types of masks and respirators (Heimbuch *et al*., 2014).

1.4.3. Sampling and studying exhaled biomaterial from the wearer

The face-mask has been investigated for sampling and studying exhaled material from the wearer. For collecting exhaled viruses, Huynh *et al*. (2008) developed a mask design made from impermeable, stretchable polyvinyl chloride and contained a centrally-located electret filter for capturing and

analysing by multiplexed PCR several respiratory RNA viruses. Of nine patients with positive nasal mucus specimens, six, five and three yielded positive masks collected over 20 coughs, 20-min-talking or 20-min-breathing, respectively. Among the limitations of the Huynh *et al*. study was the direct contact between the capturing filter and facial skin and uninvestigated potential PCR inhibition caused by the filter components, particularly for those with positive nasal mucosa but negative masks.

The Barer laboratory, focussed on TB, has established more than a decade ago a face-mask system to capture and analyse aerosols and droplets from patients with respiratory infections, proposing that it might fill some of the gaps outlined above.

Thus, direct face-mask sampling provided a potential for a safe, non-invasive, well-tolerated and cost-effective tool applied by Cheah (2010) and Williams *et al*. (2013 and 2014) for collection of exhaled Mtb, with a further potential to enable assessment of a source infectiousness.

This tool can approach AFB-smear negative and non-sputum productive cases whose diagnoses would otherwise demand the costlier, manpower-necessitating and invasive procedure in obtaining bronchoalveolar lavage (BAL) via bronchoscopies.

Cheah (2010) initiated the implementation of standard surgical masks (Figure 1.4) in quantifying Mtb aerosols, making use of real-time quantitative polymerase chain reaction (qPCR) and mycobacteriophage amplification assays. Cheah concluded, however, that the latter method was more sensitive since the former was undermined by qPCR inhibition induced by the mask components.
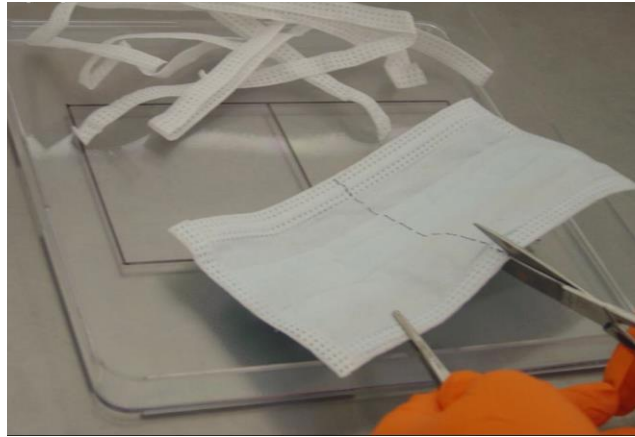
Figure 1.4 Standard surgical masks used in Cheah (2010)
This was reproduced from Cheah (2010) with the author's permission.

Williams *et al*. (2013's abstract) detected exhaled Mtb in three of five untreated patients with pulmonary TB and in one of two who were on the fifth day of treatment. All those who were sputum smear positive (four patients) had positive masks. Williams and colleagues analysed the captured material with a semi-automated platform (GeneXpert MTB/RIF, Cepheid) that delivers detection of TB and drug resistance within 2 hrs

Williams *et al*. (2014) added the use of FFP30 masks incorporated with gelatine filters (Figure 1.5) for capturing Mtb aerosols. Williams and colleagues detected the exhaled Mtb using GeneXpert positivity to which the mask gelatine inserts did not exhibit the inhibition encountered the Cheah study. The estimated sensitivity in TB patients was reported at 65%. No false positives were reported, and the results were not quantitative.

Figure 1.5 FFP30 masks incorporated with gelatine filters used in Williams *et al*. (2014)
This was reproduced from Williams *et al*., 2014 (open-access article permitting unrestricted use).

1.4.4. Other usage of masks for delivering certain materials

Oxygen masks are a special type which has been used for administrating oxygen gas or materials such as bronchodilators, sodium chloride, adrenaline, steroids or antibiotics from a storage tank or container to the respiratory tract over a certain period. These materials are usually delivered through nebulisation where the agent is converted into inspirabable aerosols.

1.5. Limitations of conventional sampling of the respiratory tract

Various types of respiratory samples can be collected either invasively (BAL, nasopharyngeal and endotracheal aspirates, transthoracic needle-biopsy, transbronchial lung biopsy) or semi-invasively (induced sputum).

Despite of their relatively-safe procedure yet invasive, cost-ineffective and manpower-necessitating, bronchoscopies used for collecting BALs and aspirates require close monitoring of subjects for several hrs, and thus they are not applicable to represent sampling from a population, particularly when a frequent or serial sampling is required.

Many studies use sputa as a surrogate for airway sampling. However, such samples are obviously contaminated with URT secretions including saliva. In addition, sputum induction is indicated in many circumstances (for example, for patients who cannot produce sputum spontaneously) and might even fail for others (for example, in pediatric groups). In terms of cost, the induction procedure has been reported to cost around 40% of what bronchoscopy does (Glenny and Pierson, 1992).

Furthermore, there are several medical conditions and circumstances for which these procedures are contraindicated. These include, for example, severe coughing, haemoptysis, acute respiratory distress and acute respiratory failure with hypercapnia, unstable cardiovascular status, thoracic, abdominal or cerebral aneurysms, hypoxia and lung function impairment, pneumothorax, pulmonary emboli, fractured ribs or other chest trauma, and high-grade tracheal obstruction.

More importantly, Nardell *et al*. (2016) demonstrated that exhaled aerosols are not fine particles of sputum as each holds a distinguishable biosignature.

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In fact, a feature that can characterize exhaled microbes is cell hydrophobicity. This has been discussed as a biological ground for rendering some mycobacterial strains more aerosolisable and consequently for successful TB transmission (Jankute *et al.*, 2017).

1.6. The microbiome of the respiratory tract

The Human Microbiome Project (HMP) was established in 2007 by the United States National Institutes of Health, rocketing understanding of the role of human microbiome in healthy and diseased states and offering an opportunity for improved management of pulmonary diseases.

In 2010, Dewhirst *et al*. established the Human Oral Microbiome Database to provide comprehensive curated information on bacterial communities inhabiting the upper respiratory tract (URT; including the oral cavity, nasal passages, paranasal sinuses, pharynx and the portion of larynx above the vocal cords). Based on analysing *16S* rRNA gene sequences, they described 619 taxa belonged to 13 phyla in the URT: Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Euryarchaeota, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, SR1, Synergistetes, Tenericutes, and TM7. Some of these were well reported previously in culture-dependent studies.

Earlier to the Dewhirst studies, Aas *et al*. (2005) sampled different anatomical sites of the oral cavity (including tongue dorsum, lateral tongue, buccal epithelium, hard and soft palates, supragingival and subgingival plaques of teeth, anterior vestibule of the maxilla and tonsils) for culture-independent analyses. Aas and colleagues reported 141 predominant species, of which >60% were not culturable. Common genera reported across these anatomical sites were *Gemella*, *Granulicatella*, *Streptococcus* and *Veillonella*.

Bassis *et al*. (2014) found that the nasal cavity is dominated by Actinobacteria and Proteobacteria which were also present in the oral cavity but at a lower abundance. Moreover, they found that the overall level of β-Proteobacteria was statistically comparable between oral and nasal cavity communities.

Although the aim of HMP was to characterise the microbiome in vital anatomical sites of the human body, the lower airways were initially excluded, reflecting the conventional views of lung sterility and to some extent the accessibility-related difficulties through invasive sampling of the LRT. This resulted in delaying a systematic exploration of the LRT microbiome.

Hilty *et al*. (2010) conducted the first study in characterizing the lung microbiome, reporting around two thousand bacterial genomes per a square cm, covering the bronchial tree surface. Hilty's group showed Proteobacteria, especially *Haemophilus spp*., more frequently detected in pulmonary samples (upper left lobe brushings) of asthmatics and COPD patients than of healthy subjects, while Bacteroidetes, especially *Prevotella spp*., were done so in the latter rather the former. However, the reported bacterial density, which was similar to that of the upper gastrointestinal tract, raised concerns whether the results reflected contamination from the bronchoscope passing through the URT.

After that, however, many studies with a careful use of the bronchoscope demonstrated that the lung microbiome topography is closely comparable to the oral one despite the former does not derive entirely from the latter (Charlson *et al*., 2011; Pragman *et al*.; 2012; Morris *et al*., 2013; Segal *et al*., 2013; Venkataraman *et al*., 2015; Bassis *et al*., 2015).

For example, Charlson *et al*. (2011) studied the microbiota from different anatomical sites of the respiratory tract with a vigilant sampling presented by collecting oral wash and oro- and naso-pharyngeal swabs, orally-introduced-bronchoscopy-assisted aspirates up to the glottis for sampling the URT, followed by serial BALs and LRT protected brush for sampling the LRT. They

found that the respiratory microbiota was similar between the upper and lower airways, but its biomass decreased with going deeply (Figure 1.6).
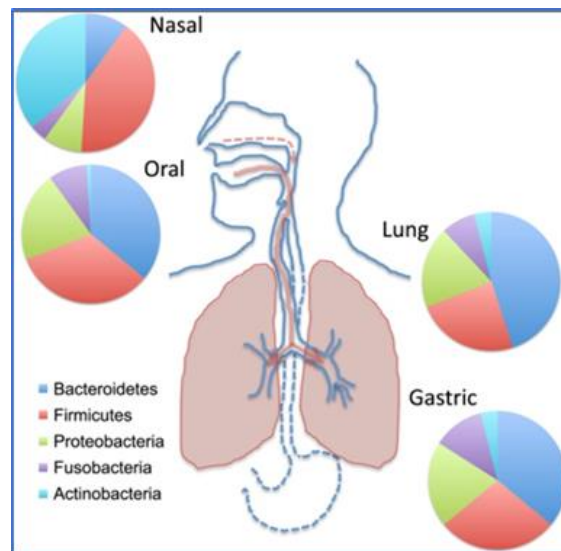
Figure 1.6 Bacterial composition at phylum level in the upper and lower respiratory tracts
This was reproduced from Huffnagle *et al*. (2017) with the author permission.

These *16S* rRNA-based studies consensually showed that, at a phylum level, Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria are harboured in the healthy lung and *Prevotella*, *Veillonella* and *Streptococcus spp*. are characteristic at genus level, in addition to facultative anaerobes.

On the other hand, Man *et al*. (2017)'s review concluded that the LRT microbiome harbours a distinguishable set from the upper one, yet they largely resemble each other. While the resemblance was demonstrated in health, Hilty *et al*. (2010) showed the oropharynx microbiome did not typically match the LRT one in diseased state. Furthermore, Dickson *et al*. (2017) demonstrated intrapulmonary regional differences in the microbiome topography.

Beside of that all, the main common limitation of these studies is their failure to isolate cultivable bacteria, where culturing "*appears fundamental for*

successful genome assemblies and for downstream functional experiments"
(Moffatt and Cookson, 2017). However, such alleged failure could be merely
a misuse of culturing conditions. For example, while anaerobes have been
described in the lung microbiome, anaerobic incubation is not routinely
performed for clinical microbiology samples, possibly contributed to the
delayed characterisation of this niche microbiome. To support this reasoning,
Venkataraman *et al*. (2015) revealed that 61% of taxonomic groups which
were detected by sequencing bacterial DNA of pulmonary specimens from
healthy subjects were identifiable by culturing when different conditions were
manipulated.

The above studies investigated *in-situ* localized respiratory tract microbiome
while the exhaled microbiome remains uninvestigated as will be detailed in
later Chapters.

1.7. Overall aim of our work

The overarching aim of the work in this project was to explore the use of face mask sampling in characterizing the microbiota naturally exhaled from the respiratory tract of healthy and diseased individuals. The general main explored ideas were:

1. Investigation of available options for improving facemask sampling

Rationale: TB is an archetypal example of airborne infection. Previous studies on facemask sampling have detected exhaled TB with a relatively-limited success. We aimed to investigate unaddressed controllable factors for optimizing the facemask methodology

2. Investigation of best instructions to the wearer for sampling the LRT

Rationale: Little is known about the biological content of exhaled aerosols and how this content might differ among different respiratory efforts. Previous studies have either investigated exhaled microbes or exhaled proteins, while none has studied these components together. We aimed to investigate the impact of instructions to the wearer on the collected exhaled material

3. Investigation of applicability of the developed tool in PJP

Rationale: Previous studies sampled environmental air of HIV-infected patients. Detectability of natural exhalation of *P. jirovecii* in HIV-uninfected and its clinical implications remained under-uninvestigated. We aimed to investigate the applicability of the developed tool for this missing group

4. Investigation of exhaled bacteriome

Rationale: Previous studies explored localized respiratory tract microbiome while naturally exhaled microbiome from human respiratory tract remained uninvestigated in health and disease. We aimed to investigate the applicability of the facemask for filling this gap

Each of these items had been characterized further throughout this project. We finally concluded ends with a general discussion of the results, outlining the faced limitations and potential future work.

Chapter Two: Development of face-mask processing techniques

2.1. Introduction

After introducing the face-mask system, we aim to build on the work established by Cheah (2010) and Williams *et al*. (2014), suggesting there are several aspects can be developed to achieve sensitive and quantitative results.

2.1.1. A potential scope for face mask improvement

From a perspective of improving the diagnostic and informative yield from face mask sampling, two groups of factors can be investigated and potentially addressed. These can be classified into biological and technical factors.

DNA based assays have been considered desirable for this study and are discussed further below. Further development of the mask system took place in parallel with this study and is reported in later Chapters.

2.1.1.1. Biological factors

Cheah (2010) conducted a feasibility study on using surgical masks to detect exhalations from 17 AFB smear-positive patients whose TB diagnoses were suspected based on clinical and radiological findings alongside the smear positivity (sputum or BAL). All the Cheah patients had positive confirmatory MGIT (Mycobacteria growth indicator tube) cultures. The employed bacteriophage and qPCR assays detected mycobacteria in 11 patients (64.7%) with mask wearing durations between 10 and 120 min. Among the 17 patients, two had NTM infection, one *M. kansasii* and a further one *M. avium-intracellulare*.

Cheah investigated several technical factors such as qPCR inhibition and the impact of decontaminating the masks with Sodium Hydroxide-N-Acetyl-L-Cystein (NaOH-NALC) prior to analysis, however despite of mentioning several potentially-contributing biological factors none was addressed.

While it cannot be excluded that those who yielded negative masks but diagnosed with TB might have been misdiagnosed, possible factors include pulmonary bacillary load of the confirmed cases, where a low load may have resulted in negative, undetectable or masked exhalations.

Eleven patients had AFB smears graded from 1+ to 2+, and six patients had scanty smears. These grades may have reflected a low intrapulmonary bacillary load.

In fact, the relation(s) between sputa and aerosols remains unknown. A number of studies highlighted a dissociation between AFB-smear positivity and infectiousness mediated by exhaled aerosols (Blahd *et al*., 1946; Catanzaro, 1982; Di Perri *et al*., 1989; Behr *et al*., 1999; Dharmadhikari *et al*., 2011).

Furthermore, intrapulmonary replication of bacilli was differently approached by other studies. For example, Garton *et al*. (2008) and Mukamolova *et al*. (2010) reported multiple phenotypes of Mtb in sputum and many non-replicating bacilli.

Of the 17 patients, Cheah reported 12 had received chemotherapy prior to sampling (within seven days) and two had a history of chemotherapy. Probably, effective anti-TB treatment resulted in diminishing or eliminating positive exhalations from these group. It is worth highlighting that all these had isolates susceptible to first line anti-TB agents.

Cheah reported that morning samples were marginally more positive than samples collected in the afternoon. However, the small sample size and the borderline difference in the quantity of mask-collected Mtb undermined confirming this.

Williams *et al*. (2014) detected positive masks in 65% of 20 patients with clinically-confirmed TB. In contrast to the Cheah ones, the Williams masks were collected prior to commencing TB treatment except for two patients

sampled on the fifth day of treatment. Although of that, however, the detection rates were comparable between both studies.

In fact, Boston (1901) who collected exhaled output from TB patients on microscope slides accommodated by a mask (Figure 1.1) reported that 38 of 50 patients yielded positive slides (stained with carbol-fuchsin for TB bacilli) with exposure time of 1–1.5 hrs. Despite more than a century separated the Boston and the Williams studies, the positivity of the earlier was reported at 76% while that for the latter was 65%. This might be accounted for advanced pathology in pre-chemotherapy patients, however, adjusting the results to patients who did not receive treatment increases the likelihood of a technical unaddressed gap. Moreover, the low sensitivity of microscopy for bacilli detection makes this possibility more remarkable. In addition, the Boston study was designed "*with the object being not to collect on the slide the spray produced by vigorous coughing*". It seems many of the Boston patients generated aerosols at highest levels of bacillary load. Indeed, Boston noted that seven of negative slides were from patients with paucibacillary contemporaneous sputa. Boston did not provide quantitative data but commented that "*In fully one-third of positive cases the bacilli were very numerous*".

While nine of Cheah's subjects had cavitary lung disease, cavitation was not mentioned for the Williams subjects.

One subject of the Cheah study was HIV-coinfected while none of the Williams was so. Turner *et al*. (2017) suggested that HIV-coinfection could reduce infectiousness in TB, owing to less occurrence of cavitation, lower sputum bacillary load, relatively shorter period of disease, and less social activities related to advanced stage debility.

Although Williams and colleagues suggested that increasing the sampling time may decrease the false negativity of the mask, the expiratory efforts during

sampling were not well studied. Different efforts may sample or generate aerosols from different parts of the respiratory tract. Cheah did not provide the subjects with instructions on performing a certain breathing activity during sampling. Cheah only instructed the patients to briefly remove the mask if they needed to expel sputum. Similarly, Williams further instructed those who did not make any vocal effort to cough once and repeat '*Peter*' ten times.

The mask does not differentiate in collection between droplets and aerosols. This mixed collection was mentioned in terms of the mask ability to provide data on TB transmission rather providing data on the anatomical part being sampled and whether it was the upper or lower respiratory tract.

Some cellular features, including morphology, hydrophobicity and hydrophilicity, were reported in rendering some microorganisms aerosolisable.

Parker *et al*. (1983) and Falkinham (2003 and 2015) demonstrated significant correlations between hydrophobicity of *M. avium* and other NTM members and their propensity to enter into aerosols generated from aqueous media.

Jonsson *et al*. (2007 and 2013) and Park *et al*. (2015) reported that *M. abscessus* isolates responsible for chronic colonisation or infection of the respiratory tract were usually of rough strains. In fact, rough strains of *M. abscessus* lack polar glycopeptidolipids and have been demonstrated more hydrophobic than the smooth strains and possibly more aerosolisable or transmissible.

On the other hand, although Bryant *et al*. (2016) studied the possibility of *M. abscessus* infections by aerosol transmission, they did not mention a link with rough-smooth strains.

On these bases, Jankute *et al*. (2017) suggested that enhanced hydrophobicity of Mtb is likely linked to its successful aerosol transmission.

In contrast, Tseng and Li (2005) evaluated the collection efficiencies of four aerosol-samplers (Andersen impactor, AGI-30 impinger, gelatine or nuclepore filters) using four different bacteriophages as substitutes for human viruses. They found the collection efficiencies of the four samplers were for hydrophilic viruses 10–100 times higher than hydrophobic viruses, and that the morphology of the viral particles affected these efficiencies. While the Tseng and Li results can be interpreted technically, they might reflect the role of these features in the aerosolizability of respiratory viruses.

Many of previously mentioned factors are not naturally, practically nor clinically controllable. For example, it is a challenge to delay or alter treatment of patients for mask collection, unless a mask supply with a modestly-trained personnel are available, and the factors that render microorganisms aersolisable cannot be altered. On the other hand, a low pulmonary bacillary load might be manipulated by improving the assay sensitivity and its detection limit. Therefore, improving the yield from face mask sampling practically starts from addressing technical and controllable factors.

2.1.1.2. Technical factors

Cheah (2010) investigated several technical factors within two aspects: the sensitivity and quantitative potential of the mask tool. Cheah analysed the surgical masks with qPCR and mycobacteriophage assays and found that the mask analytical procedure and the mask materials induced a significant PCR-inhibition activity with less impact on the mycobacteriophage assay. However, Cheah found the mask decontamination with NaOH-NALC prior to analysis significantly affected the sensitivity of the mycobacteriophage assay.

These drove Williams and colleagues (2014) to add the use of FFP30 masks incorporated with gelatine filters and to analyse them on the WHO recommended GeneXpert platform. However, after these steps were implemented the detection rates remained comparable between the two studies, reported at ~65%. In addition, the results were not quantitative.

Therefore, it was essential to start by addressing some of the imitations of the Williams *et al*. (2014) study. The developmental nature of the Williams work did not involve optimizing the used technical methodologies in terms of the sensitivity and quantitative aspects of the downstream molecular assay, nor involved determining optimum duration and most efficient respiratory effort for sampling.

Williams and colleagues were unable to obtain positive masks in 35% of 20 patients with clinically-confirmed TB. TB has been known the archetypal example of airborne infections (Riley, 1974; Roy and Milton, 2004). Recent RASC studies demonstrated positivity rates of Mtb exhalations at 93% (Wood *et al*., 2016; Patterson *et al*., 2017). However, the RASC required extensive infrastructure and thus is limitedly-applicable in different clinical settings.

Therefore, when the mask approach fails to detect Mtb aerosols from such a high percentage, the first possibility to be addressed is technical. While this false-negativity is attributable to several factors, one of the most important is

the recovery rate of the material exhaled by these patients and captured on their masks.

There are many potentially controllable variables that might alter the yield from face mask sampling. These can be classified as biotechnical factors and can be summarised as the following:

A) Related to the face-mask:
- mask design e.g. space between internal surfaces and oronasal cavity
- mask type e.g. FFB30, N95 and surgical types
- capturing surface e.g. gelatine, cellulose and polyvinyl alcohol (PVA)

B) Related to processing the face-mask
- transforming captured material to analysable material
- DNA extraction method
- downstream analysis method
- transport and storage conditions

C) Related to the sampling process:
- optimum duration
- time of day
- sampling environment
- processor or investigator

D) Related to the sampled subject:
- breathing activity or expiratory efforts
- individual productivity of exhaled output
- medical state
- personal characteristics including possible personal microbiome

E) Related to result interpretation e.g. the gold standard and study design

Each of these variables can be characterised further. This Chapter deals mainly with item B.

While it was originally intended to include sampling from suspected TB cases in Leicester during this study, the incidence of such cases was not sufficient to support the present work and alternate sampling was undertaken. Nonetheless, the methods established here were deployed in a separate study to which this author contributed (Williams *et al*., 2018).

2.1.2.   Viable versus non-viable microbial detection

Molecular-based (DNA) analyses have been increasingly utilised in medical research and diagnostics. DNA detection of Mtb-carrying aerosols in room air of respiratory isolated TB patients was performed (Mastorides *et al*., 1999) however the molecular based nature of detection which was not quantitative at that stage or viability-discriminative was criticised (Nardell, 1999 in comment on Mastorides *et al*., 1999) but these concerns were addressed (Sandin's response to Nardell, 1999). The field of molecular diagnostics has indeed further developed, bringing new insights from propidium monoazide (PMA) or ethidium monoazide (EMA) treatment of DNA samples.

PMA is a cell membrane-impermeable dye that covalently intercalates accessible nucleic acids. This renders DNA strands unelongatable with polymerase and subsequently non-amplifiable.

Culture-independent methods such as PCR can detect DNA whether it was free or extracted from a bacterium or cell. It is not necessarily that this bacterium was intact, and if it was, it is not necessarily it was viable, and if it was, it is not necessarily it was colony-forming or infectious. Furthermore, DNA can be detected from persister cells that do not respond to antimicrobial challenges (Lewis, 2010). On the other hand, culture-dependent methods can detect only culturable cells which must be viable and colony-forming. It must be recognised, however, that culturability is different from viability. For example, Mukamolova *et al*. (2010) revealed a population of non-replicating Mtb bacilli in sputum culture after this was challenged with resuscitation-promoting factors. Therefor, culture-independent methods can be more informative due to involvement of a wide spectrum of statuses while culture-dependent ones can be more sensitive to a certain status.

In this Chapter, a series of experiments was carried out focusing on intact mycobacterial cells taken at mid-exponential phase in order to target viable

bacilli. It is noteworthy that for diagnostic assays based on DNA detection the viability is not generally considered though it could be important when the purpose is, for example, to monitor a drug response for reaching a final diagnosis or to estimate a source infectiousness.

2.1.3. PCR fluorescence chemistries for real-time quantification and *IS6110* as a target

PCR has been introduced as a cost-effective and reliable method to replicate a defined segment of DNA by means of thermal cycling (Bartlett and Stirling, 2003). qPCR has then been deveopled as a quantitative assay for amplifiable DNA, based on ethidium bromide fluorescence (Higuchi *et al*., 1993).

Two main chemistries have been frequently applied for real-time quantification with PCR and were used in this study, SYBR-Green and TaqMan. The former is characterized by its reversible nature (allowing a melting curve analysis) whereas that for the latter is irreversible. TaqMan was derived from devising a thermostable-polymerase-producing *Thermus aquaticus* with a Pac-Man principle-based fluorogenic oligonucleotide probe specific to a hybridisable complementary target. Thus, each TaqMan-based amplicon initiated by gene-specific primers is represented by a single fluorescent molecule. On the other hand, SYBR-Green is a non-specific, double-stranded DNA (dsDNA)-intercalating asymmetrical cyanine dye, presenting each amplicon initiated by specific primers as multiple SYBR-Green molecules (Figure 2.1). While SYBR-Green is more sensitive with higher false-positivity (primer-dimers and non-specific amplicons), more cost- and time-effective, TaqMan is more specific with high false-negativity (probe-binding failure) and applicable for multiplex assays (Dorak, 2006; Haldar, 2014; Kralik and Ricchi, 2017).

TAQMAN PROBE-BASED ASSAY CHEMISTRY

1. Polymerization: A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe, respectively.

2. Strand displacement: When the probe is intact, the reporter dye emission is quenched.

3. Cleavage: During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.

4. Polymerization completed: Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

SYPR GREEN I DYE ASSAY CHEMISTRY

1. Reaction setup: The SYPR Green I Dye fluoresces when bound to double-stranded DNA.

2. Denaturation: When the DNA is denatured, the SYPR Green I Dye is released and the fluorescence is drastically reduced.

3. Polymerization: During extension, primers anneal and PCR product is generated.

4. Polymerization completed: When polymerization is complete, SYPR Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.

Figure 2.1 TaqMan vs SYBR Green fluorescence chemistry workflows
This was reproduced from https://www.thermofisher.com/uk/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/taqman-vs-sybr-chemistry-real-time-pcr.html

For real-time detection rather than quantification of MTC, GeneXpert MTB/RIF assay is run by an automated system integrating DNA extraction (by sonication) and amplification, targeting a segment containing the 81bp core region of the bacterial RNA polymerase *rpoB* gene with its mutations (predicting rifampicin susceptibility in the former and resistance in the latter) (Lawn and Nicol, 2011). This assay, however, does not differentiate between species of MTC species (comprising Mtb, *M. africanum*, *M. canetti*, *M. bovis* including BCG strain, *M. caprae*, *M. pinnipedii*, *M. microti*, *M. mungi*, and *M.*

orygis and *M. suricattae*). Moreover, the assay has been reported detecting NTM with high bacterial load (Pang *et al*., 2017). Therefore, a modified version has been recently developed targeting further the insertion sequences 6110 and 1081 (Chakravorty *et al*., 2017).

The insertion sequence 6110 (*IS6110*) is a mobile genetic element that has been used as a molecular target of MTC in clinical specimens since it was described by Thierry and colleagues in 1990. Although the number of *IS6110* copies differs in genomes of different MTC members (0-21 copy, Table A.4) and an *IS6110*-related element was identified in one strain of *M. smegmatis* (Coros *et al*., 2008), *IS6110* has been appreciated as the most abundant element in Mtb genome and the exclusive one to MTC members. Other insertion sequences such *IS1081* and *IS1547* lack these characteristics.

In a comprehensive comparison study, the GeneXpert assay showed lowest analytical sensitivity among 14 molecular assays targeting MTC in BALs, and was at best one log fold less sensitive than the rest (Akkerman *et al*., 2013). In addition, Akkerman and colleagues showed that the intertest variability of the GeneXpert was persistent even when the storage conditions were considered, although they hypothetically attributed this variability to cellular lysis due to frequent freezing/thawing cycles.

2.1.4. Hypotheses and objectives

The overall aim of the work in this Chapter was to investigate options for improving facemask processing.

We hypothesized that the established facemask processing methods were sub-optimal for the detection and quantification of captured Mtb.

Objectives:

1. To evaluate the recovery rate of the Williams *et al*. (2014) protocol
2. To improve the processing protocol of masks and report the technical developments for detection and quantification of captured Mtb
3. To compare different DNA extraction protocols for isolating mycobacterial DNA for quantification by qPCR
4. To compare the performance of qPCR assays with different chemistries for quantifying Mtb
5. To identify the limit of detection of the developed protocol

2.2. Materials and methods

2.2.1. General materials and disinfection methods

Unless stated otherwise, plastics were obtained from Corning Incorporated (USA), VWR International Ltd (UK), Greiner Bio-One Ltd (UK) and Eppendrof (UK) while individual chemicals and other reagents were obtained from Sigma-Aldrich Company Ltd (UK), QIAGEN (UK) and Fisher Scientific Ltd (UK). The culture media were products of BD Biosciences (UK).

Microbial sterilization of media, containers and reagents was achieved by autoclaving at 121°C at 15 psi for 15 min and by microfiltration with 0.2μm-microfilters (Nalgene, UK) for non-autoclavable solutions, unless indicated otherwise. Microbial sterilization was preceded by washing with detergents, followed by irradiation with high-dosage of UV (9,999x100 μJ) using Stratalinker UV Crosslinker (Stratagene, USA) when biomolecular decontamination was sought.

2.2.2. Growth media and reagents

2.2.2.1. Albumin – Dextrose – Catalase enrichment (ADC)

Use: Growth supplement for liquid media

7.5g of bovine serum albumin fraction (V), 3.0g Dextrose (D-Glucose), 1.28g Sodium Chloride and 6.5mg catalase were dissolved in 150mL distilled water. The solution was sterilized by microfiltration and stored at 4˚C.

2.2.2.2. Tween 80

Use: Tween 80 (Polysorbate 80) is used as an anti-clumping agent for mycobacteria species grown in liquid media

10g of Tween 80 stock solution was dissolved in distilled water to a final volume of 100mL giving a final concentration of 10% w/v. The solution was sterilized by microfiltration and stored at 4°C.

2.2.2.3. Middlebrook 7H9 broth

Use: Liquid nutrient medium for mycobacteria growth

4.7g of Middlebrook 7H9 broth powder was dissolved in 1L Duran bottle with 900ml distilled water containing 2.5g glycerol. The solution was then sterilized and supplemented with 10% v/v ADC and 0.05% v/v of 10% w/v Tween 80.

2.2.2.4. Middlebrook 7H10 agar

Use: Solid nutrient medium for mycobacteria growth

19g of Middlebrook 7H10 agar powder was dissolved in 900mL distilled water containing 6.25g glycerol. The agar was heated and stirred until complete dissolution then sterilized. The medium was supplemented before solidification with 10% v/v ADC, poured in Petri dishes in a laminar flow cabinet (Walker Safety Cabinets Ltd., UK) and stored at $4^{o}C$

2.2.3. Optical density (OD) measurement

Optical density was considered by measuring the absorbance of 1mL of culture in a 1.5mL cuvette at wavelength of 580nm (OD_{580}) against a blank of relevant medium. The measurement was done using Sanyo SP75 UV/Vis spectrophotometer (Watford, UK) and Jenway 6300 spectrophotometer (Stone, UK) for Category II and Category III laboratories, respectively. The letter cuvettes were sealed with autoclave tape and Nescofilm (Bando Chemical, Japan). Dense cultures with readings ≥1.0 were ten-fold diluted with sterile 7H9 broth and the OD measured.

For *M. smegmatis*, OD_{580} of 0.7 was ~1.6×10^8 CFU/mL, and for *M. bovis* BCG OD_{580} of 0.9 was ~9.7×10^7 CFU/mL.

2.2.4. Preparation, cultivation and storage of mycobacteria

2.2.4.1. Mycobacterial strains used

The used mycobacterial strains are listed in Table 2.1.

Table 2.1 Mycobacterial strains used

Strain	Description	Source
M. smegmatis MC^{2}155	Non-pathogenic strain (Category II)	Laboratory stock (ATCC 700084)
M. bovis BCG Glaxo	Attenuated TB vaccine strain (Category II)	Laboratory stock (GlaxoSmithKline)
M. tuberculosis H37Rv	Virulent strain (Category III)	Laboratory stock (WR Jacobs lab strain)

2.2.4.2. Glycerol stocks of mycobacteria

In order to preserve mycobacteria for long-term use, Middlebrook 7H9 broth culture grown to mid-exponential phase (OD_{580} = 0.5–0.8) was added to equal amount of 65% v/v glycerol, aliquoted in 1.5mL micro-centrifuge tubes and stored at -80°C.

2.2.4.3. Cultivation of *M. smegmatis*

A -80°C frozen stock (1mL) was thawed at room temperature to inoculate 5mL of Middlebrook 7H9 broth in 30mL polypropylene universal container (Sterilin, UK). This was incubated with shaking rate of 200rpm at 37°C overnight. The resultant culture was used to inoculate 25mL fresh Middlebrook 7H9 broth for a final OD_{580} of 0.05 to be incubated in 125mL conical glass flask under the same conditions.

2.2.4.4. Cultivation of *M. bovis* BCG

The same cultivation method applied for *M. smegmatis* was used for BCG except that the incubation was static for a period of 5-6 days.

2.2.4.5. Cultivation of Mtb H37Rv

All Mtb work was carried out in Category bio-containment level III suite according to the University of Leicester Code of Practice. -80°C stocks were kindly supplied by Mrs Wegrzyn. The frozen stock was thawed in Class 2 microbiological safety cabinet and used to inoculate 50mL Middlebrook 7H9 broth in 250mL polypropylene conical flask. This was double bagged, incubated with shaking rate of 100rpm at 37°C until reaching early exponential phase (OD_{580}= 0.4) and sub-cultured as mentioned before.

2.2.5. Artificial inoculation of the mask inserts

Gelatine filters (3μm pore size; Sartorius Stedim Biotech GmbH, Germany) were cut into three equal portions using a cutting-guide template. Each one-third was spiked with a known volume and concentration of Middlebrook 7H9 broth culture. The contaminated inserts were left to dry into 50mL propylene conical-base tube at room temperature.

2.2.6. Gelatine filter processing methods

2.2.6.1. Gelatine filter dissolution by heating (Williams *et al*., 2014)

Filters were heated to 95°C for 10min until completely dissolved. 2mL of the lysate was then transferred to a 1.5mL micro-centrifuge tube and centrifuged at 10,000xg for 10min. The supernatant was removed, and the pellet resuspended in 1mL of molecular grade water.

2.2.6.2. Gelatine filter hydrolysis with alkali

1.5mL of 0.5% w/v NaOH (125mM) was added to filters in 40mL crystallizing dishes at room temperature. The hydrolysis was completed within 15min then the lysate was neutralized with an appropriate volume of 1M hydrochloric acid (HCl) to minimize potential inhibitory effects on PCR amplification. The lysate

was transferred to 2mL O-ring seal screw cap tube for further processing or storage.

2.2.6.3. Gelatine filter digestion with collagenase

Collagenase A (COLLA-RO Roche, Sigma, UK) was dissolved in distilled water and sterilized by ultrafiltration with Vivaspin 500 (0.2µm pore size Polyethersulfone Membrane; Sartorius Stedim Biotech GmbH, Germany) and UV irradiation (specified earlier). 1900µL of 50µg/mL collagenase buffer (containing 50µg/mL collagenase A, 50mM N-Tris-methyl-2-aminoethanesulfonic acid (TES), 0.36mM Calcium chloride, pH 7.4) was added to gelatine filters in 40mL crystallizing dishes and incubated at 37°C for 15min. The lysate was transferred to 2mL O-ring seal screw cap tube for further processing or storage.

2.2.7. DNA extraction methods

2.2.7.1. DNA release by boiling

Cell suspensions were transferred to 1.5mL microfuge tubes, centrifuged at 13,000xg for 10min and the resultant pellets were resuspended with 200µL of PCR-grade water (Life Technologies Ltd. (Invitrogen), UK). The suspensions were boiled in a heat block set at 100°C for 15min, left to cool down at room temperature for 5min then centrifuged at 15,000xg for 10min. The resultant supernatants were used as DNA extracts.

2.2.7.2. DNA extraction by Chelex100–Nonidet P40

The in-house extraction protocol outlined by van der Zanden (1998) was used with few modifications. Cell suspensions were transferred to microfuge tubes, centrifuged at 13,000xg for 10min and the resultant pellets were resuspended with 100µL of Chelex suspension containing 50% w/v Chelex100, 1% w/v Nonidet P40 Substitute and 1% w/v Tween 20. The samples were incubated

in a heat block set at 100°C for 30min then centrifuged at 15,000xg for 10min. The resultant supernatants were used as DNA extracts.

2.2.7.3. DNA extraction by bead-beating (glass beads)

The in-house extraction protocol outlined by Larsen (2000) was used with few modifications. Cell suspension was transferred to 2mL O-ring seal screw cap tubes and centrifuged at 13,000xg for 10min. The supernatant was discarded, and the pellet suspended with 100µL of Tris(hydroxymethyl)aminomethane-Ethylenediaminetetraacetic acid buffer (Tris-EDTA; 20mM Tris, 2mM EDTA, pH 8). Lysing matrix B (MP-Biomedicals, UK) was added to the lysate so a thin layer of Tris-EDTA was left above the beads. The sample was vortexed using Vortex-Genie 2 (Scientific Industries, Inc., USA) for 1min then incubated in ice-box for 1min. This step was repeated then the sample incubated in a 95°C heat-block for 5-10min. The lysate was microcentrifuged for 2min, and the vortexing/ice incubation step repeated. The sample was then microcentrifuged and the supernatant used as DNA extract.

2.2.7.4. Optimized DNA extraction protocol

The sample was transferred to 2mL O-ring seal screw cap tube and centrifuged at 15,000xg for 10min. The supernatant was discarded, and the pellet resuspended with 100µL of Tris-EDTA (20mM Tris, 2mM EDTA, pH 8) and 100µL Chelex suspension (specified before). 0.25g of lysing matrix B (specified before) was added to the lysate. The samples were homogenized by Hybaid RiboLyser (Hybaid, USA) in Category II laboratory or FastPrep-24 Classic Instrument (MP Biomedicals, UK) in Category III laboratory for 45sec at 6.5m/sec speed and incubated in ice-box for 2min. This step was repeated. The lysates were centrifuged for 2min at 15,000xg and the homogenization/ice incubation step repeated. The samples were then centrifuged for 2min at 15,000xg. The supernatants (DNA extracts) were

directly used or applied in Category III work to Vivaspin 500 (specified before) and centrifuged for 2min at 15,000xg where the flow-through was collected and used as DNA extract.

2.2.8. Mycobacteria quantification methods

2.2.8.1. Microscope-assisted quantification using cytometer

A Petroff-Hausser counting chamber (Hausser Scientific Co., USA) was used for quantifying mycobacterial cells suspension at concentration of 10^7–10^8 cell/mL. The chamber and cover slip were cleaned and dried. The cell suspension was ten-fold diluted in phosphate buffered saline (34mM KH$_2$PO$_4$, 33mM K$_2$HPO$_4$, pH 7.4) and a drop of the diluted specimen was delivered to the chamber. Mycobacterial cells of density between 8–80 cells per square were counted under the 40-power dry objective in 10 large squares. The number of cells per mL of the original suspension was calculated as follows:

$$Number\ of\ cells.mL^{-1} = Mean_{sq} \times DF \times F_{sq}$$

where:

$Mean_{sq}$: *Average cell number per squre*

DF: *Dilution factor of quantified specimen*

F_{sq}: *Factor of large squre* $= 1.25 \times 10^6$

2.2.8.2. CFU counting

Cell suspension of Middlebrook 7H9 broth culture was serially ten-fold diluted in 7H9 broth. 20µL from each dilution was plated in triplicate onto duplicate 7H10 agar plates separated into four sectors. The plates were sealed with Nescofilm and incubated at 37oC until mycobacterial colonies were visually countable. The counting was considered on sectors having 10–100 colonies and based on the following equation:

$$CFU.mL^{-1} = Mean_{sct} \times DF_{sct} \times 50$$

where:

$Mean_{sct}$: *Average number of colonies per sector*

DF_{sct}: *Dilution factor used to cultivate colonies of this sector*

2.2.8.3. Real-time quantitative polymerase chain reaction (qPCR)
Preparation of qPCR standards

Absolute quantification with qPCR requires running DNA standards with a known concentration of the target gene or fragment to interpolate unknown samples from a standard curve generated by plotting the log of target concentration versus threshold cycle (Ct) (Ramakers *et al*., 2003, Ruijter *et al*., 2009).

Genomic DNA of Mtb H37Rv was kindly provided by Dr Lazar-Adler of this Department and used for preparing the standards. DNA concentration was measured using Nanodrop 1000 Spectrophotometer (Thermo Scientific, UK) as follows. 1µL of molecular-grade water was pipetted onto the lower measurement pedestal as a blank. 1µL of the DNA was then loaded onto the pedestal and the absorbance was read at 260nm (A_{260}).

The concentration was calculated based on the equation derived from Beer-Lambert law, as the following:

$$\boldsymbol{DNA\ concentration\ (\mu g.mL^{-1})} = A_{260} \times 50 \times Dilution\ factor$$

considering dsDNA concentration equals to 50 µg/mL at A_{260} of 1.

For calculating the total number of DNA copies, the following equation was used:

$$\boldsymbol{Number\ of\ DNA\ copies.\mu L^{-1}} = \frac{\text{DNA concentration (ng.}\mu L^{-1})}{\text{Genome mass (ng)}}$$

The genome mass is equal to molecular weight (MW) of genome divided by Avogadro constant (6.23×10^{23}). The MW equals to the size of genome (base pair) multiplied by the average MW of DNA base-pair (649 Dalton).

For Mtb H37Rv*:*

- Genome MW = 4,411,539 x 649 = 2.86×10^9 g/mole
- Genome mass = 2.86×10^9 / 6.23×10^{23} = 4.60×10^{-15} g = 4.60×10^{-6} ng
- The standards were serially ten-fold diluted with molecular-grade water, starting from 10^7 genome/µL (equivalent to 46ng/µL) to 10^2 genome/µL.

Target genes, primers and probes

Mycobacterial genome targets used for qPCR and the oligonucleotide primers and probe run for each are listed in Table 2.2. The specificity of the primer pairs was verified by performing BLAST analysis at the NCBI GenBank using Primer BLAST tool with the mRNA, genomes and nr Databases. All primers were obtained from Integrated DNA Technologies (UK) and the probes were from Life Technologies Ltd. (Invitrogen) (UK).

Table 2.2 Mycobacterial target genes, primers and probes

Primer/probe designation	Oligonucleotide sequence	Amplicon size (bp)	qPCR Target	Copies per mycobacterial genome	Reference
MYCO*16S*	Forward: 5'-GAAACTGGGTCTAATACCG-3'	171–173	*16S*	0–2	Cheah *et al.*, 2010
	Reverse: 5'-ATCTCAGTCCCAGTGTGG-3'				
IS6110	Forward: 5'-AGCGTAGGCGTCGGTGAC-3'	74	*IS6110*	0–25	Akkerman *et al.*, 2013
	Reverse: 5'-GGGTAGCAGACCTCACCTATGTGT-3'				
	Probe: 5' 6-FAM TCGCCTACGTGGCCTTT-3' MGBNFQ				

Definition of abbreviations: MYCO*16S*= Mycobacterial *16S*; *IS6110*= Insertion sequence 6110; 6-FAM= Fluorescent reporter dye; MGBNFQ= Minor groove binder non-fluorescent quencher

Throughout this work, molecular assays developed or optimised in the laboratory were referred to as 'in-house' to be distinguished from those commercially available or published and reproduced here without modifications.

In-house MYCO*16S* qPCR SYBR-Green Assay

SensiFAST SYBR No-ROX Kit was provided by BIOLINE (UK) as a 2x mastermix containing DNA polymerase, SYBR-Green I dye, deoxyribonucleotide triphosphate (dNTP)s and SYBR-Green buffer of Tris-Cl, KCl, NH4Cl, MgCl2 and Q-Bond.

25µL PCR mixture of each reaction tube was prepared in 0.1mL Rotor-Gene PCR tubes (QIAGEN, UK) containing 12.5µL of 2X SensiFAST SYBR No-ROX, 1µL of 10µM MYCO*16S*F forward, 1µL of 10µM MYCO*16S*R reverse primer, 1µL of DNA template and 9.5µL of DNase-RNase free water.

The cycling conditions were one holding cycle at 95°C for 15min for polymerase activation, 40 amplification cycles at 95°C for 15sec for denaturing, 60°C for 30sec for annealing, 72°C for 20sec for extension with acquisition at cycling A and 82°C for 20sec for extension with acquisition on FAM/Green channel (470nm) on green channel at cycling B. Melting condition was set at 60°C to 95°C rising 1°C per cycle to determine the specificity of the amplicons. PCR-grade water was used as negative control of amplification to detect contaminating DNA.

In-house 10µL MYCO*16S* qPCR SYBR-Green Assay

A similar protocol to the detailed above, excepting that DNA template for all reaction tubes was 10µL after adjusting the amount of DNase-RNase free water.

In-house *IS6110* qPCR SYBR-Green Assay

PCR mixture contained 12.5µL of SensiFAST SYBR No-ROX, 1µL of 10µM *IS6110*F primer, 1µL of 10µM *IS6110*R primer, 1µL of DNA template and 9.5µL of PCR-grade water. Cycling conditions were one holding cycle at 95°C for 15min, 40 amplification cycles at 95°C for 15sec, 68°C for 30sec, 72°C for 20sec and 82°C for 20sec with acquisition on FAM/Green channel (470nm). Melting condition was set at 68°C to 95°C rising 1°C per cycle. Molecular-grade water was used as a negative control of amplification.

In-house 10µL *IS6110* qPCR SYBR-Green Assay

The same conditions of the detailed above, excepting that DNA template was 10µL and the reaction volume was brought to 25µL with 0.5µL DNase-RNase free water.

In-house qPCR *IS6110* TaqMan Assay

The primary protocol was established by Akkerman *et al*. (2013) to be run on LightCycler 480 Instrument II (Roche) and optimized as below for Rotor-Gene 6000 real-time DNA analysis system on Corbett PCR machine.

30µL PCR mixture contained 15µL of TaqMan universal PCR master mix +AmpErase UNG (Applied Biosystems, UK), 1µL of 10µM *IS6110*-forward, 1µL of 10µM of *IS6110* primer, 0.5µL of 10µM TaqMan *IS6110* probe, 10µL of DNA template and 2.5µL molecular-grade water. Cycling conditions were one holding cycle at 50°C for 2min, one holding cycle at 95°C for 10min, 45 cycles at 95°C for 15sec and 60°C for 6sec with acquisition on FAM/Green channel (470nm), and one holding cycle at 40°C for 20sec. DNase-RNase free water was used as negative control of amplification.

Multiplex MTC qPCR assay

Multiplex qPCR assay with real-time hybridization-fluorescence detection for MTC based on TaqMan polymerase chemistry was run using a commercial kit from InterLabService (Russia) under the brand name "AmpliSens MTC-FRT PCR kit". 25µL PCR mixture contained 15µL of reaction mix prepared per the manufacturer's instructions and 10µL of DNA template. A kit-provided DNA buffer was used as a negative control for amplification. The cycling conditions were as recommended by the manufacturer for amplification on Rotor-Gene 6000 and run on the Corbett machine.

qPCR performance assessment

The most important factors considered when evaluating a qPCR performance are the dynamic range, precision and sensitivity (Dorak, 2006; Haldar, 2014; Kralik and Ricchi, 2017). **The first** can by evaluated through the efficiency (slope) and coefficient of determination (R-squared; R^2). A minimum of 5 serial log dilutions of the molecular target are required to generate a standard curve. A slope describing best-fit values of −3.322 reflects an efficiency of 100%. R^2 value is a statistical term describes the goodness of fit, or in other words, how good an Y value is at predicting an X. The Ct by definition is an intersection between an amplification curve and a threshold line. Therefore, R^2 of 1 indicates that Y(Ct) = X. qPCR efficiency refers to how efficiently the PCR polymerase duplicates the template DNA during every cycle in a concentration-independent manner (100% efficiency being ideal). **The second** parameter can be detected through calculating the standard deviation (SD) of a minimum of 3 replicates (<0.167 (1 Ct / 6 SD) being ideal to indicate ability of quantifying a 2-fold dilution in >99.7% of samples with 100% efficiency). **The third** can be approached with a statistical test analysis. Poisson rather than Gaussian distribution is technically expected. Poisson distribution principles should be applied for a suitable number of replicates

having low copy number of the molecular target. In fact, a large number of replicates are required to result in a statistical significance and to overcome this distribution limitation (Dorak, 2006; Haldar, 2014; Forootan *et al*., 2017; Kralik and Ricchi, 2017).

All the assays were run in technical triplicates and analysed on Rotor-Gene 6000 real-time DNA analysis system (Corbett Life Science, QIAGEN). The Ct was set by Rotor-Gene 6000 Series software 1.7. Lower limit of detection (LLD) was 100 copies per reaction (lowest concentration amplifiable and differentiable from the negative control). The analyses were accepted when R^2 was ≥0.98, efficiency was ≥0.70 and negative control of amplification showed <100 copy/reaction. The slope correct option was selected. The quantitative readings were accepted (i.e. considered legitimate and repeatable) when the coefficient of variation of the triplicates was less than 20%. This indicated that the difference in Ct values was <1 (Dorak, 2006). Ct of different assays was uniformly set at a single value when comparability was sought.

2.2.9. Statistical analysis

Data of this Chapter were analysed using Microsoft Excel 2016 of Office ProPlus (Microsoft Corporation, USA) and GraphPad Prism version 7 (GraphPad Software Inc., USA), as appropriate. The used statistical tests are stated in due place. A p value of 0.05 was considered the significance threshold for all statistical analyses. Definitions of asterisks displayed to denote the degree of statistical significance are listed in page xxx.

2.3. Results

2.3.1. Optimisation of the recovery rate of Williams *et al*. (2014)'s method was necessary

Although many protocols for assaying Mtb DNA have been established, it was necessary to review procedures appropriate to assays applied to cells captured on the mask inserts (gelatine filters).

To evaluate the recovery rate of Williams *et al*. (2014)'s method, a set of filters contaminated with *M. smegmatis* ($\sim$2x10^5 CFU) were processed per this alongside the same volume of water contaminated directly with the same inoculum (also heated). The non-pathogenic *M. smegmatis* mc^2 155 was used since it is equivalent in size and morphology to Mtb (Gordon and Smith, 1953). DNA extracted from the pellets by boiling was quantified using the in-house MYCO*16S* SYBR-Green assay as were the supernatants.

It was found that the recovery rate of the heating protocol was less than 1% (0.61%) of the direct inoculum extract (Figure 2.2). Direct analysis of free DNA in the supernatants revealed almost the same quantity (91%) as was found in the inoculum pellet extracts (Figure 2.2).
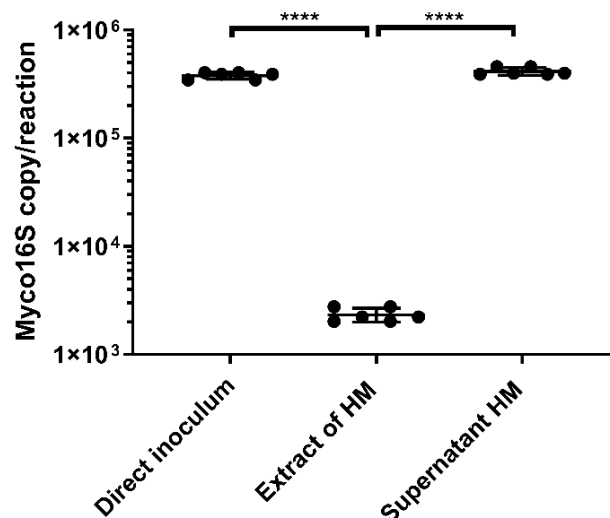
Figure 2.2 Extracts of Williams *et al*. (2014)'s method show poor recovery rate
HM= Heating method of Williams *et al*. One-way ANOVA with multiple comparisons was applied. Error Bars= SD, n= 6.

2.3.2. Gelatine filter hydrolysis with alkali and digestion with enzymes

The use of sodium hydroxide as an alternative to heating to dissolve gelatine was explored and compared to the established heating method. Gelatine was found readily hydrolysable at room temperature with NaOH. A set of filters contaminated with *M. bovis* BCG (~4x105 CFU) were hydrolysed with 125mM NaOH (selected as the lowest concentration found to result in complete hydrolysis at room temperature). DNA was extracted from the hydrolysed filters in parallel with samples processed as per the Williams *et al*. (2014) protocol.

Figure 2.3 demonstrates that there was a significant improvement in DNA yields where hydrolysing the filters with NaOH increased mycobacteria recovery rate by ~3.8 folds.
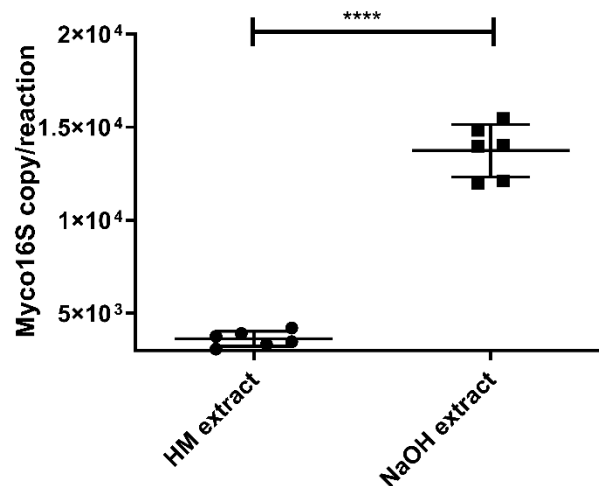
Figure 2.3 Comparison of the recovery rate of filter heating and filter hydrolysis with NaOH
HM= Heating method of Williams *et al*. (2014). DNA was extracted by boiling and quantified using the in-house MYCO*16S* SYBR-Green assay. Unpaired t-test was applied. Error Bars= SD, n= 6.

Hatagishi and co-workers (2014) treated the same commercial filters with collagenase at 37°C for a period of one hr to later extract viral nucleic acids. When this was compared to NaOH hydrolysis, it was noted that the latter produced slightly more repeatable results with a coefficient of variation of less than 20%. To support later protein biomarker studies (Chapters Three and Four) the digestion conditions were optimized (detailed in 2.2.6.3).

It was found that both gelatine hydrolysis with NaOH (125mM) and the optimized digestion with collagenase (50 µg/mL) resulted in a recovery rate of more than 75% of BCG inoculum ($\sim$9.7x10^2 CFU) (Figure 2.4).
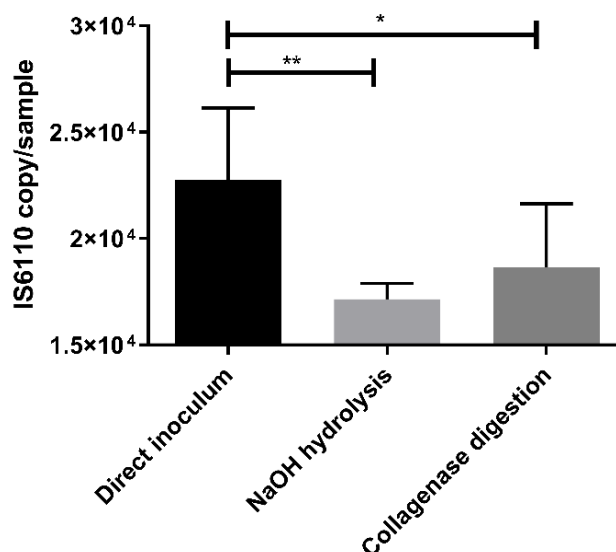
Figure 2.4 NaOH hydrolysis of gelatine and collagenase digestion of gelatine show good and comparable recovery rates
One-way ANOVA with multiple comparisons was applied. Error Bars= SD, n= 6.

It was interesting to note that there was no statistically significant difference between the yields of gelatine hydrolysis with NaOH and that of gelatine digestion, while within less than 0.5 log fold there was a statistically significant difference between each of these yields and that of the direct inoculum.

2.3.3. Standardizing and optimising DNA extraction protocol for isolating mycobacterial DNA

2.3.3.1. Combination of extraction protocols

Mycobacteria possess a tough, lipid-rich cell wall (Figure 2.16) and lysis is required for DNA extraction. While DNA isolation by sample boiling renders samples sterile and is simple, time- and cost-effective, it may not be optimally efficient (2.3.4).

After cell wall lysis is actioned, for example by bead-beating, the released elements include metal cations (i.e. Mg^{+2} and Ca^{+2}) that can mediate a degradative activity of nucleases cleaving the phosphodiester bonds between monomers of extracted nucleic acids.

Tris-EDTA was involved to solubilize and preserve the nucleic acids, where the Tris-base buffers the pH and EDTA chelates co-extracted magnesium cations.

Chelex 100, a styrene-divinylbenzene co-polymer containing iminodiacetic acid groups [$HN(CH_2CO_2H)_2$] another Mg^{+2} chelating agent, was found in this study combinable to Tris-EDTA.

Adding non-ionic, non-denaturing detergent, namely Nonidet P40 Substitute (NP-40 octylphenoxypolyethoxyethanol) and polyoxyethylene 20 sorbitan monolaurate (commercially known as Tween 20) was found combinable, with the aim of eliminating proteinaceous contaminants.

Figure 2.5 shows that this combination had significantly (p<0.0001) improved DNA yields from mycobacteria.
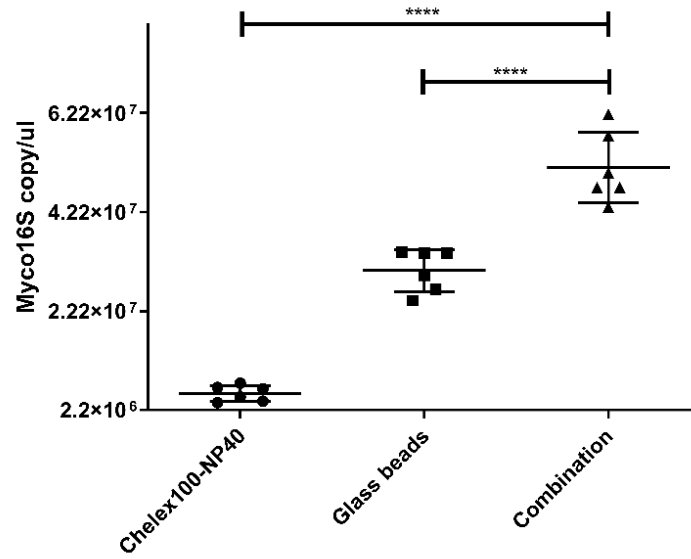
Figure 2.5 Combination of extraction protocols improved DNA yield
DNA was extracted from *M. smegmatis* broth culture (~2.7X10^7 CFU) using DNA extraction by Chelex100-Nonidet P40, DNA extraction by glass-beads and DNA extraction by combination (The optimized protocol). One-way ANOVA with multiple comparisons was applied. Error Bars= SD, n= 6.

2.3.3.2. Optimized bead beating

Three systems were assessed, and settings were adjusted according to the manufacturers' recommendations to achieve an equivalent speed and number of cycles. For example, 2700 RPM was considered equivalent to 4.5 m/sec.

It was found that using the Hybaid RiboLyser resulted in highest qPCR signals (Figure 2.6). Subsequent to this, the RiboLyser has been discontinued and has been replaced with a FastPrep-24 Classic Instrument (MP Biomedicals, UK); this was found to deliver comparable results (data not shown).
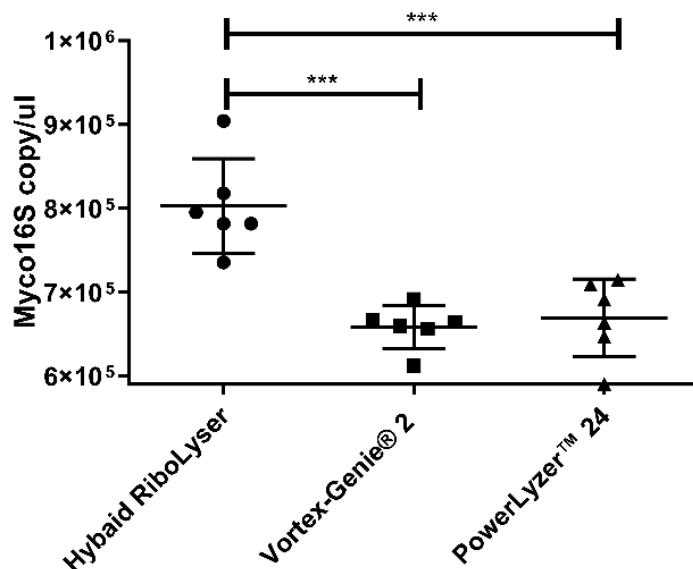
Figure 2.6 Sample homogenization with Hybaid RiboLyser resulted in highest qPCR signals
DNA was extracted from BCG broth culture (50μL, OD_{580}= 0.77) using glass-bead beating and quantified with Myco*16S* qPCR. The used systems: Vortex Adapters for Vortex-Genie 2 (MO BIO, QIAGEN), PowerLyzer 24 Bench Top Bead-Based Homogenizer (MO BIO, QIAGEN) and Hybaid RiboLyser (Hybaid, USA). One-way ANOVA with multiple comparisons was applied. Error Bars= SD, n= 6.

2.3.3.3. Comparison of commercial lysing matrices for bead–beating extraction

Twelve different types of glass beads from MP Biomedicals (USA) (Table 2.3) were evaluated since they were not tested before for DNA extraction from mycobacteria-contaminated gelatine filters.

Table 2.3 MP Biomedicals lysing matrices used	
Lysing matrix	**Composition**
A	Garnet matrix and a 0.25in ceramic sphere
B	0.1mm silica spheres
C	1mm silica spheres
D	1.4mm ceramic spheres
E	1.4mm ceramic spheres, 0.1mm silica spheres and a 4mm glass bead
F	1.6mm aluminium oxide particles and 1.6mm silicon carbide particles
G	1.6mm silicon carbide particles and 2mm glass beads
H	2mm glass beads and 2mm yellow zirconium oxide beads
I	2mm yellow zirconia beads and a 4mm black ceramic sphere
J	2mm yellow zirconia beads and 1.6mm aluminium oxide particles
K	0.8mm zirconium silicate beads
Y	0.5mm Yttria-Stabilized Zirconium Oxide beads

DNA of BCG-contaminated filters ($\sim$2x10^6 CFU) was extracted using the glass-bead protocol with changing only the lysing matrix.

Quantification showed 0.1mm silica spheres (lysing matrix B) superior to all other types of beads except 1.4mm ceramic spheres (lysing matrix D) (Figure 2.7).
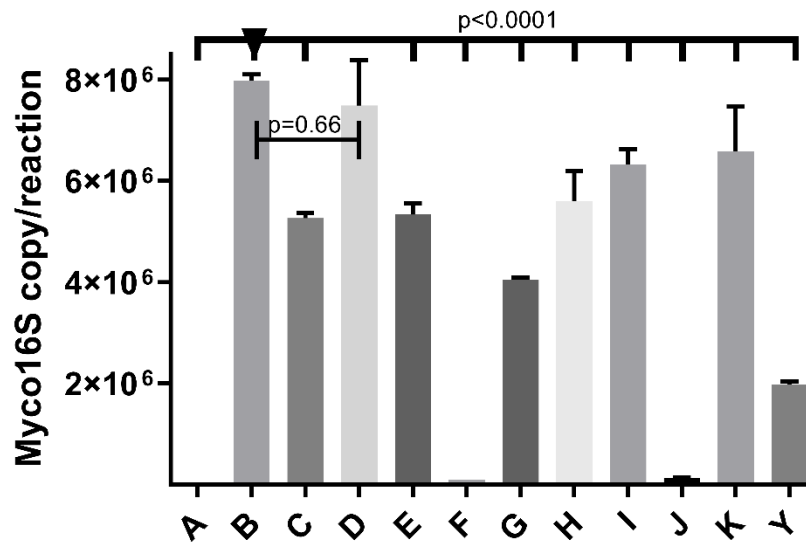
Figure 2.7 Lysis matrices B and D yielded highest qPCR signals
The letters on x axis indicate the brand name of compared lysis matrices. DNA was quantified with Myco*16S* qPCR. The results were normalized according to DNA volume. Lower limit of detection= 100 copy/reaction. One-way ANOVA with multiple comparisons was applied. Error Bars= SD, n= 6.

2.3.3.4. Optimizing the time of bead beating

Increasing the time of bead beating was assessed to investigate whether this could further improve the DNA yield from mycobacteria-contaminated gelatine filters.

Hybaid RiboLyser and Fast Prep-24 were applied to BCG samples ($\sim5x10^6$ CFU) with a total duration of 360, 240, 180 and 120 sec per the whole protocol, or 90, 60, 45 and 30 sec per run, respectively. These two most efficient homogenizers used in this study, offer for homogenization a time range option of 1–45 and 1–60 sec, respectively. For periods longer than 45 sec, multiple runs were applied.

Figure 2.8 shows that 45 sec gave satisfactory yields with a trend indicating that 30 sec was inferior.
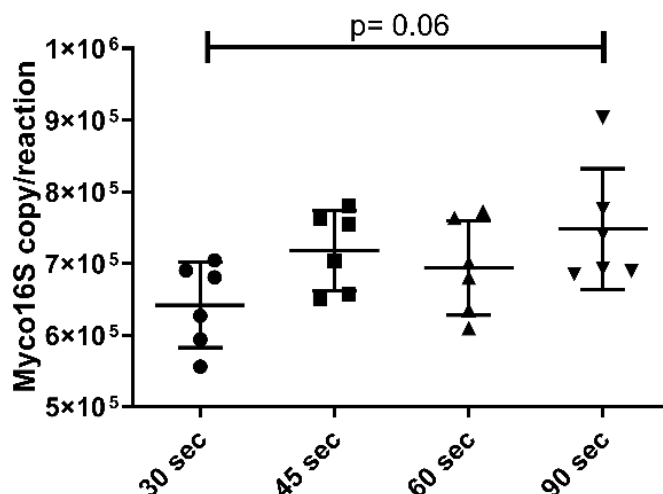
Figure 2.8 Four duration times of homogenization yielded comparable qPCR signals
DNA was quantified with Myco*16S* qPCR. One-way ANOVA with multiple comparisons was applied.
Error Bars= SD, n= 6.

2.3.3.5. Optimizing the ratios of Chelex, Nonidet and Tween 20 suspension

Different ratios of Chelex-100, Nonidet P40 Substitute, and Tween 20 were assessed.

Figure 2.9 shows that 50% w/v Chelex-100, 1% w/v Nonidet P40 Substitute, 1% w/v Tween 20 resulted in highest qPCR signals from BCG-contaminated gelatine filters (~4×10^7 CFU). Increasing or decreasing this ratio significantly reduced yield.
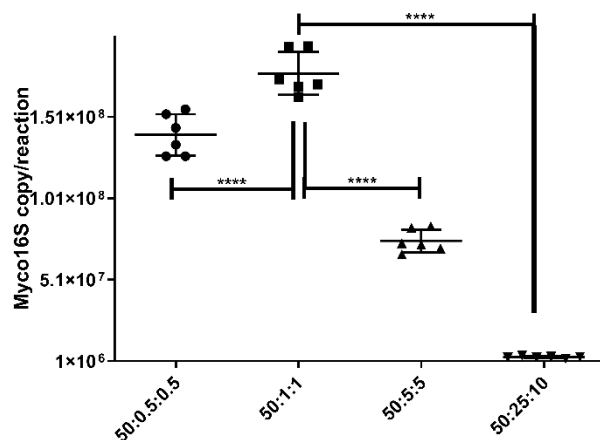
Figure 2.9 50% w/v Chelex-100, 1% w/v Nonidet P40 Substitute, 1% w/v Tween 20 yielded highest qPCR signals

The shown concentrations on x axis are % of w/v Chelex-100, w/v Nonidet P40 Substitute and w/v Tween 20, respectively. DNA was quantified with Myco*16S* qPCR and the results were normalized according to DNA volume. One-way ANOVA with multiple comparisons was applied. Error Bars= SD, n= 6.

2.3.3.6. Sample sterility

The Advisory Committee on Dangerous Pathogens has classified the biosafety level of H37Rv as a Category III dangerous biological agent which requires level-3 biocontainment precautions.

In fact, the glass-bead beating protocol described by Larsen (2000) involves, as many other DNA isolation methods, a boiling step for two main purposes: improving thermal cell lysis conditions and inactivation of virulent bacilli. While processing sample outside bio-containment level III suite requires sample to be free of live Mtb, this must be balanced against target degradation.

Three heating conditions were compared using otherwise exact DNA extraction steps. Figure 2.10 shows that heating the filter extracts at 95°C for 10 min can significantly reduce the DNA yield compared to extracts treated with either lower temperature degrees (80°C for 10 min) or with no exposure to a heating source.
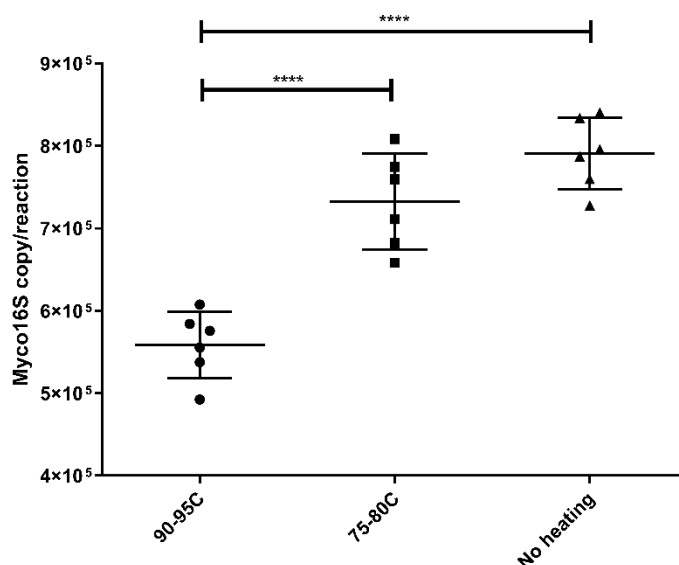
Figure 2.10 Pre-extraction DNA heating at higher than 80 degrees can compromise DNA
DNA was extracted from BCG-contaminated filters (50µL, OD$_{580}$:0.77) using glass-bead beating and quantified with Myco*16S* qPCR. One-way ANOVA with multiple comparisons was applied. Error Bars= SD, n= 6.

The usability of heating for inactivation of mycobacterial bacilli was further assessed on *M. bovis* and Mtb. It was found that 500µL *M. bovis* BCG ($\sim$3x10^7 CFU) were inactivated by incubation at a dry heat block set at 100°C for 10 min, resulting in $\log_{10}$ reduction of 8 (100%), whereas Mtb H37Rv (same inoculum) were not, showing a $\log_{10}$ reduction of 2 (99%).

Therefore, use of filtration post lysis was explored with Mtb. We hereby validate the safety of DNA extracted with the optimized protocol to be handled outside bio-containment level III suite.

Six samples (500µL each) of Mtb H37RV 7H9 broth culture (OD$_{580}$= 0.8) were extracted through the optimized DNA extraction protocol for Category III work (2.2.7.4). The supernatants (before microfiltration) were cultured on 7H10 agar plates alongside the flow-through DNA extracts and the original broth culture.   The plates were statistically incubated at 37°C and periodically examined until 8 weeks.

The plates showed 7.85x10^8 CFU, 4.47x10^1 CFU and no growth, for the broth culture, post-homogenization supernatants and DNA extracts, respectively. The 2.2.7.4 protocol provided a $\log_{10}$ reduction of 7 (99.99999%) after bead-beating and $\log_{10}$ reduction of 8 (100%) after microfiltration. Although microfiltration in this validation was applied once, it must be applied twice to avoid any potential filtration-related failure in sterilization. Vivaspin 500 0.2µm pore-size filters were designed for ultrafiltration concentration and did not undergo quality control for sterilization purposes. In addition, the validation was performed on solid media based on the requirements of the local safety manager, while liquid media could be more sensitive to a single organism.

Finally, serial 10-fold dilutions of BCG were processed through lysis and microfiltration to determine loss of target attributable to the latter step. Up to two-fold reduction in signal was found at high cell concentrations but with low input (~6,000 CFU) this was not significant (Figure 2.11).
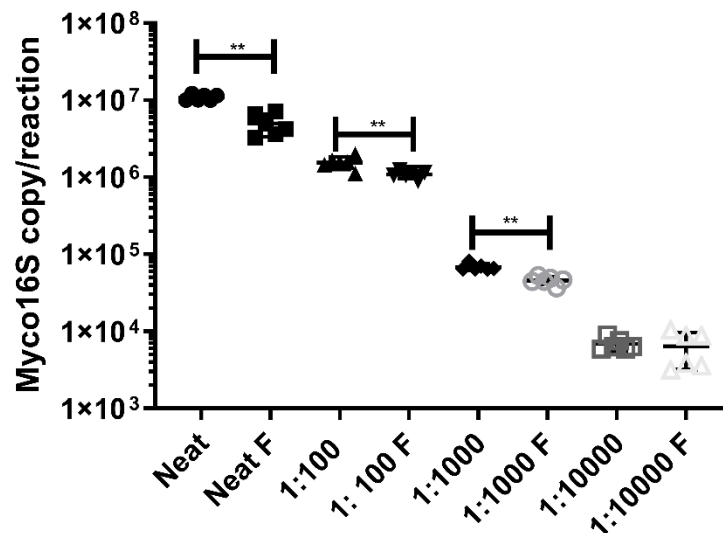
Figure 2.11 Microfiltration shows no significant impact on DNA yield of samples with lowest concentration

Neat is DNA extracted from BCG inoculum (~1.5x10^6 CFU). The neat was 10-fold diluted and DNA was extracted from dilutions without and with microfiltration (F). DNA was quantified by In-house 10µL MYCO*16S* RT-PCR SYBR-Green Assay. Paired t-test was applied. Error Bars= SD, n= 6.

2.3.4. Assessment and comparison of common genomic DNA isolation protocols and commercial kits for mycobacteria

Fifteen DNA extraction methods and commercial kits were compared for yield determined by qPCR. The compared protocols are listed in Table A.1 (note "A" designates Appendix).

Face-masks can be expected to be paucibacillary samples. Thus, 100µL of *M. bovis* BCG (OD$_{580}$=0.5-0.7) was used to contaminate the filters and DNA extraction was carried out by the described methods for in-house isolation and according to the manufacturers' instructions for the commercial kits. The extracts were quantified by the in-house MYCO*16S* qPCR SYBR-Green assay. A single threshold value was set for uniformity and comparability. The average quantitative readings were normalized per the volume of DNA yielded from the corresponding protocol. The in-house optimized protocol assigned to of 100%. The relative yields are displayed in Figure 2.12.
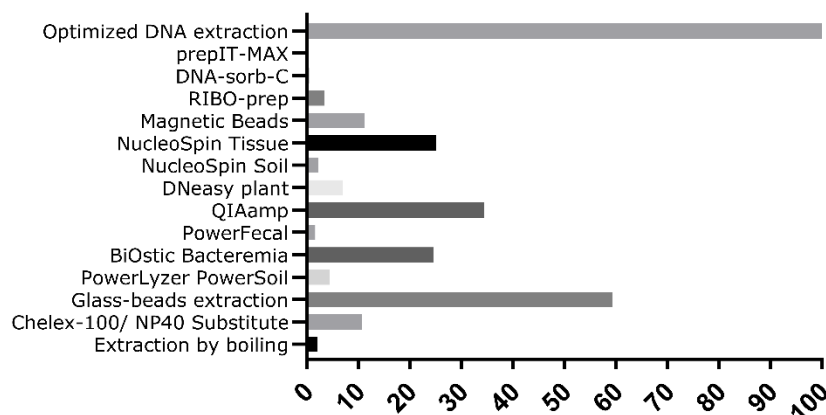
Figure 2.12 Comparison of yields quantified by myco*16S* of 15 extraction methods and kits
The calculated yields are displayed in relative percentage to the optimized protocol.

Overall, while the in-house optimized extraction protocol provided the highest yield, it was the third most time- and cost-effective (Table A.3) and produced relatively impure DNA outside the recommended ranges (A260/A280: 1.8–2.0 and A260/A230 ≥2; Table A.2) (Glasel, 1995; Sambrook and Russell, 2001).

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2.3.5. Comparison of three molecular assays for detection of MTC

The two most sensitive assays identified by Akkerman *et al*. (2013) for detecting Mtb in BAL were evaluated alongside an optimized SYBR-Green assay. The molecular target of the three assays was the *IS6110* element. These were:

I.   Multiplex AmpliSens real-time qPCR assay (MTC-FRT)

II.   In-house 10ul *IS6110* qPCR TaqMan assay (TaqMan)

III.   In-house 10µL *IS6110* qPCR SYBR-Green assay (SYBR-Green)

The number of *IS6110* copies varies between different genomes of MTC members (0-21 copy) as demonstrated in Table A.4. Mtb H37Rv genomic DNA (one genome has 16 *IS6110*-copies) was quantified to prepare standards as described in 2.2.8.3. Serial 10-fold dilutions (covering 10^6 to one genome equivalent) were prepared and analysed in technical triplicates per the three different assays on the Rotor-Gene 6000 platform.

The dynamic range efficiency and R^2 associated with the generated curve of each assay of the three assays are presented in Table 2.4.

Table 2.4 Performance of the three molecular assays

	R^2	Slope	Efficiency
TaqMan	0.99614	-3.210	1.05
MTC–FRT	0.99396	-3.179	1.06
SYBR-Green	0.98747	-3.933	0.80

The three assays were able to detect the lowest four concentrations input (100, 10, 5 and 1 genome copy per reaction or what was equivalent to 1600, 160, 80 and 16 *IS6110* copies per reaction). The obtained Ct values of these inputs are shown in Figure 2.13.
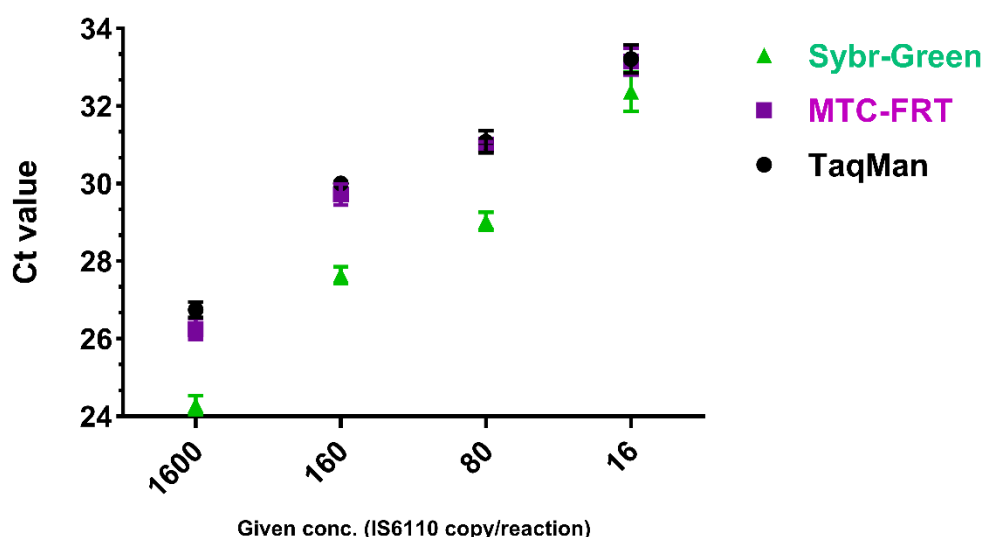
Figure 2.13 Lowest Ct values obtained from MTC-FRT, Sybr–Green and TaqMan assays
The values were calculated by Rotor-Gene 6000 Series software 1.7 at 0.0132 single threshold with $R^2 \geq 0.98$ and efficiency ≥ 0.80. (Error Bars= SD, n= 3)

In order to evaluate the precision parameter of each assay, the standard deviation (SD) and coefficient of variation (CV) of Ct values obtained from the technical triplicates were calculated. The results for the lowest concentration inputs are displayed in Table 2.5.

Table 2.5 Precision parameters of the three molecular assays

IS6110	TaqMan			MTC–FRT			SYBR-Green		
copy	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
1600	26.74	0.20	0.75	26.26	0.27	1.01	24.29	0.24	0.99
160	30.00	0.08	0.27	29.72	0.27	0.91	27.64	0.22	0.78
80	31.08	0.28	0.91	31.01	0.07	0.21	29.03	0.23	0.78
16	33.21	0.36	1.09	33.15	0.34	1.03	32.37	0.51	1.56

Overall, the three assays showed comparable but not identical performance, and all were able to detect one copy of H37RV genome with high repeatability.

2.3.6. Detection limit of the optimized face-mask processing protocol

Lower limit of detection is the lowest concentration which signal can be feasibly and reliably distinguished from the analytical noise (Armbruster and Pry, 2008).

For calculating the detection limit of the optimized processing protocol (comprising gelatine filter digestion, DNA extraction and qPCR detection) of mask samples, a set of gelatine filters (6 technical replicates) were contaminated with *M. bovis* BCG (OD_{580}=0.7) and quantified by microscopy (cytometer), CFU determination, in-house 10µL qPCR *IS6110* TaqMan assay and in-house 10µL *IS6110* qPCR SYBR-Green assay (Figure 2.14A). The inoculum was diluted to 1:100,000, 1:200,000 and 1:1,000,000, and 100µL of each was used to contaminate the filters. The filters were processed per the optimized protocol.

The optimized protocol detected 11 CFU and 260 cytometer counted bacilli per sample (110 CFU/mL and 2600 cell/mL, respectively) (Figure 2.14D). A further dilution of 1:2,000,000 was trialed but only culture was positive (40 CFU/mL).
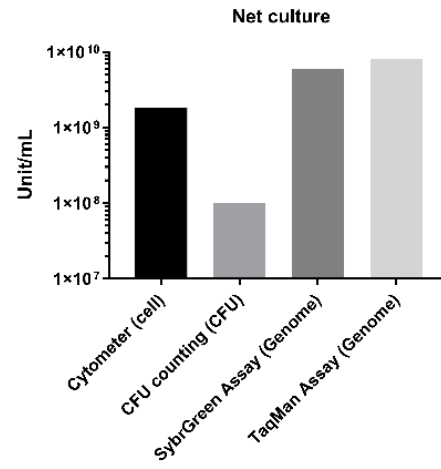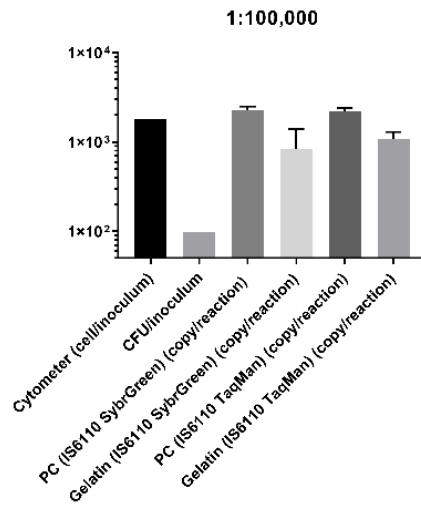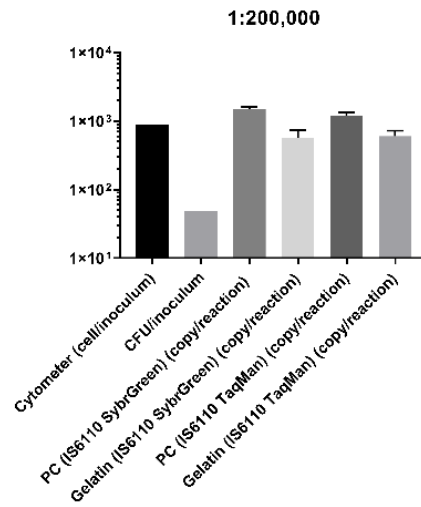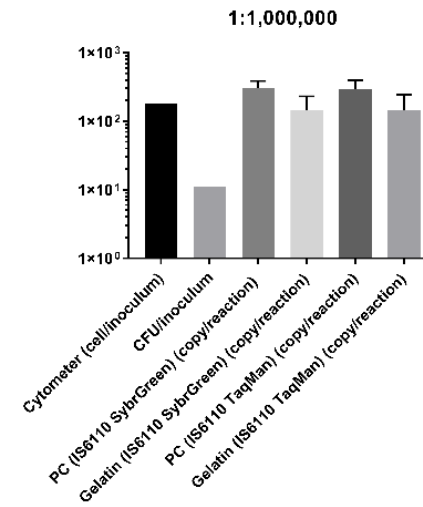
Figure 2.14 Detection limit of face-mask processing protocol
The positive control (PC) is the quantity of inoculum used for contaminating the face-mask samples. (Error Bars= SD, n= 6)

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Armbruster and Pry (2008) based on guideline EP17 of the Clinical and Laboratory Standards Institute calculated the limit of blank (LOB) and limit of detection (LOD) as follows:

$$\boldsymbol{LOB} = \ Mean_{blank} + 1.645 \ \times \ SD_{blank}$$

M. bovis BCG genome has only one copy of *IS6110* (Table A.4). Ct is defined as the intersection between an amplification curve and a threshold line. The blank filters were negative for *IS6110* (no sigmoid amplification curve) and their signals were not recognizable above noise (Figure 2.15), therefore the LOB was considered as 0 genome copy/reaction since unmeasurable noise signals are attributable to the reagents, and by definition "*a blank sample should never be positive in PCR*" (Kralik and Ricchi, 2017). Hence:

$LOD = LOB + 1.645 \ \times \ SD_{low\ concetration\ sample}$

$LOD = 0 + 1.645 \ \times 99.58$

$LOD = 163.81 \ genome\ copy.\ reaction^{-1}$

To meet the functional sensitivity criterion of a CV≤20%, the higher concentration (600.67 genome copy/reaction) equivalent to 49 CFU per filter (490 CFU/mL) achieved this and can be used to define the limit of quantification (LOQ).
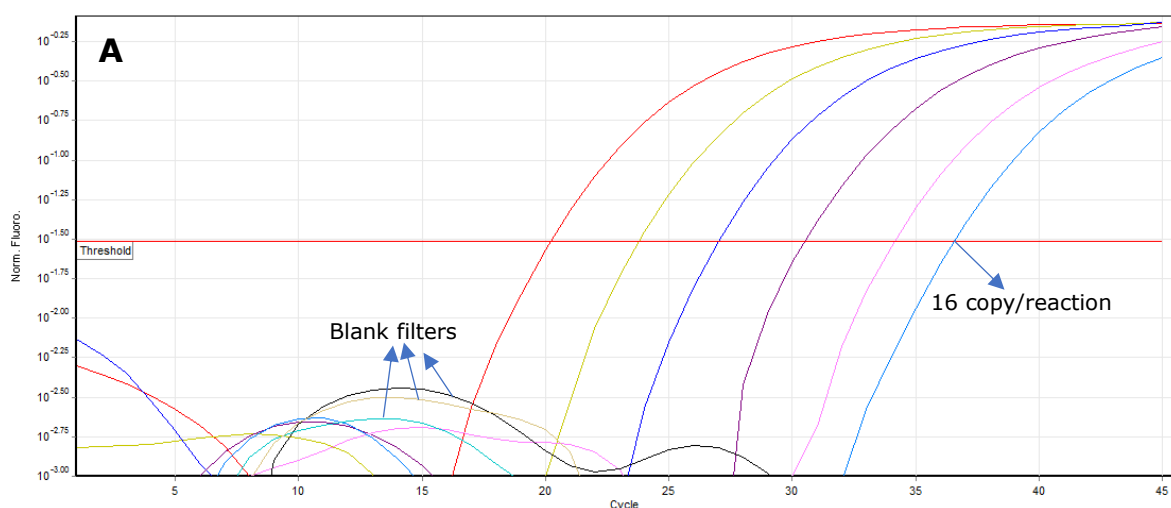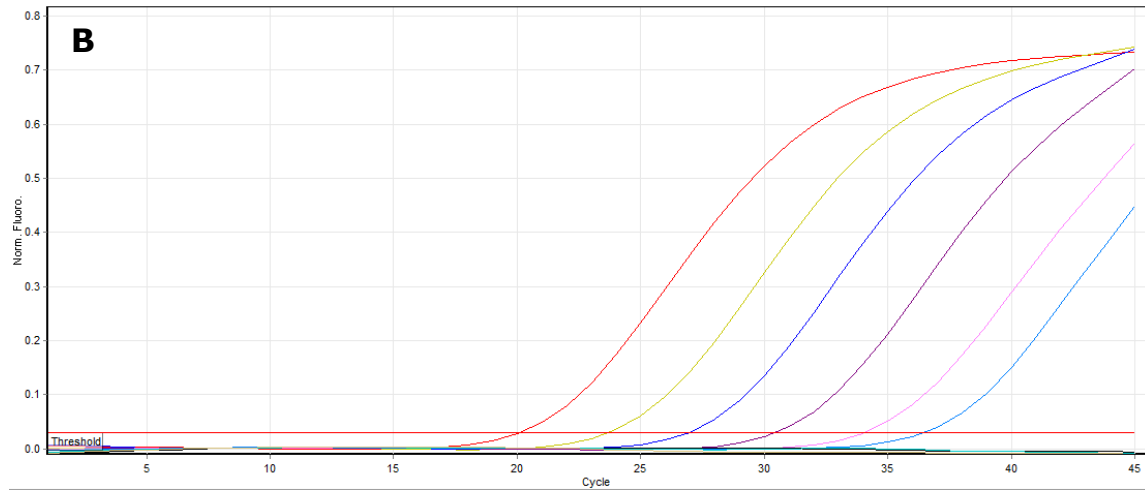
Figure 2.15 Amplification signals of blank filters
Blank filters were analysed with in-house *IS6110* TaqMan assay. The standard curves were obtained from using serially diluted H37Rv MTB DNA. (A) log scale; (B) linear scale

2.4. Discussion

2.4.1. Optimisation of the recovery rate of Williams *et al*. (2014)'s method was necessary

It was suggested that the processing protocol of mask samples can be developed to improve detection and quantitative aspects of collected Mtb aerosols. The first step was to investigate the recovery rate of the protocol established by Williams *et al*. (2014). A protocol by definition means the processing method of transforming the filter solid state to an analysable material. It was found that the recovery rate of that protocol was less than 1% of the inoculum, indicating a poor recovery of collected bacilli.

Williams and colleagues were unable to obtain positive masks in 35% of 20 patients with clinically-confirmed TB. TB has been known the archetypal example of airborne infections (Riley, 1974; Roy and Milton, 2004). Therefore, when such a sampling approach fails to detect Mtb aerosols from such a high percentage, the first possibility to be addressed is technical. While this false-negativity is attributable to several factors, one of the most important is the recovery rate of the material exhaled by these patients and captured on their masks. In a separate study where the optimised technique here was deployed, the detectability had been rocketed, revealing around 92% of 24 patients and 87% of 192 face-masks (Williams *et al*., 2018).

It is probable that almost all the captured material was lost in the supernatant which was not devised in the Williams *et al*. (2014) study. Indeed, the heating step itself is a method of DNA extraction. If those supernatants were kept, they were likely to be positive in a higher frequency. Unfortunately, this possibility could not be retrospectively tested.

One could argue that Williams's group (2014) were able to detect Mtb exhaled from the remaining 65% using the heating protocol. The ability to recover heated material might indicate a high bacterial load on the positive masks for

which the Williams study did not provide quantitative data and/or high sensitivity of the GeneXpert assay used in that study. The aim here, however, was not to assess the downstream assay itself.

Thus, the clinically-confirmed cases with negative masks might have involved producers of Mtb aerosols with low-quantities that were further poorly recovered, assuming the criteria of cultured mycobacteria hold for exhaled ones.

It could be argued that direct contamination of the mask with mycobacteria is different from Mtb aerosolization. In addition, the latter is not necessarily composed only of CFUs or of intact cells. This evaluative experiment, however, was carried out on a relatively low cell concentration comparable to an exhaled dose (Williams *et al*., 2018), and more importantly, it was designed to evaluate the recovery rate of captured mycobacteria and not the capturability itself.

2.4.2. Gelatine filter hydrolysis with alkali and digestion with enzymes

Gelatine as well other proteins can be hydrolysed mediated by heat, acids, alkali or enzymes.

Regarding the first, the reversible effect of hydrogen ions concentration on gelatine solubilization has been studied at least one century ago (Patten and Johnson, 1919). Adding water molecules can hydrolyse peptide bonds, facilitated by heating. Upon cooling and depending on the concentration of gelatine molecules, the heat-mediated hydrolysis can be reversed where the triple-helical structure is partially reformed (Yoshioka *et al*., 1998; van den Bosch and Gielens, 2003; Gómez-Guillén *et al*., 2002). In fact, gel consistency has been known to be directly proportional with the quantity of undegraded proteinaceous molecules (Bogue, 1920 cited in Croom, 1953). This reversibility and subsequent viscosity were probably contributed to the poor recovery of the heating protocol (95°C) used by Williams *et al*. (2014). Boiling occurs at 100°C, while the gelatine starts to denature at 40±1°C and to (irreversibly) degrade at 300±10°C with a secondary degradation stage at 420±10°C (Bozec and Odlyha, 2011; Hayashi and Oh, 1983).

NaOH is a routinely-used reagent since its first use described by Petroff (1915) for decontaminating clinical TB specimens, particularly sputa, when cultivated. NaOH biocidal activity is mediated through hydroxide free radicals after being dissociated from sodium cations to oxidize lipids, proteins and DNA. Different NaOH concentrations and exposure times have been evaluated by several studies (Satapathy *et al*., 2014; Chatterjee, 2013; Burdz *et al*., 2003) with the aim of achieving a balance between removing non-target microbes and preservation of mycobacteria, most crucial for paucibacillary samples. The concentration used here was not validated for decontamination of mask samples because cultivation was not intended. Timely dissolution at room temperature and preservation of the DNA target were the aim here.

NaOH hydrolyses protein, in general, producing the sodium salt of the carboxylic acid group and an amine. This first-order hydrolysis is irreversible since there are no free hydrogen cations to catalyse peptide bonds formation (Croome, 1953).

DNA might be exhaled either as free molecules or as an intracellular component releasable at alkaline pH after cell membranes destroyed (containing lipids and proteins). NaOH denatures DNA through breaking the hydrogen bonds between guanine and thymine by the ability of hydroxide electron charge to bind to protons of these bonds (Shin and Day, 1995; Wang *et al*., 2014).

Thus, use of minimum time and concentration, followed by neutralisation was required here. Rigaud *et al*. (1987) reported 5-10 times lower hybridization efficiency for DNA transferred in 400mM NaOH than the one transferred in 200mM NaOH with 1M ammonium acetate. Broad *et al*. (1988) showed that exposing DNA to 400mM NaOH before biding to Zeta probe membrane affects hybridization efficiency in a manner that is time- and concentration-dependent, recommending using low concentration (200mM) followed (after binding) by a brief exposure to 400mM NaOH, without providing an explanation. In fact, further to NaOH inhibition of PCR by denaturing DNA, fluorescence emission of molecules is dependent on pH and salt concentration of the mixture.

Thus, the lower concentration of 125mM NaOH applied gave good recovery that was roughly four-fold better than the established heating method (Williams *et al*., 2014). However, while NaOH can further minimize the impact of non-target DNA (PCR competitors; Kalle *et al*., 2014), NaOH is not selective.

Regarding the enzymes, denaturation occurs at levels of protein structure. Gelatine is commercially manufactured by partial hydrolysis of collagen of which at least 28 different types have been identified, and this destroys the

tertiary, secondary and, to a lesser extent, its primary structure (Schrieber and Gareis, 2007; Ricard-Blum, 2010; van den Bosch and Gielens, 2003). Collagen is composed of multiple repetitions of Gly-Pro-Hyp peptides building its triple-helical structure (Cremer *et al.*, 1998; Brinckmann, 2005). Clostridial collagenase, used in this study, can degrade the polypeptide chain at many sites through hydrolysing the c-terminal bonds (Nezafat *et al.*, 2015). This catalytic activity has been observed to be pH- and temperature-dependent, where the former appears to be proton-linked and the latter to optimize its kinetic energy in colliding the substrate (Fasciglione *et al.*, 2000). Hence the use of collagenase buffer at 37°C results in optimal digestion. In fact, the use of collagenase has the privilege of being substrate-specific, preventing exposing target proteins and other NaOH-susceptible molecules to extreme pH. One could argue that using mammalian collagenase is better to study exhaled bacteriome. However, this has a little capacity to cleave gelatine (McCroskery *et al.*, 1973) and as almost any other experiment a balance is required based on the target.

Finally, it was interesting to note statistically significant differences between apparently comparable quantities (Figure 2.4). Distinguishing between statistical significance and practical importance has been a long-standing subject of discussion (Kirk, 1996; Amrhein *et al.*, 2019). Not every statistically significant difference implies a practical importance and not every practical important finding necessitates posing a statistical significance for which the effect size is a key (Peeters, 2016). The former can be found even with very small differences when the sample size is large enough. However, while there are mathematical equations to regulate defining the former, the latter is solely based on experience. Accepting or rejecting a difference using this criterion could be a subject for the bias factor. Therefore, the most rigorous manner may be achieved by accepting an experience that is supported statistically.

Since PCR is a doubling process, a log fold difference equates to around 3.33 amplification cycle difference ($2^{3.33} \approx 10.06$). Such difference for a quantified target has been considered practically important (Dorak, 2006). This difference, however, was not shown in Figure 2.4. Thus, it cannot be ignored that there was a statistically significant difference, however such difference is not clinically important. One could argue that repeatable and accumulated statistically significant differences of less than one log fold can result in a practical importance. Probably therefore other authors consider a difference above 0.5 log clinically important (Gale, 2000; Jennings *et al*., 2012).

In summary, both NaOH and collagenase were considered satisfactory methods for gelatine hydrolysis giving comparable yields for mycobacteria-contaminated filters.

2.4.3. Standardizing and optimising DNA extraction protocol for isolating mycobacterial DNA

While the robust mycobacterial cell wall (Figure 2.16) offers an advantage of manipulating a sample driven from a microbial community, this robustness hinders cell lysis.
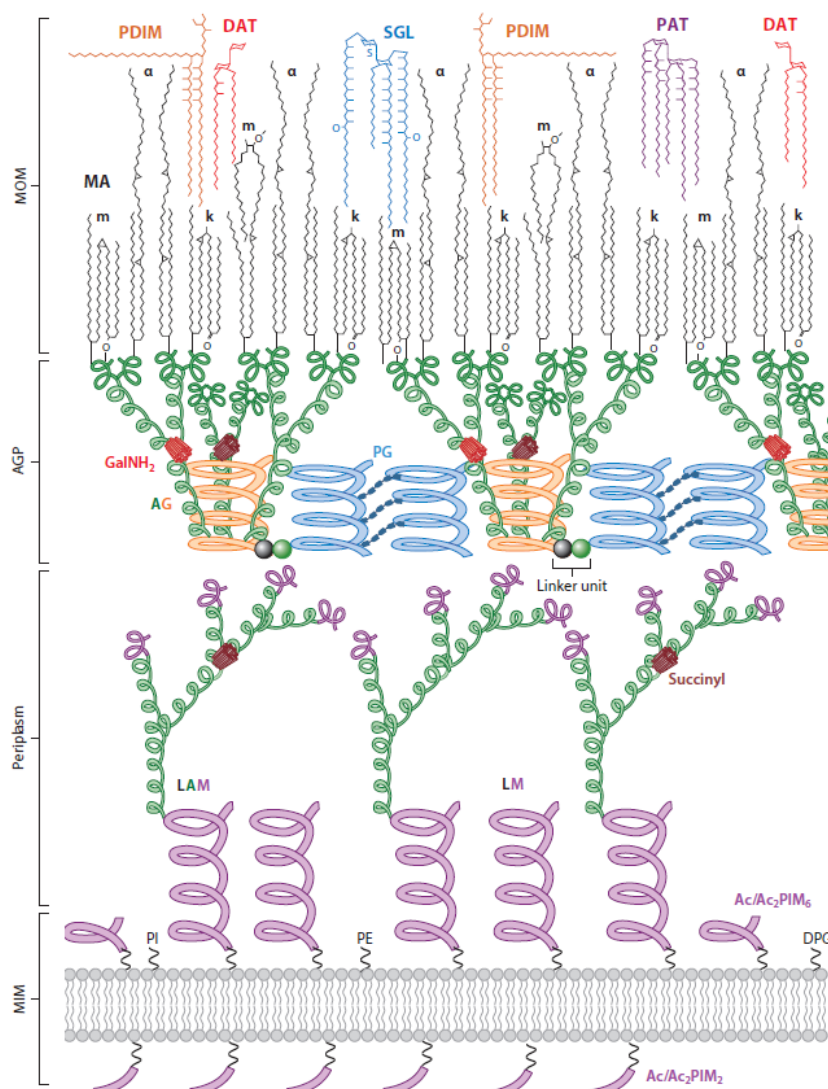
Figure 2.16 Schematic representation of mycobacterial cell wall
"*Abbreviations: Ac/Ac2PIM2, tri-/tetra-acylated phosphatidyl-myo-inositol-dimannoside; Ac/Ac2PIM6, tri-/tetra-acylated phosphatidyl-myo-inositol-hexamannoside; AG, arabinogalactan; AGP, arabinogalactanpeptidoglycan complex; DAT, diacyltrehalose; DPG, diphosphatidylglycerol; GalNH2, galactosamine residue; k, keto; LAM, lipoarabinomannan; LM, lipomannan; m, methoxy; MA, mycolic acids; MIM, mycobacterial inner membrane; MOM, mycobacterial outer membrane; PAT, polyacyltrehalose; PDIM, phthiocerol dimycocerosate; PE, phosphatidylethanolamine; PG, peptidoglycan; PI, phosphatidyl-myo-inositol; SGL, sulfoglycolipid*". This was reproduced from a drawing by Jankute et al. (2015) with permission from Annual Review of Microbiology.*

A series of experiments was carried out to improve the overall performance of the extraction while also attending to sterilisation of the extracts to maintain laboratory safety. The first was to combine two widely used in-house methods which combination has not been mentioned before (to the best of our knowledge). The main devised principle was in combing an efficacious mechanical disruption of mycobacterial cell wall composed mainly of lipids and carbohydrates (Figure 2.16) to chemical inhibition of co-released degradative enzymes. In addition to removing contaminating proteins and other potential PCR-inhibitors, the added detergents can break lipid-protein interactions, enhancing the lysis further.

Lysis matrices with large diameters are usually intended for grinding while smaller ones are used for homogenization. The finding here (Figure 2.7) that the smaller the diameter the bead, the more the efficient extraction is in agreement with the Kaser *et al*. (2009) finding that "*cell solubilization was best when using beads of small diameter*". Similarly, while there was a tendency of shortening the homogenization time below 30 sec to result in a decrease of DNA yield, the Kaser group found that "*harshest conditions result in highest yields*" referring to homogenization time and speed. Although this can result in highest DNA quantity, it might affect its quality through the applied shearing forces. Therefore, the applicability depends on the intended use. For this work, the protocol was successfully optimised for a downstream qPCR.

Laboratory acquisition of TB is a recognised hazard (Alonso-Echanove *et al*., 2001) with some special concerns relating to safe DNA extraction (Bemer-Melchior *et al*., 1998 cited in Bemer-Melchior and Drugeon, 1999; Doig *et al*., 2002; Somerville *et al*., 2005; Warren *et al*., 2006). Published advice is highly inconsistent (van Embden *et al*., 1993; Bemer-Melchior and Drugeon, 1999; Doig *et al*., 2002; Somerville *et al*., 2005 and Warren *et al*., 2006).

Mtb DNA integrity has been reported to be unaffected by boiling (100°C) for five min (Bemer-Melchior and Drugeon, 1999) or heating (80°C) for two hrs (Warren *et al*., 2006) while it can be sheared if the former was extended to 30 min (Zwadyk *et al*., 1994). These discrepancies indicate that a sterilization method should be locally validated.

Accordingly, following demonstration that exposure to lethal heating resulted in significant signal loss, use of filtration was examined. Critically this was found not to affect extracts from low cell numbers and therefore may not affect assay sensitivity.

2.4.4. Assessment and comparison of common genomic DNA isolation protocols and commercial kits for mycobacteria

After optimising the protocol, comparing it to different extraction methods was suggested to assess its yield and fieldwork suitability.

Compared to 15 DNA extraction methods, the in-house optimized protocol has provided the highest yield determined by qPCR, been found the third most time- and cost-effective and produced relatively impure DNA outside the recommended ranges (Glasel, 1995; Sambrook and Russell, 2001).

A universally standardised DNA extraction protocol for mycobacteria is currently unavailable. The widely used phenol-chloroform/ethanol method in research laboratories is time-consuming (2 days) and does not suit clinical settings.

In fact, several studies with different types of samples have evaluated different protocols, however this has not been done so for on mycobacteria-contaminated filters. The yield can vary based on the sample type and mycobacterial load. Furthermore, Amaro *et al*., (2008) showed a degree of difference based on mycobacterial species, even between Mtb and *M. bovis*.

Efficient mycobacterial DNA extraction is a challenge for paucibacillary samples (Ruiz-Fuentes *et al*., 2015; Thakur *et al*., 2011). Therefore, this parameter should be determined experimentally. For example, protocol (15) was commercially claimed more efficient than bead-beating. However, this was not proven upon testing and the employed kit was ultimately discontinued. Similarly, Kaevska and Slana (2015) recommended using MoBio kits in extracting mycobacterial DNA from water samples for retrieving high DNA yields. The sample type is different; however, both are paucibacillary. Moreover, Macovei *et al*. (2015) used these kits for MiSeq Illumina sequencing to reveal that the URT harbours NTM in health. These kits, however, were not found more efficient than the developed protocol.

By contrast to Pan *et al*. (2013)'s findings showing Mtb DNA extraction by magnetic beads is more efficient than by using Qiagen kits, the latter were found here again more efficient than the former.

In fact, some manufacturers instruct to transfer a certain volume from a step to another. This is probably to meet the instrument capacity ensuring, for example, proper DNA binding to silica, and to avoid saturating the given amounts of reagents. However, such transfer results in a material loss. Furthermore, repeating a transfer step is not clinically feasible, and even if it was, it would ultimately result in a further material loss. DNA can bind to internal surfaces of tubes and pipette-tips (polypropylene).

Bead-beating has been known efficient for extracting mycobacterial DNA (Zhang and Ishaque, 1997; Odumeru *et al*., 2001; Corti and Stephan, 2002, Tell *et al*., 2003). Some suggested that this could be substituted by a combination between heating and sonication (Aslanzadeh *et al*., 1998). A shock treatment like a freezing followed by heating has been recommended by some studies (Aldous *et al*., 2005). In addition to its role in minimizing heat-related degradation, the ice-incubation step of over-heated samples by mechanical disruption might resemble to some extent such treatment.

Molecular assays such as PCRs are highly dependent on DNA extraction method (Nakatani *et al*., 2004; Nagdev *et al*., 2010). The optimised protocol, however, should not be claimed suitable for all molecular-based applications. This protocol did not result in highest purity required, for example, in sequencing studies. Even for qPCR-based applications, some suggested that successful DNA detection depends on its quality (Buck *et al*., 1992; Thakur *et al*., 2011) where impure DNA has been found to result in a higher limit of detection (Aldous *et al*., 2005). Others like Radomski *et al*. (2013) found that merely high purity was not sufficient to achieve accurate quantification for mycobacterial samples harbouring significant amount of host DNA. Indeed,

Radomski's group reported that qPCR results were only reliable when the quantity of non-target DNA was low, and the purity was high.

Nucleic acids have absorbance wavelength at 260nm. The obtained poor ratio of A260/A280 might indicate presence of proteins, particularly aromatic amino acids, and other contaminants absorbing near 280nm. Furthermore, Alkaline pH can over-represent this ratio (Wilfinger *et al*., 1997). On the other hand, the obtained poor ratio of A260/A230 might be due to EDTA or other molecules like carbohydrates absorbing near 230nm. Apart from the instrument itself where the wavelength accuracy is essential, the nucleic acid composition can also affect the overall ratio as each nucleotide has its own absorbance wavelength.

This limitation can be circumvented by adding a purification or dilution step. Some recommend using silica technology for purification since DNA can selectively bind to silica membrane at high salt concentrations. However, this can result in material loss, particularly if the input was scarce, and subsequently may undermine assay sensitivity.

DNA quality was only evaluated through estimating its purity. Other approaches include gel electrophoresis to estimate its integrity.

The challenge lies in finding a balance between extraction efficiency (yield), clinical feasibility, time- and cost-effectiveness and suitability for the downstream application. The optimised protocol has been shown compared to 15 methods the most yieldful for qPCR and the third time- and cost-effective.

In fact, this protocol is strived in making use of DNA material with minimal steps. The simplicity presented by using one tube throughout the process can, alongside being time- and cost-effective, minimize DNA loss due to multistage transfers in both manual and automated protocols.

One could argue that an automated platform is more feasible (due to the automation nature) and appropriate (due to its "gold-standard" reproducibility in clinical settings). However, the efficiency parameter is more important in reducing false negativity introduced, for example, by limited manipulability on input material. In addition, such infrastructure is not always an available option, particularly in low-resource settings. Even in high-resource ones, the yield of two automated methods (QIAsymphony DNA Kit and NucliSens easyMAG) were compared, independently to this study, to the optimised protocol and the latest proved its superiority (C Williams, personal communication).

The optimized protocol is not exclusive to mycobacteria and could be applied for other cells given the personalised requirements are met. For instance, while the shear forces are efficient for rigidly-walled cells (Amaro *et al*., 2008; Macovei *et al*., 2015), they can shear DNA of thinly-membraned ones (de Lipthay *et al*., 2004).

Finally, and most interestingly, each step of this series showed a statistically significant difference that may not be interpretable as practically important i.e. differences less than one log fold. However, the accumulated improvements had resulted in a practical significant finding (Figure 2.12).

2.4.5. Comparison of three molecular assays for detection of MTC

The two most sensitive TaqMan-based assays highlighted by Akkerman *et al*. (2013) and a SYBR-Green-based assay (all targeting *IS6110*) were compared and their performance evaluated on the available qPCR platform. The comparison was made over a range of template dilutions since a single-point comparison can lead to a misleading conclusion.

Pure H37Rv genomic DNA was used rather than DNA extracted from gelatine filters because the objective was to compare the net analytical sensitivity of these assays rather the clinical sensitivity of the mask system.

The SYBR-Green assay showed an earlier Ct than the TaqMan assays, but this did not reflect a better performance. In general, Ct values from reactions run with different reagents cannot be directly compared to make an effective judgment on reagents performance. One reason is that artifacts from reaction mixture can result in template-independent changes. The shown difference was less than 3.33 Ct and the three assays can be deemed comparable.

Indeed, the data of dynamic range and R^2 value (Table 2.4) indicate that the performance of the three assays are satisfactory. These values are a subject of pipetting errors. However, these errors should have had similar effects across all assays, unless one claims the SYBR-Green assay is more sensitive to such errors. It is unclear why MTC-FRT, TaqMan-based chemistry, resulted in marginally (insignificant) lower performance than the in-house TaqMan assay. This might be related to the master mix composition (e.g. salts, pH, reference dye) which was not fully disclosed.

The intertest variability of the three assays at the highest used dilutions was within 2 standard deviations, indicating their precision was high, repeatable and comparable.

The three assays were able to detect the lowest tested concentration equivalent to one H37RV genome or 16 copies of *IS6110* element (Figure 2.13), indicating the three assays sensitivity to this low concentration. This is in agreement with the Akkerman's findings for the two TaqMan-based assays, indicating possible inter-laboratories reproducibility (a minimum six laboratories are required to confirm this; Kralik and Ricchi, 2017) despite the used thermal cyclers were different (Rotor-Gene-6000 vs Roche LC480) with cycling conditions adjusted for each.

A further dilution (equivalent to 4 *IS6110* copies) was trialed. This was detectable only by the TaqMan assay (Ct=34.03) but its inclusion affected the dynamic range (efficiency=1.27; slope= -2.81) and the linearity ($R^2=0.97$) therefore was excluded. The signal resulted from this input on the SYBR-Green assay was representing a non-specific product (primer-dimer) per the melting curve analysis (Figure 2.17). Theoretically and according to the Poisson distribution, the limit of detection of a qPCR cannot be <3 copies of the molecular target (Kralik and Ricchi, 2017).
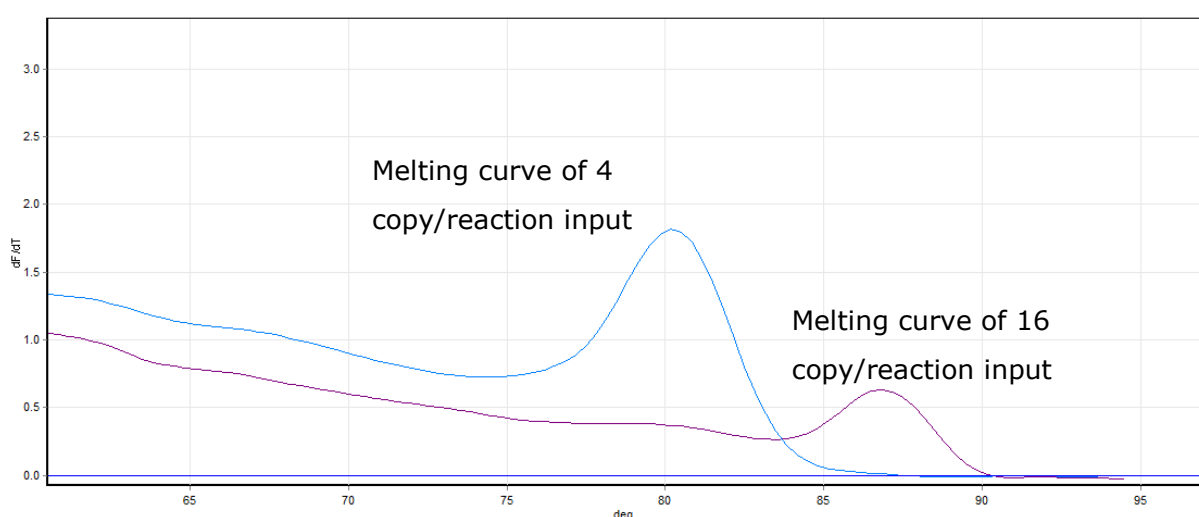
Figure 2.17 Melting curve analysis of SYBR-Green assay for lowest input concentration

Akkerman and colleagues did not explain why different assays resulted in different analytical sensitivities or why the same assay showed different sensitivities with strains containing different numbers of *IS6110*. Their only comment was "*Only the MP MTB assay yielded unexpected results. This kit had an analytical sensitivity with the dilution of the one-copy IS6110 strain, which was better compared to that of the five-copy IS6110 strain*". It is plausible that strains with higher copy numbers resulted in higher sensitivity and lower limit of detection in the Akkerman study. However, it seems that a merely presence of a template does not suffice for its detection by qPCR (an example shown in 4.3.5). In addition to absence of inhibitors, non-specific templates can compete for reaction compounds (Kalle *et al.*, 2014).

Akkerman's group found that molecular assays with lower DNA template (5µL) showed higher detection limit than those with higher DNA input (10µL). While using different DNA extraction protocols could be a reasonable explanation, increasing DNA volume per reaction has both advantages and disadvantages. The increase can result in lowering the detection limit, particularly for non-abundant targets and less homogenous extracts, and in minimizing pipetting errors and related variabilities. However, it can potentially increase the load of PCR contaminants, particularly for impure extracts, which could include inhibitors. In addition, such an increase consumes larger amounts of the extract itself, which is undesirable if small amounts are available or requirable in future, as well as consumes larger amounts of reagents. It might be worth mentioning that a Multiplex AmpliSens reaction costs more than five times of what TaqMan and SYBR-Green ones do.

2.4.6. Detection limit of the optimized face-mask processing protocol

A comprehensive series of experiments had been carried out with the purpose of optimizing a protocol for extracting mycobacterial DNA from simulated mask samples.

Several terms have been used to describe how far one could go with the reliability of a measurand. The aim lies in defining capability, limitations and fit for purpose. The terminology includes sensitivity, analytical sensitivity, functional sensitivity, limit of detection, instrument detection limit, method detection limit, limit of blank, limit of quantification and practical quantification limit. It is not uncommon for one or more terms to be interchangeably used while they should not. What complicates the situation further is that there is no one method for calculation. Available ways include visual evaluation-based approach, signal-to-noise approach and the approach of standard deviation of the response and the slope. The traditional approach in estimating LOD requires a larger sample size of replicates from the blank for determining their mean and SD values and calculating the LOD as the mean +2 SD (Armbruster and Pry, 2008). The large sample size is intended to robust the statistical confidence of this estimated parameter. However, Armbruster and Pry acknowledge that "*clinical laboratories can validate these parameters using a smaller number of samples* and *likely will use only one analyser* and *one lot of reagents*". A requirement of six replicates has been deemed a minimal number (Kralik and Ricchi, 2017).

Different quantification methods were applied for the used inoculum. A microscope-assisted one can detect cells whether they were viable or dead. Cultivation methods can detect viable cells that are further able to form colonies. PCR methods can detect DNA whether it was free or extracted from a bacillus. It is not necessarily that this bacillus was intact, and if it was, it is not necessarily it was viable, and if it was, it is not necessarily it was colony-

forming or infectious. Therefore, the applied methods reflected these, resulting in different quantities of the same inoculum (Figure 2.14A).

After optimizing gelatine filter treatment, DNA extraction and molecular-based quantification, the face-mask processing protocol shows a capability to detect less than 165 mycobacterial genomes or 11 colony-forming units equivalent. Once again, one could argue the filter was directly contaminated rather than through aerosolization. Whether or not this can pose a difference, it is likely then to account for sampling, not processing. This experiment was addressing the detection limit of material recovery rather than that of capturability.

Mycobacterial cultivation, for which the method was not optimized, was more sensitive to a more diluted inoculum. However, not all of materials exhaled by TB patients are culturable (Fennelly *et al*., 2012). The described method was validated *in vitro* for downstream qPCRs. Since the exhaled material is expected to include culturable and unculturable material, this protocol should be more retrieving than a culture-based technique, given the mask captures what is exhalable. Technically, the detection rate of the optimised protocol has been comparable to that of RASC, and thus the former can eschew the need for the latter's extensive infrastructure in terms of microbial detection (Figure 1.3). It should be investigated, however, whether captured materials potentially contain PCR inhibitors undermining the detection efficiency of the developed protocol. A more accurate picture can be obtained when the protocol is validated on materials collected from TB patients, and this has been proven successful (Williams *et al*., 2018).

2.5. Concluding remarks

- Three main variables of processing mycobacteria-contaminated face-masks have been identified and optimised. These were 1) solubilization of the capturing surface 2) DNA extraction 3) DNA analysis

- Gelatine filter solubilization can be achieved either with NaOH hydrolysis or collagenase digestion. Choosing a solubilization method, however, depends on the question being asked. For preservation of NaOH-susceptible particles, treatment with collagenase is suggested

- Mycobacterial DNA extraction from gelatine filters can be achieved with the in-house optimised extraction protocol which combines bead-beating and chemical lysis. Choosing an appropriate DNA extraction method, however, depends on the question being asked. The optimised protocol was the most efficient among 15 protocols compared for qPCR detection and quantification of mycobacterial DNA from gelatine filters

- Mycobacterial DNA detection and quantification from gelatine filters can be achieved with TaqMan based assays i.e. Multiplex AmpliSens real-time qPCR assay and in-house 10ul *IS6110* qPCR TaqMan assay. However, the latter was less expensive

- The developed processing protocol showed a capability to recover from the mask insert and detect what was equivalent to 11 CFU (~164 genome copy/reaction), provided this was captured, and to quantify what was equivalent to 49 CFU (~601 genome copy/reaction)

- The developed aspects are aligned with recovery of gelatine-captured mycobacteria rather than with capturability

- High temperatures posed a detrimental impact on the face-mask yield

- The mask sensitivity has been significantly improved, yet this remains to be confirmed in a formal diagnostic study

- Further work should investigate whether the ability of the mask to capture can be improved in terms of the mask design and other technical variables such as the mask type and the capturing surface

Chapter Three: A healthy volunteer study: novel insights into face-mask sampling

3.1. Introduction

After reporting the technical development of processing face-mask samples and on basis of the evidence-based ability of the mask to detect Mtb exhaled by TB patients (Cheah, 2010; Williams *et al.*, 2013 and 2014), we suggested that the material captured on the face-mask consists of aerosols and its composition might differ between various respiratory activities.

Aerosol, when used in this work to refer to exhaled air particles, is intended to include both aerosols and droplets since the mask system was not designed to differentiate between them.

3.1.1. Background on biological content of aerosols

Many respiratory infections are well-known to be acquired and transmitted via the airborne route. In epidemiology literature and broader, this type of spread is mapped to respiratory efforts capable to generate aerosols carrying the aetiological agent. Typical examples of such efforts are breathing, talking, coughing and sneezing. Little is known, however, about how the generation and composition of the air particles differ between these activities, particularly in terms of their biological content.

The importance of volatile organic compounds of breath in providing diagnostic clues has been known since the Hippocratic age. However, the objective breath analysis was initiated in the late 17th century with the work of Lavoisier and Laplace who were the first analysers of gaseous components in exhaled air. Detection of other volatile compounds was introduced with colorimetric assays employed by Nebelthau in the mid-18th century for demonstrating acetone excretion in diabetics (Phillips, 1992) followed by Anstie's work in 1874 by isolating ethanol from breath.

In the late-18th century, the work of Koch (Koch, 1883 cited in Wells *et al*., 1948) showing animals developing TB-like disease after inhalation of sprayed bacilli then of Flugge (1897 and 1899) describing sprays of small droplets during different respiratory activities dropped the attention to airborne microorganisms, the starting point for investigations of non-volatile exhaled particles.

Different techniques and tools have been employed to study these exhaled particles. Boston (1901), as introduced earlier, studied exhaled output on microscope slides in TB. Winslow and Robinson (1910) applied artificially-infected mouth experiments to investigate some quantitative aspects of aerosolization by studying "*the extent of the bacterial pollution of the atmosphere*". Bourdillon *et al*. (1941) developed a slit sampler for collecting and counting exhaled bacteria, with a recovery rate of sampling smallest bacteria-carrying particles reported at >94%. Duguid (1945) re-applied artificially-infected mouth, nose and throat experiments under different breathing patterns to investigate the direct origin of exhaled particles; however, the used technical principles limited interpretation of the findings to particles generated in the URT. These particle counts were significantly higher than the estimated colony counts, suggesting that small particles which had a significant contribution did not harbour CFUs.

Other tools for sampling exhaled Mtb were developed by Anderson (Anderson impactor; 1958), Loudon and Roberts (Cough box; 1967 and 1968) and Marple *et al*. (MOUDI; 1981 and 1991); and these were detailed earlier.

Exhaled breath condensate (EBC) was a technique developed by Sidorenko *et al*. (1980) who devised the condensation nucleus counter of Landsberg (1938) based on Aitken's dust counter (1923). Technically, EBC is based on cooling

exhaled breath where the biological content is mainly composed of water vapor with a small fraction of volatile and non-volatile macromolecules. The EBC was devised to detect inflammatory biomarkers and exhaled metaboliome (Peralbo-Molina *et al*., 2017; Konstantinidi *et al*., 2015's review; Bannier *et al*., 2019's review), exhaled salivary proteins (Griese *et al*., 2002) and exhaled microbes (Jain *et al*., 2007; Kawada *et al*., 2008; Zakharkina *et al*., 2011; Xu *et al*., 2012; May *et al*., 2015; Zheng *et al*., 2018).

Scheideler *et al*. (1993) detected exhaled non-volatile particles in the form of inflammatory proteins, such as interleukin-113, soluble interleukin-2 receptor protein, light chain (sIL-2R), interleukin-6, and tumor necrosis factor-α, in breath condensates hypothesized to be generated from the extracellular respiratory tract lining fluid (RTLF), partially from the nasooropharyngeal tract and partially from the LRT.

After that, Papineni and Rosenthal (1997) studied using optical particle counter and electron microscope the size distribution of exhaled particles with different breathing patterns (oral breathing, nasal breathing, coughing and talking). Papineni and Rosenthal found that coughing was productive of the largest droplet concentration while nasal breathing the least. More interestingly, X-ray dispersive analysis of a particle residue showed contents of potassium, calcium and chloride, which supported an RTLF origin of these particles.

With the beginning of 20th century and after Mastorides *et al*. (1999) detected DNA of Mtb-carrying aerosols in the room air of isolated TB patients, Fennelly and colleagues (2004) devised the Anderson sampler to develop the CASS tool for quantifying culturable Mtb exhaled by TB patients, as detailed earlier.

Edwards *et al*. (2004) showed that isotonic saline in nebulized aerosols administered to human participants had diminished particle emission in high producers of exhaled particles while marginally increased emission for low producers. On the other hand, administration of surfactant simulant consisting of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) or 1-palmitoyl-2-oleoylsn-glycero-3 phosphoglycerol increased the emission by around five times.

Watanabe *et al*. (2007) tried to answer why inhaling saline can change the exhaled biomaterial, using a calf lung-model with a cough machine. They found that the charge-mediated gelation of mucin or mucin-like macromolecules near the RTLF-mimetic surface was reversible, altering the physical properties of this surface and reducing its breakup.

In fact, studies concerned with respiratory efforts usually compare the particle size rather the particle biological content. For instance, Xie *et al*. (2009) concluded no significant difference in the size distributions of droplets produced during coughing and during talking, assuming the residence time of aerosol settlement on a sampling surface equals to the free fall time at their experimental conditions for all their captured droplets. Similarly, Morawska *et al*. (2009) and Chao *et al*. (2009) compared exhaled particle concentrations and droplet size distributions between different breathing patterns.

However, few other studies tried to investigate some aspects of the biological content of the exhaled particles. Almstrand and co-workers (2009) observed an increased ratio of surfactant protein to phospholipid in forced exhalations produced by asthmatics and cystic fibrosis (CF) patients compared to healthy individuals. In 2012, Almstrand's group found the ratio of unsaturated to saturated phospholipids was lower in exhaled particles collected by their

design, later called particles of exhaled air (PExA or PEx) technique, from asthmatics than those collected from healthy controls. In addition, they found that molecular ions from phosphatidylcholine and phosphatidylglycerol, and protein fragments discriminated between the asthmatics and the healthy groups.

Larsson *et al*. (2012) validated the suitability of PEx to detect surfactant protein A (SP-A) and albumin as two pretentious components of the RTLF, while the albumin content was not detectable in paired EBC samples. They showed that both SP-A and albumin were correlated to the PEx mass.

Bredberg *et al*. (2012) conducted the first proteomic analysis on PEx composition of RTLF proteins. Due to the small amounts of their analysed material (1−1.5µg), they were able to identify only the most abundant proteins comparable with published proteomic studies on BALs. Bredberg and colleagues interpreted their results as supporting to the hypothesis that PEx are formed in the LRT, by identifying typical proteins of pneumocytes type II and Clara cells (SP-A, SP-B, SP-C and CC16), and by matching more than 80% of identified proteins in PEx to those previously detected in BAL. They were able to characterize 124 proteins in PEx. Similarly, Östling *et al*. (2017) after that explored 207 different proteins exhaled from asthmatics with different lung-clearance indices and from healthy controls. Östling and colleagues found differentially abundant proteins in PEx, with a potential to sub-phenotyping asthma pathobiologically.

Bredberg *et al*. (2013) found that smokers yielded a larger extent of protonated and sodiated phospholipids than non-smokers in their PEx samples. Larstad and colleagues (2015) showed that SP-A mass content of exhaled particles was lower in patients with chronic obstructive pulmonary

diseases (COPD), the smokers' lung disease, than in healthy subjects, while the albumin content was similar in both groups.

Biologically, SP-A is produced by pneumocytes type II (Figure 3.1) while albumin is synthesized by hepatocytes then hematologically distributed as one the main serum proteins.
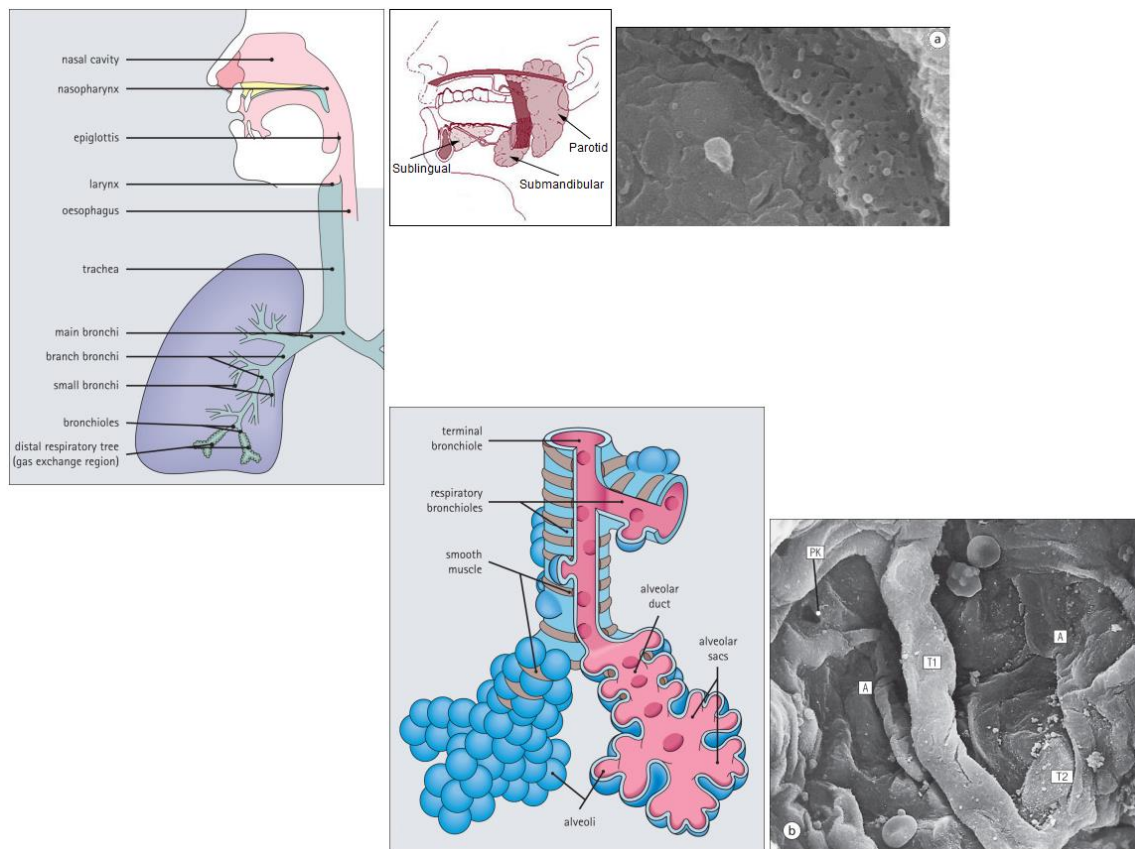
Figure 3.1 Human Respiratory Tract
(a) A scanning electron micrograph looking into the glandular epithelium of a Parotid gland. The acinar cells of the parotid and other salivary glands secret α-amylase. Humans have three paired major salivary glands (parotid, submandibular, and sublingual), as well as hundreds of minor salivary glands. (b) A scanning electron micrograph of an alveolar sac looking into two of the alveoli (A) that open into it. The two alveoli are separated by a wall, which is covered by pneumocyte-type I cytoplasm (T1). On the left is a pore of Kohn (PK), and a round pneumocytes-type II (T2 which produce SP-A) can be seen in the right alveolus. This was reproduced from Lowe *et al*. (2019) with the permission of Elsevier

Albumin was thought to be a marker of increased alveolar capillary permeability. However, increased albumin concentration was not observed in asthmatics' airways sampled by PEx and thus it was hypothesized that albumin

might be actively regulated to modify the oncotic pressure for balancing the fluid levels in the airways during a change in hydrostatic pressure (Larsson *et al.*, 2015). In fact, Larsson and colleagues investigated using the PEx technique a number of markers of small airway inflammation in asthmatics. They found that the amounts of exhaled particles were lower after exposure to birch pollen in asthmatics and in those with birch pollen allergy, while their concentrations of SP-A and albumin were not significantly changed.

Ericson *et al*. (2016) investigated PEx biomarkers of chronic rejection of lung transplantation in the form of bronchiolitis obliterans syndrome (BOS). Ericson and colleagues found that SP-A and SP-A to albumin ratio were lower in BOS patients compared to BOS-free subjects, and that stable lung transplant recipients had lower albumin content but higher amounts of exhaled particles than healthy controls.

Larsson *et al*. (2017) studied the effect of exhalation flow on phospholipid composition in PEx. In this study, Larsson and colleagues found that inhalations from low lung volumes increased the mass of exhaled particles by more than 450% and surfactant lipid DPPC by ~9wt% compared to slow exhalations, while forced exhalations increased that by 150% but only ~3wt%, respectively. The effect of these respiratory efforts on exhaled palmitoyl-oleoyl-phosphatidylcholine (POPC) was also studied but with less prominent findings.

3.1.2. Different breathing activities have been studied to collect PEx

Almstrand and co-workers (2009 and 2012) devised a type of instructed breathing patterns (ten forced exhalations) to demonstrate their PEx design for collecting and analysing exhaled endogenous particles (Figure 3.2).
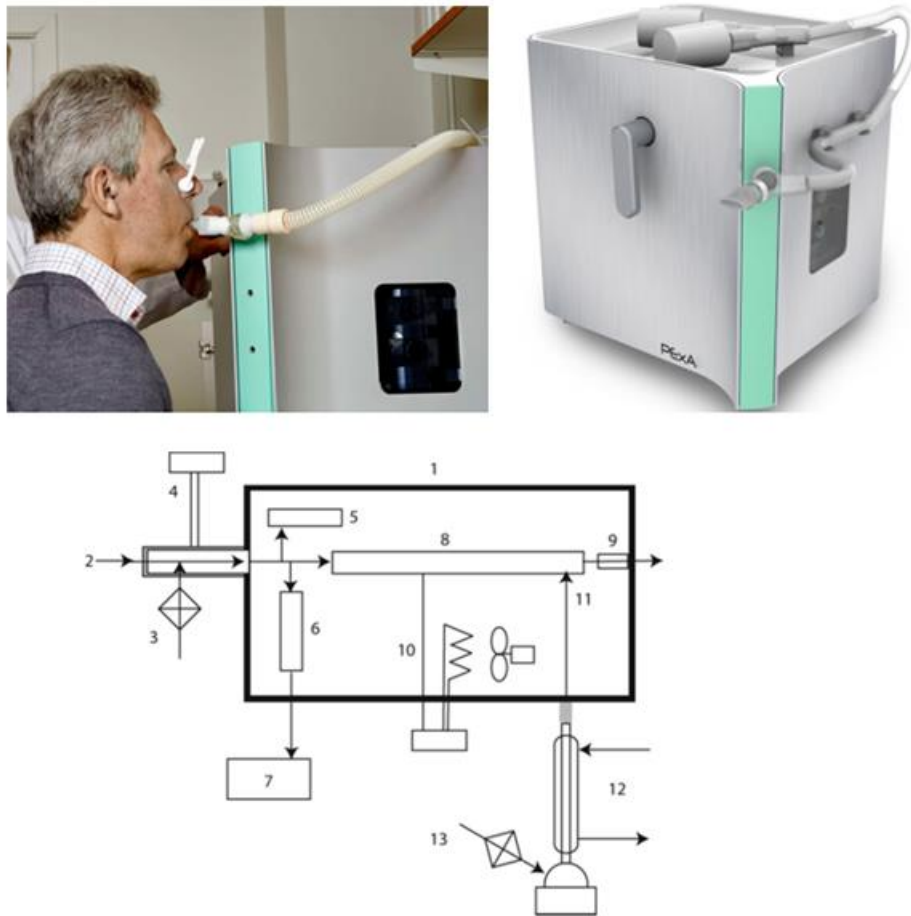
Figure 3.2 Particles of exhaled air (PExA) technique
(1) Thermally insulated box (52 × 46 × 121 cm) (2) Mouthpiece through which the patient inhales and exhales. Inhalation of room air takes place through the particle filter (3). The mouthpiece temperature is maintained by a temperature controller (4) using a Pt-100 thermometer and a heating tape surrounding the mouthpiece. (5) Particle counter/sizer. (6) Inertial impactor. (7) Vacuum pump serving the impactor. (8) Tubular reservoir for exhaled air. (9) Flow meter for measurement of excess air venting into the room. (10) Box temperature control consisting of a Pt-100 thermometer attached to the tubular reservoir, an electrical heater, circulation fan, and a controller. (11) Moist air inlet, with a flow slightly larger than the combined flow of the particle counter (5) and the impactor (6). (12) Air humidifier consisting of a flask containing distilled water, heated by a heating mantle. Clean, filtered air enters from 13 and becomes saturated with water vapor at ∼50 °C. This air then passes through the gas cooler, kept at 36°C by circulating water from a thermostat bath. The excess water is lost by condensation until the dew point is reached. This was reproduced from Almstrand *et al*., 2009 with permission from Copyright Clearance Center.

In 2010, Almstrand's group compared the concentration and size distribution of particles exhaled in three manoeuvres of slow deep and slow shallow breathing. They found that an exhalation from Functional Residual Capacity (FRC) to Residual Volume (RV) followed by inspiration to Total Lung Capacity (TLC) and exhalation back to FRC resulted in highest number of exhaled particles while normal tidal breathing resulted in lowest number compared to instructed breathing patterns.

After that, Larsson *et al*. (2012) applied a pattern of instructed breathing composed of three steps: full exhalation to RV then a brief hold, rapid inhalation to Vital Capacity (VC) and an exhalation back to RV at a given peak flow (1−1.5 L/sec), to detect SP-A and albumin in PEx samples.

Bredberg *et al*. (2012) applied the forced exhalation pattern for collecting PEx to investigate these particles' proteome. Similarly, in 2013, Bredberg's group used this pattern to compare the PEx composition of phospholipids between smokers and non-smokers.

Holmgren *et al*. (2013) studied the effect of breath holding at low and high lung volumes on the amounts of exhaled particles. They found that breath holding at the former (TLC) resulted in low concentrations of PEx while holding at the latter (RV) yielded high concentrations.

Larstad and colleagues (2015) applied for PEx collection another pattern of instructed breathing composed of four steps with a nose clip in place: full exhalation to RV then a brief breath holding, rapid inhalation to VC, normal exhalation and tidal breathing to a given particle concentration (<150 particles/L). They found the particle number concentration was lower in COPD patients compared to healthy individuals, relating that to hyperinflation and

bronchioles destruction resulting in a limited ability of those patients to exhale to low lung volumes.

Ericson *et al*. (2016) applied for PEx collection another pattern: exhalation to RV, rapid inhalation to TLC and deep relaxed exhalation to investigate the serviceability of PEx in providing biomarkers for chronic rejection of lung transplantation in the form of BOS.

Ljungkvist *et al*. (2017) compared PEx and SensAbues device for collecting exhaled methadone from patients on methadone maintenance treatment. They found methadone concentration in PEX was less than 1% of that of the SensAbues device, concluding that the former sampled the LRT while the latter did the URT. Moreover, they found that applying different breathing patterns did not increase methadone amount from the latter as it did for the former.

In addition to the above, Larsson *et al*. (2015) applied another pattern: maximal exhalation to RV then a brief holding of breath, maximal inspiration and relaxed exhalation, to investigate PEx markers of small airway inflammation in asthmatics. After that, Larsson *et al*. (2017) postulated via studying the effect of exhalation flow on phospholipid composition that inhalations from low lung volumes can sample small airways by a suggested airway re-opening mechanism while forced exhalations can sample central or upper airways by high air velocity where exhaled DPPC was found diluted or degraded.

3.1.3. Coughing has been studied in infectiousness and broader

Another respiratory activity is coughing, and this is usually linked to disease rather than to health. Coughing has been recognised as a typical clinical symptom of TB, the classic example of airborne infections (Riley, 1974; Roy and Milton, 2004).

Loudon and Spohn (1969) described a lack of association between cough frequency and infectiousness of TB patients. This was based on correlating the nocturnal cough rate of untreated cases with the results of tuberculin skin test in their household contacts. However, Turner *et al*. (2014's abstract; 2018) found a reduction in nocturnal cough rate in active TB cases compared to that of the diurnal one and correlated 24 hrs cough frequency with sputum culture positivity.

It sounds the criteria of coughing can impact on the biological content of the exhaled material. For example, Fennelly *et al*. (2012) associated the production of cultivable aerosols of TB patients with their subjectively-assessed force of coughing.

Analysis of cough sound has been studied outside TB. Toop *et al*. (1989) compared resting and exertional cough in asthmatic and healthy paediatric groups and found that the post-exercise cough sound change could be used in approaching asthma diagnosis.

Measuring the cough sound with the Leicester cough monitor has been validated as a non-invasive analyser of cough frequency over prolonged periods by Birring *et al*. (2008)'s and Lee *et al*. (2012)'s works.

Several studies have described physiological parameters like cough flow, oesophageal pressure, gastric pressure and electromyography as objective assessors of cough intensity or cough sound power (Fontana *et al*., 1997; Smith *et al*., 2012; Lee *et al*., 2015a and 2017a). Moreover, Pavesi *et al*. (2001) suggested the reliability of their computerized cough acquisition system based on cough sound analysis in measuring the intensity feature.

Compared to slow exhalations, Larsson *et al*. (2017) found that the mass of exhaled particles increased by more than 600% while the sampled mass of DPPC or POPC was not increased when their subjects were instructed to inhale to TLC and perform a Valsalva manoeuvre against a closed glottis then producing two to three forceful coughs (with end-respiratory volume between RV and FRC).

3.1.4. Vocalising activities have been studied in infectiousness

Talking, as an example of respiratory and vocalising activities, has been mentioned among the modes of dissemination of airborne infections including TB and others.

Loudon and Roberts (1967) studied the numbers and sizes of particles exhaled during coughing and during talking. In 1968, Loudon and Roberts extended their experiments to cover singing after they noted a TB outbreak report describing a higher rate of tuberculin converters in contacts with an index case at a choir practice than those in contact with that case at other activities.

Loudon and Roberts found that the total number of exhaled droplets was more than 40,000, 10,000 and 4,000 during coughing, talking and singing, of which around 50%, 5% and 35%, respectively, remained airborne as droplet nuclei for 30 min.

3.1.5. Suggested mechanisms of aerosolization

The generation mechanism of aerosols (and droplets) remains poorly understood and multiple acting mechanisms have been suggested.

In fact, two main approaches have been employed to investigate the origin of aerosols and the mechanism of aerosolization. Exploring the biological content of the exhaled material and matching it with that of the RTLF, and relating a certain marker in the exhaled material to lung volumes and capacities during different respiratory activities.

For example, Papineni and Rosenthal (1997) suggested that respiratory droplets are generated by sheer forces resulted from respiration. This mechanism, however, has been proposed without definitive proof. Johnson and Morawska (2009) found that subjects who inhaled rapidly or exhaled deeply increased the concentration of their exhaled particles than those who exhaled rapidly. Their findings were supportive to the hypothesis that the source of exhaled particles is the fluid film burst resulted from opening the terminal airways during inhalation. However, these authors (Johnson *et al*., 2011) also considered, through studying the size distribution of exhaled particles in a logarithmic trimodal model (oral, laryngeal and bronchiolar), that turbulence and wind shear are nonetheless relevant to particle generation higher up in the respiratory tract.

Almstrand and co-workers (2010) postulated that small, non-rigid airway opening following closure has an important role in generating these endogenous particles from terminal bronchioles, based on studying exhaled particle number concentrations generated by different depths of exhalation. In fact, the suggested mechanism of distal airways closure, beginning in the LRT and progressing toward the URT with decreased lung volumes, was first

discussed by Dollfuss *et al*. (1967) after correlating the concentrations of orally-administrated ^{133}xenon measured by scintillation counters with the changes of different lung volumes.

Fabian *et al*. (2011) also concluded that exhaled aerosols might be generated from the upper and lower respiratory tract. Having accounted the lower generation to opening collapsed terminal bronchioles and alveoli, Fabian's group suggested that the breathing activity itself plays a key role in generating aerosols from the lower airways.

3.1.6. Limitations of PEx and EBC compared to the mask

The current sampling tools of exhaled materials, including the mask, are limited by several factors. These include little available information about aerosolization, no conclusive evidence for the origin of aerosols, available tools are not specific to an anatomic site and the lack of a standard method for sampling including collection, preservation, processing and analysis.

Compared to mask sampling, among the main limitations of PEx collection are its required infrastructure and therefore limited applicability in different clinical settings. In addition, the used vacuum evaporation to improve the PEx recovery rate could undermine the quantification results (Larsson *et al*., 2012). The PEx sampling design involves long tubings where temperatures of some parts are not controlled at 35°C, the degree used to minimize condensation and maintain the size of exhaled particles. These particles are exposed further to sharp turns that can contribute to loss of exhaled large particles. Furthermore, the PEx technique does not sample natural exhalation output and nasal breathing is excluded by clips applied in the current design. In contrast to PEx, the mask does not interfere with the size range of collected material.

EBC shares with PEx the limitations presented by the infrastructure requirements and their implications and more importantly, sampling deliberate rather natural output, where nasal breathing is further excluded and almost all available designs sample oral breathing. Furthermore, even for studies exploring EBC biomarkers, little information is available on markers for interstitial lung disease and the feasibility of biomarkers not related to oxidative stress has not been tested yet. In addition, the EBC samples are subject to concentration artifacts resulted from evaporation. Thus, a recent

review carried on more than 220 EBC studies concluded poor reproducibility of investigated biomarkers in EBC (Konstantinidi *et al*., 2015).

3.1.7. Hypotheses and objectives

Previous studies have either investigated exhaled microbes in disease or exhaled proteins and lipids, while none has studied these components together or investigated their potential associations. More importantly, such studies have explored aspects of the biological content in deliberate or artificial aerosolization, while none has done so far in natural exhalation output from different breathing activities.

The overall aim of the work in this Chapter was to determine the effects of different respiratory efforts on the material collected by face-mask sampling.

Assuming the sampling matrix makes no contribution, we hypothesized that:

1. Different respiratory activities vary in their biological output, and a pattern exhaling largest amounts of SP-A and albumin could be most productive of aerosols of an LRT origin, improving the efficiency of the face-mask as a non-invasive tool for sampling the LRT

2. The quantity of material collected by mask sampling would be time-dependent

3. Different breathing activities could yield microbiota reflecting that of the source zone to a greater or lesser extent

Using gelatine containing masks, the specific objectives were:

1. To detect, quantify and compare the amounts of selected protein biomarkers in masks across four different breathing patterns collected for 15, 30 and 60 min.

2. To detect, quantify and compare the bacteria exhaled in masks across four different breathing patterns collected for 15, 30 and 60 min, by assaying *16S* rDNA at bacterial Domain and selected phylum levels

3. To investigate potential time-dependent features in the results with additional studies on potentially contributing proteins.

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3.2. Materials and methods

3.2.1. Recruitment of healthy volunteers

Fifteen healthy volunteers, (10 female, ages 18–48 years), were recruited between September and October 2015. All were non-smokers with no recent history (<8 weeks) of respiratory infections or antibiotic treatment. The study information sheet, the participant informed consent and questionnaire confirming healthy status were provided under the governance of local departmental ethical approval (Ref: 2369-ma680-3i).

3.2.2. Face-mask assembly

The face-mask (Figure 3.3) was assembled with gloved hands and sterile forceps inside a laminar flow cabinet (Walker Safety Cabinets Ltd., UK) from an FFP1 mask (Moldex 2380 Smart FFP1 Non-valved respirator, Scientific Laboratory Supplies, UK), gelatine filter (specified in 2.2.5), metal holder and pins (manufactured in the workshop of the University and sterilized per 2.2.1). The gelatine filter was cut (6cm in diameter) to fit the metal holder using a cutting plate specifically designed in the workshop and sterilized per 2.2.1. The assembled face-masks were further disinfected by exposure to UV in the cabinet for one hr and individually packed in resealable plastic bags.
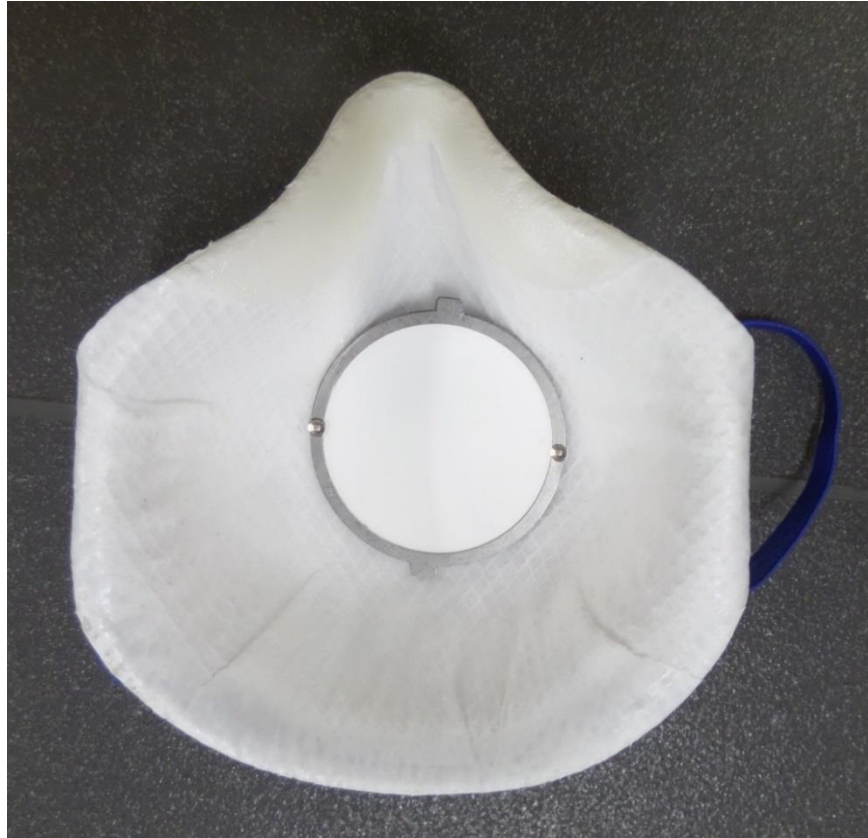
Figure 3.3 Face-mask system
The assembled mask is composed of an FFP1 face-mask, gelatine filter (6cm in diameter), metal holder and pins.

3.2.3. Mask sampling

The mask samples were collected during four respiratory activities performed under supervision with the volunteers seated at rest. These were 1) normal tidal breathing (NB), 2) instructed breathing (IB) involving one deep oral exhalation down to RV every min, 3) intermittent coughing (IC) performed as one deep cough produced every min and 4) reading-out loud (RL) where the volunteers were instructed to read-out a defined piece of text at a standardised average volume and rate. The sampling was conducted over 15, 30 and 60 min using one face-mask for each activity (the 60-min interval was excluded for the RL group as it was judged not clinically feasible particularly for patients with respiratory infections). The RL participants were trained to maintain their sound wave amplitude at the range of 70–75 decibels using a

decibel-meter. The rate of reading was controlled by a group leader to maintain the group reading rate at the normal speech rate. The volunteers were sampled in groups of three to five per session in one room. Room air was simultaneously sampled at 5 L/min as a background control. A one-minute interval timer was provided as required.

Direct contact with the gelatine filters was carefully avoided throughout. Following sampling, the face-masks were placed back in resealable plastic bags and transported to the laboratory for immediate processing.

3.2.4. Processing of face-mask samples

3.2.4.1. Extraction of exhaled material

Exposed masks were removed from their bags and placed open in the laminar flow cabinet until the gelatine filters were dry to visual inspection (~30 min). The filters were then removed from the holders using sterile forceps and processed by collagenase digestion (2.2.6.3). The resultant lysates were centrifuged at 15,000xg for 10min, the supernatants and pellets collected separately, transferred into 2mL O-ring seal screw cap tubes, and stored at -20°C if not processed on the same day. The supernatants were used for protein assays and the pellets for bacterial DNA analyses.

3.2.4.2. DNA extraction and propidium monoazide (PMA) treatment

The pellets described above were resuspended with 200µL molecular grade water and divided into two 100µL aliquots. One of these was directly subjected to DNA extraction using the semi-automated QIAcube system with QIAamp DNAmini kit (QIAGEN, UK) for Gram-positive bacteria, while the other was extracted after exposure at 20mM to the photoreactive dye PMA and UV (PMA-Lite LED Photolysis Device; both from Biotium, USA) per the manufacturer's instructions. PMA binds to exposed DNA and is excluded from intact cells. UV cross-links the dye to DNA and prevents subsequent PCR amplification so only

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DNA extracted from intact cells is amplified. PMA was applied in an attempt to remove background signals from the gelatine filters which were not free from bacterial DNA. This finding was confirmed in personal communication with the manufacturer (Sartorius, Germany).

3.2.4.3. qPCR assays

Preparation of qPCR standards

Genomic DNA of *Streptococcus pneumoniae,* Mtb H37Rv*, Bacteroides fragilis, Burkholderia mallei* and *Moraxella catarrhalis* were used to prepare qPCR standards for phylogenetic quantification assays of Firmicutes, Actinobacteria, Bacteroidetes, Beta-proteobacteria and Gamma-proteobacteria, respectively. Genomic DNA of Mtb and *B. mallei* were kindly provided by Dr Lazar-Adler. Genomic DNA of *S. pneumoniae*, *B. fragilis* and *M. catarrhalis* was extracted from 10mL pure culture of each relevant bacterium (A.2.1) using QIAamp DNA mini kit according to the manufacturer's instructions. The rest of qPCR standards preparation method was as described in 2.2.8.3 with changing the parameters for each genome accordingly.

Target genes and primers used in qPCR

While there are *16S* primer pairs specific for Firmicutes, Actinobacteria and Bacteroidetes, none is currently available with sufficient specificity and sensitivity for entire Proteobacteria. Therefore, *16S* primers targeting beta and gamma classes of this phylum were selected. Oligonucleotide sequences (Table 3.1) were tested using *in silico* PCR to confirm the primer pairs had best possible values of sensitivity and specificity based on Ribosomal Database Project (RDP) matches. It must be acknowledged, however, that *in silico* findings are not necessarily applicable *in vitro*, even for published primers (Henriques *et al*., 2012).

Table 3.1 Target genes and primers used for the real-time assays				
Primer designation	**Target taxon**	**Oligonucleotide sequence**	**Taxon-specific oligonucleotide position***	**Reference**
16S 338F	All bacteria (*16S*rDNA)	5' ACTCCTACGGGNGGCNGCA 3'	338−356	Free, 2005
16S 515R		5' GTATTACCGCNNCTGCTGGCAC 3'	515−536	
Firm928F	Firmicutes	5' TGAAACTYAAAGGAATTGACG 3'	928−948	Bacchetti De Gregoris *et al.,* 2011
Firm1040R		5' ACCATGCACCACCTGTC 3'	1040−1056	
Act920F	Actinobacteria	5' TACGGCCGCAAGGCTA 3'	920−935	Bacchetti De Gregoris *et al.,* 2011
Act1200R		5' TCRTCCCCACCTTCCTCCG 3'	1200−1218	
Bact934F	Bacteroidetes	5' GGARCATGTGGTTTAATTCGATGAT 3'	934−958	Guo *et al.,* 2008
Bact1060R		5' AGCTGACGACAACCATGCAG 3'	1060−1079	
Beta359F	Beta-proteobacteria	5' GGGGAATTTTGGACAATGGG 3'	359−378	Ashelford *et al.,* 2002 Muhling *et al.,* 2008
Beta682R		5' ACGCATTTCACTGCTACACG 3'	682−701	
γ871F	Gamma-proteobacteria	5' TAAGTHGACCGCCTGGGGAGT 3'	871−891	Muhling *et al.,* 2008
γ1202R		5' CGTAAGGGCCATGATG 3'	1202−1217	Bacchetti De Gregoris *et al.,* 2011
* Equivalent position within *E. coli* genome per PRIMROSE (Ashelford *et al*., 2002)				

In-house SYBR-Green based qPCR assays

Table 3.2 Cycling conditions

	Amplification§				Melting¶
	Denaturation	Annealing	Extension	Acquisition*	
Firmicutes	95°C for 15 sec	62°C for 30 sec	72°C for 20 sec	78°C for 20 sec	62°C to 95°C
Actinobacteria	95°C for 15 sec	62°C for 30 sec	72°C for 20 sec	82°C for 20 sec	62°C to 95°C
Bacteroidetes	95°C for 15 sec	65°C for 30 sec	72°C for 20 sec	82°C for 20 sec	65°C to 95°C
Beta-proteobacteria	95°C for 15 sec	62°C for 30 sec	72°C for 20 sec	82°C for 20 sec	62°C to 95°C
Gamma-proteobacteria	95°C for 15 sec	60°C for 30 sec	72°C for 20 sec	78°C for 20 sec	60°C to 95°C

§ 40 cycles preceded by a holding cycle (95°C for 15 min)

* on FAM/Green channel (470nm)

¶ rising 1°C per cycle

For the universal bacterial *16S* rDNA targeted assay, 25µL PCR mixture of each reaction tube was prepared in 0.1mL Rotor-Gene PCR tubes containing 12.5µL of 2X SensiFAST SYBR No-ROX (specified earlier), 1µL of each 10µM *16S* 338F and 10µM *16S* 515R (Table 3.1), 1µL of DNA template and 9.5µL of DNase-RNase free water. Cycling conditions were: one holding cycle at 95°C for 15min, 40 amplification cycles at 95°C for 15sec, 64°C for 30sec, 72°C for 20sec and 82°C for 20sec with acquisition on FAM/Green channel (470nm). Melting was set at 64°C to 95°C rising 1°C per cycle. Following these steps, conditions for the phylum- and class-level assays are given in Table 3.2.

No template controls (molecular grade water) were included with each assay and all were run in technical duplicates analysed on a Corbett Life Science Rotor-Gene 6000 real-time DNA analysis system. The lower limit of quantitation (LLQ) was assigned as 10^3 copies per reaction (Figure 3.10). The analyses were accepted when R^2 was ≥0.98, efficiency was ≥0.70 and the no template control indicated <100 copy/reaction. The slope correct option was selected. The quantitative readings were accepted when variation between replicates was <20% of the mean. The Ct for all these assays was set at 0.04 for uniformity and comparability. Copy numbers per sample were adjusted according to the total volume of DNA extract.

3.2.4.4.   Albumin titration in exhaled aerosols

The concentration of albumin in exhaled aerosols (extracts of 3.2.4.1) was measured using Human Albumin ELISA Kit [E-80AL] (Immunology Consultants Laboratory, USA) per the manufacturer's instructions. The absorbance of the enzyme-linked immunosorbent assay (ELISA) reaction was read using EL808 Ultra Microplate Reader (BIOTEK, USA) at 450nm wavelength. The albumin standard calibrator was provided by the manufacturer and an additional calibration standard was prepared by serial dilutions to 3.12 ng/mL. Albumin

concentrations of the samples were interpolated from a standard curve generated using GraphPad Prism version 7 (specified before).

3.2.4.5. SP-A titration in exhaled aerosols

The concentration of SP-A in the aerosol extracts was measured using SEA890Hu ELISA Kit for Surfactant Associated Protein A (Wuhan USCN Business Co., China) per the manufacturer's instructions. The absorbance of ELISA was read using EL808 Ultra Microplate Reader at 450nm wavelength. The SP-A standard calibrator was provided by the manufacturer and an additional calibrator was prepared at 23.44 pg/mL. SP-A concentrations of the samples were interpolated from SP-A standard curve using GraphPad Prism software (specified before).

3.2.4.6. Lysozyme activity detection in exhaled aerosols

Active lysozyme of the aerosol extracts was quantified based on fluorescence intensity using ENZchek Lysozyme Assay Kit (Fisher Scientific Ltd, UK) per the manufacturer's instructions. The fluorescence at excitation/emission wavelength of 494/518nm was read with Varioskan Flash Multimode Reader (Thermo Scientific, UK) using SkanIt Software version 2.4.5 (Thermo Scientific, UK). Lysozyme concentration of the samples were interpolated from lysozyme standard curve using GraphPad Prism software (specified before).

3.2.4.7. Protease activity detection in exhaled aerosols

Active protease of the aerosol extracts was quantified using RayBio Protease Activity Assay Kit (RayBiotech Inc., USA) per the manufacturer's instructions. This assay was designed for the quantitative detection of proteases in protein samples. The fluorescence was measured with Varioskan Flash Multimode Reader (specified before) using excitation/emission wavelength at

485/530nm. Protease concentrations of the samples were interpolated from protease standard curve using GraphPad Prism software (specified before).

3.2.4.8. DNase quantitation and activity detection in exhaled aerosols

The concentration of human DNase in the aerosol extracts was measured using Deoxyribonuclease 1 ELISA kit for *Homo sapiens* E1127h (WUHAN EIAab Science Co. Ltd, China) per the manufacturer's instructions. This assay was designed as an indirect sandwich ELISA for detecting human DNase by the specific immunogen (Met38~Gln258; UniProt No.: P24855) so that human DNase can be distinguished in terms of detection from DNases of other species. The absorbance of ELISA was read using EL808 Ultra Microplate Reader at 450nm wavelength. The DNase standard calibrator was provided by the manufacturer. DNase concentrations of the samples were interpolated from DNase standard curve using GraphPad Prism software (specified before).

Active DNase of the aerosol extracts was tested using DNase Detection Kit (Jena Bioscience GmbH, Germany) per the manufacturer's instructions. This assay was designed for fluorescence-based detection of DNase activity. The real-time fluorescence was measured on Corbett Life Science Rotor-Gene 6000 system (specified before).

3.2.4.9. α-Amylase activity detection in exhaled aerosols

Active α-amylase of the aerosol extracts was detected using EnzChek Ultra Amylase Assay Kit (Fisher Scientific Ltd, UK) per the manufacturer's instructions. α-Amylase from human saliva (A1031, Sigma-Aldrich, UK) was used as a standard. The fluorescence was measured with Varioskan Flash Multimode Reader (specified before) using excitation/emission wavelength at

502/512nm. α-Amylase concentrations of the samples were interpolated from the standard curve using GraphPad Prism software (specified before).

3.2.4.10. Lower limit of detection

The LLD of all the above assays was the lowest concentration differentiable from zero, determined as the lowest concentration of the standard assayable and recognizable from the negative control. The negative control samples (n= 10) had undetectable amounts of the target, i.e. below LLD. All values calculated as zeros were considered below LLD.

For describing the data summary (median and interquartile range (IQR)) excluding ratios and percentages, zeros were presented as 0.01, unless stated otherwise.

3.2.5. Statistical analyses

On advice from Dr Viskaduraki of the Bioinformatics and Biostatistics Hub at the University, large data sets were analysed using a mixed linear regression model using STATA (Intercooled) version 15.0 (Timberlake Consultants Limited, StataCorp LLC, USA), unless stated otherwise. Unlike repeated measures ANOVA, this model is appropriate where there are missing values (presented as no data available). The above method gave exact p values for each variable and the cut-off point of 0.05 was the default false discovery rate controllable by applying the Bonferroni correction (Kenward and Roger, 1997).

3.3. Results

Protein targeting assays were used as potential biomarkers for the origins of exhaled material collected and in attempts to understand some of the time-dependent features in the bacterial assays reported in later sections.

3.3.1. SP-A and albumin collected with different respiratory activities

These proteins, known to be abundant in LRT lining fluid, were selected to determine which breathing pattern might exhale them most profusely. SP-A was detected in all samples (Table A.5), while albumin was undetectable in 13 of 110 samples and at low levels in a further 36 (Table A.6). Background controls were negative in both cases and the results are presented in Figure 3.4.

Reading-out loud (RL) stood out as producing highest outputs of both biomarkers at 15 and 30 min. Although there was a trend to higher outputs of albumin with intermittent coughing (IC), this did not reach significance at the $p < 0.05$ level.
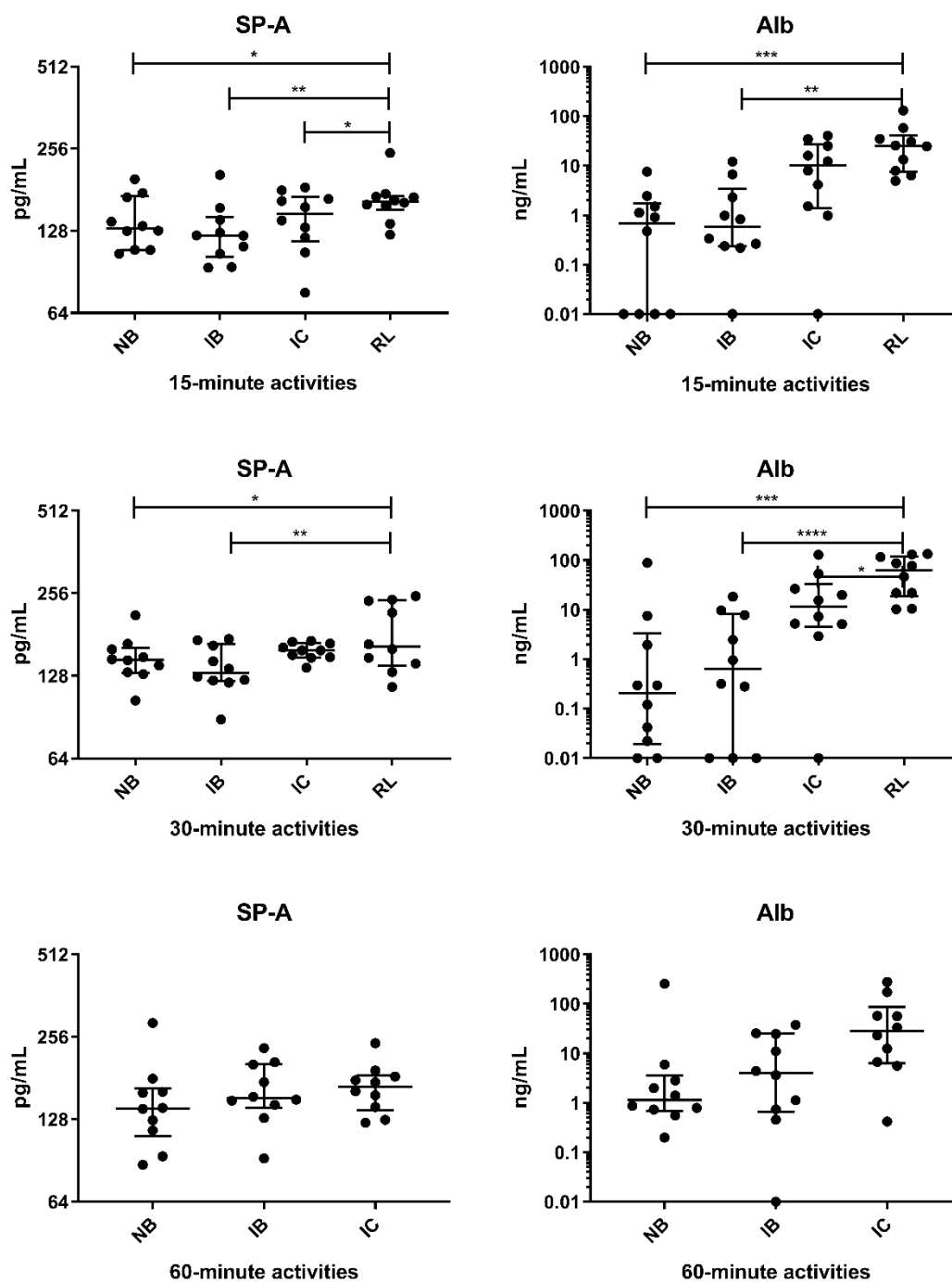
Figure 3.4 Comparison of the quantity of exhalable SP-A and albumin (Alb) in different respiratory activities

Mixed linear regression was applied. Error bars= IQR; n=10.

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The quantitative results (Figure 3.5) showed, not surprisingly, that increasing the sampling time from 15 to 60 min resulted in a statistically significant increase in the quantity of exhaled SP-A and albumin. Increasing sampling time, however, from 15 to 30 min or from 30 to 60 min did not result in statistically significant increases.
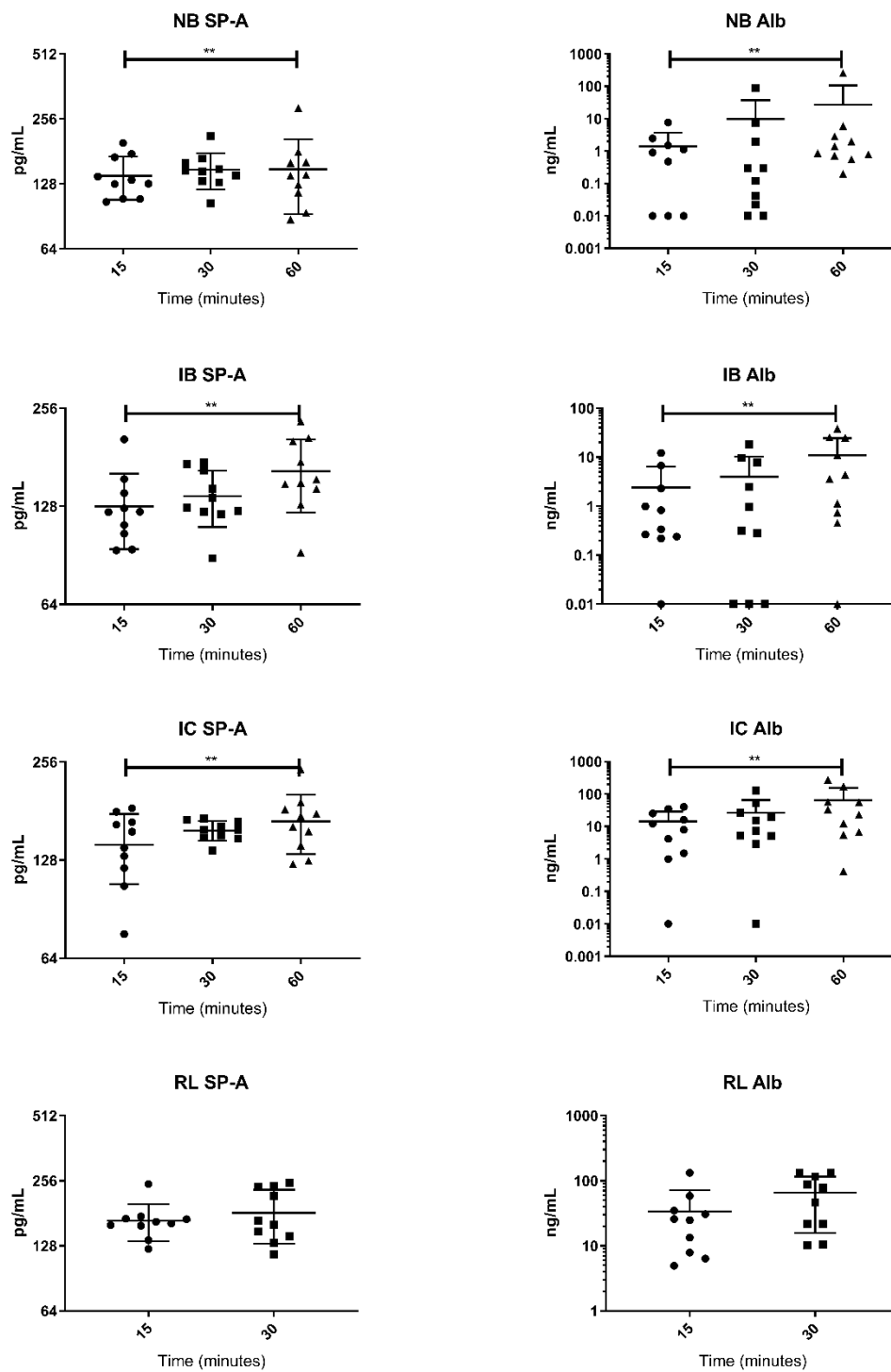
Figure 3.5 Comparison of the quantity of exhalable SP-A and albumin of different sampling time periods

Mixed linear regression was applied. Error bars= IQR; n=10.

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Regression analysis against time showed a linear trend in all cases (Figure 3.6) but only the relationship for SP-A exhaled by instructed breathing (IB) reached statistical significance (Table A.24).
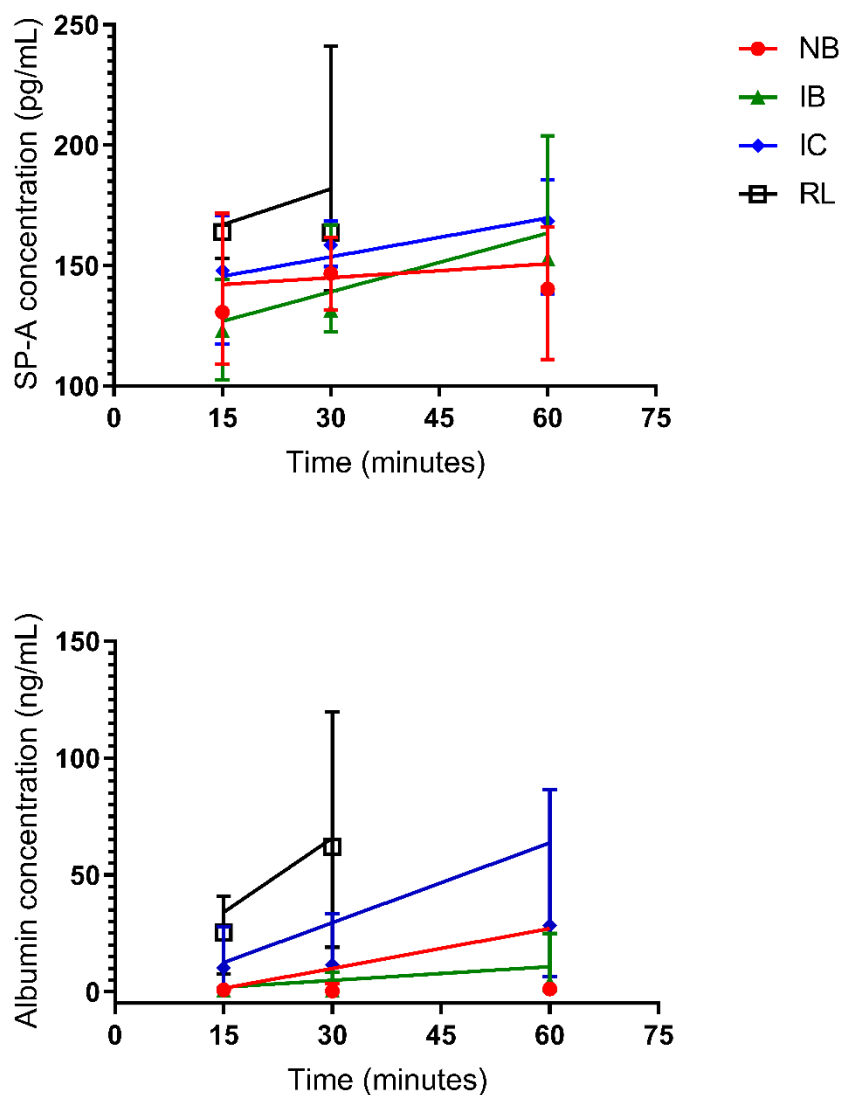
Figure 3.6 Linear regression of exhaled SP-A and albumin over time
(Error bars= IQR; n=10).

3.3.2. Protease assays

One possible explanation for the lack of statistically significant regression in the amounts of exhaled SP-A and albumin could be due to protein-signal degrading factors. The face-masks were therefore investigated for the presence of protease.

All samples were analysed for active protease using a fluorescein isothiocyanate (FITC)-labeled casein substrate. Activity was detected in 95 of 110 samples (Table A.7). Background controls were negative.

No statistically significant difference was found in the quantity of exhaled protease between different respiratory activities or between different time periods (Figure 3.7).

The quantities of exhaled SP-A and albumin were tested for correlation with that of exhaled protease. Notwithstanding a borderline positive correlation found between the quantities of active protease and SP-A exhaled during IB over 30 min ($r= 0.64$, $p= 0.05$), no statistically significant correlation was found between the quantity of the former and that of SP-A or albumin exhaled during other breathing patterns at the three different time periods. The full results are presented in Table A.28.
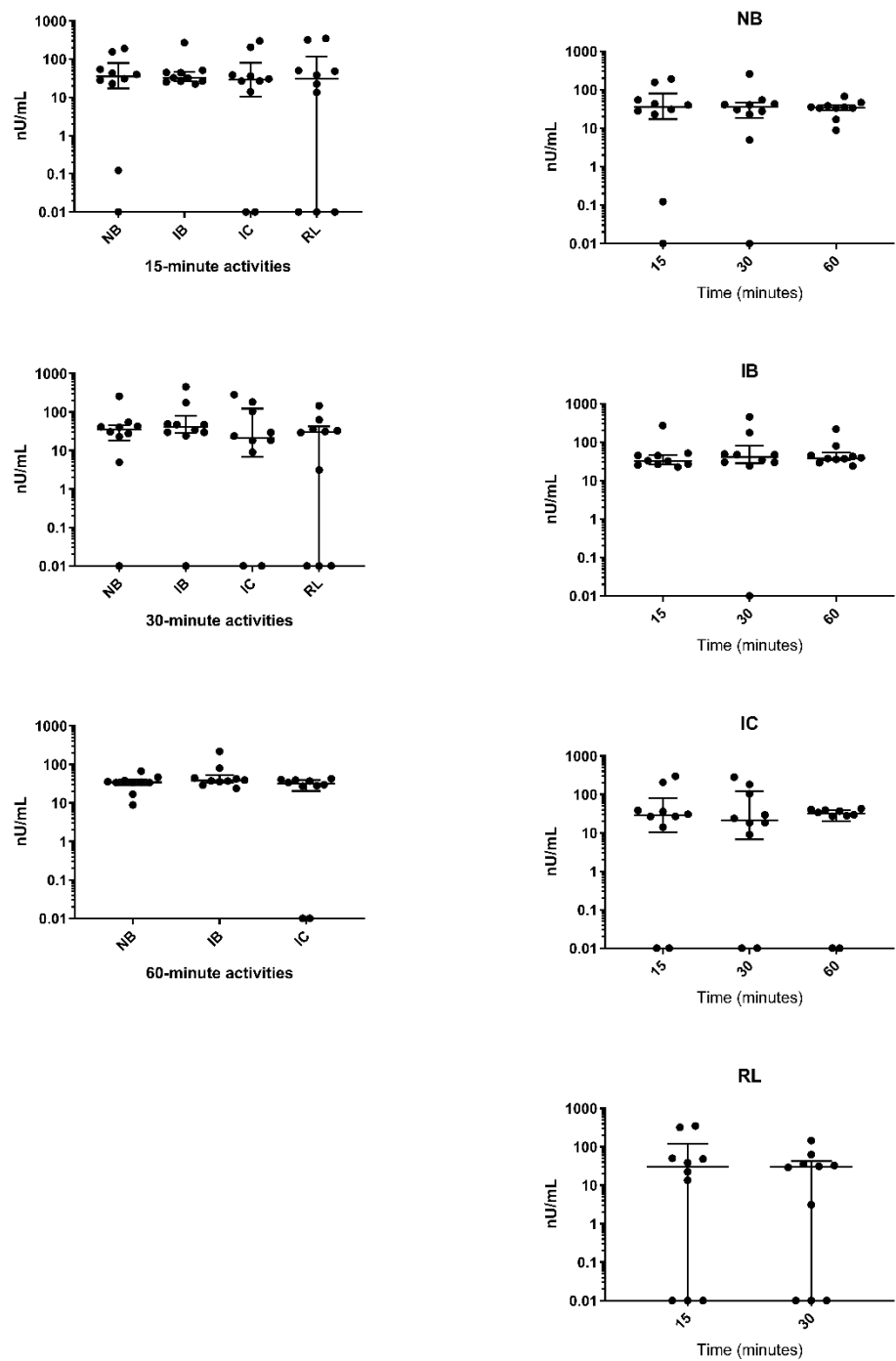
Figure 3.7 The quantity of exhaled protease does not change between different respiratory activities and time periods

Mixed linear regression was applied. Error bars= IQR; n=10.

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3.3.3. Salivary α-amylase assays

This activity was assayed as a biomarker potentially indicating the level of contamination of mask samples with the oral cavity secretions; a boron-dipyrromethene (BODIPY)-labeled starch derived fluorogenic substrate was used. Activity was detected in 98 of 110 samples and at low levels in a further seven (Table A.8). Background controls were negative.

IC and RL consistently produced the highest signals of exhaled α-amylase, with the latter higher. As found with exhaled SP-A and albumin, increasing the sampling time from 15 to 30 min or from 30 to 60 min did not result in statistically significant increases, while increasing that from 15 to 60 min resulted in a statistically significant increase (Figure 3.8).
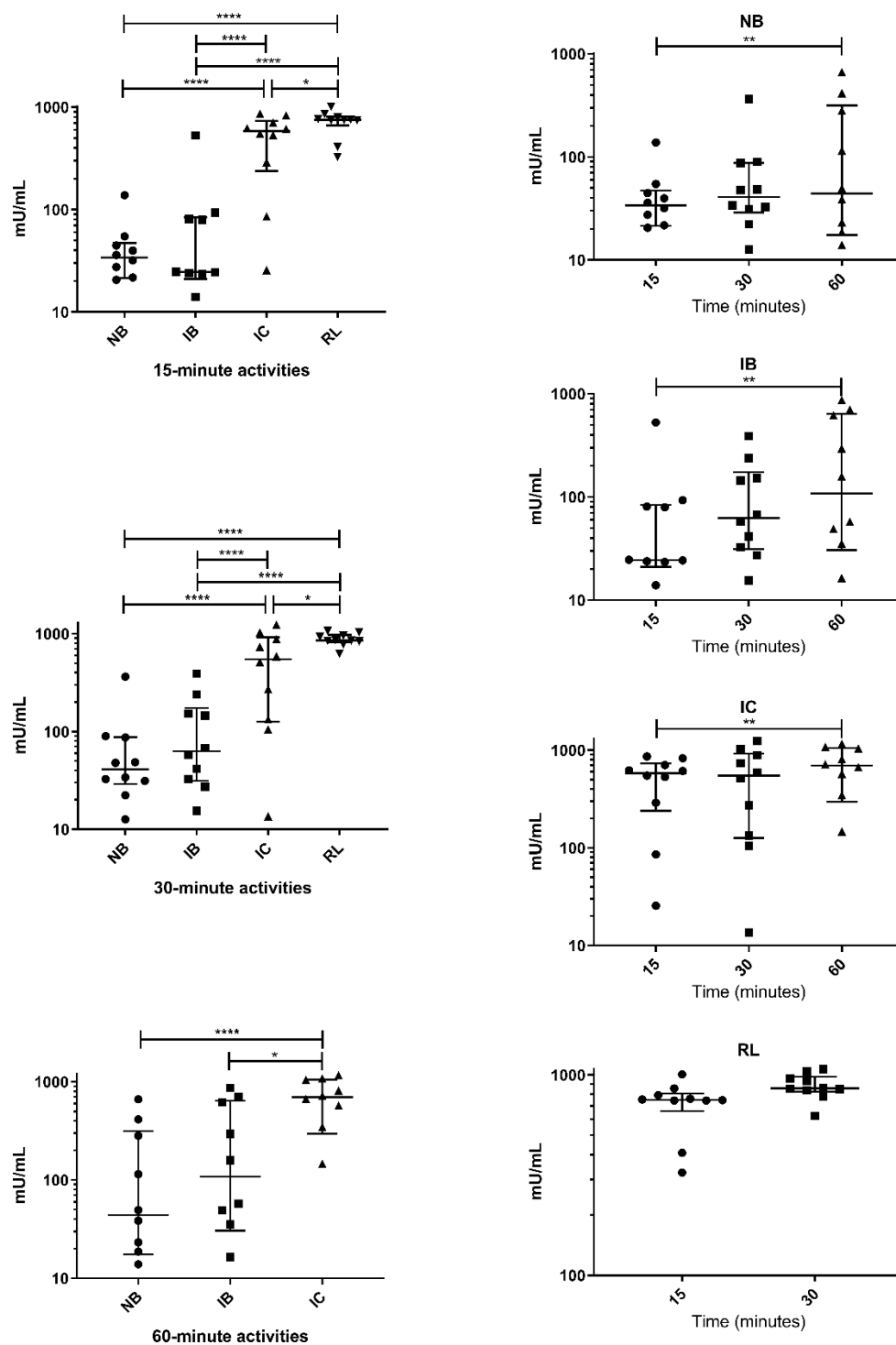
Figure 3.8 The amounts of exhaled active α-amylase of different respiratory activities and time periods

Mixed linear regression was applied. Error bars= IQR; n=10.

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A linear trend with time was found again, only reaching significance with IC (Figure 3.9 and Table A.25).
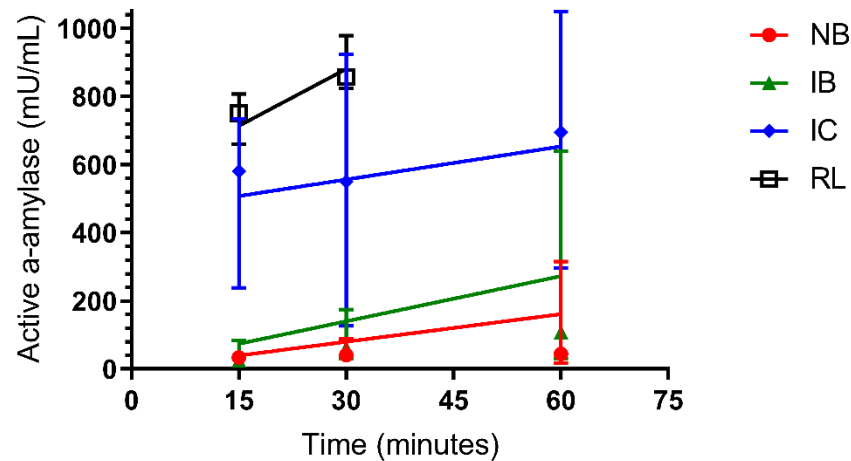
Figure 3.9 Linear regression of exhaled active ɑ-amylase over time
(Error bars= IQR; n=10).

The results were examined for correlations between protease and ɑ-amylase. Significant correlations were found in normal breathing (NB) samples at 15 and 30 min (r= 0.70, p=0.03; r= 0.78, p=0.01; respectively). The full results are presented in Table A.29.

3.3.4. Correlations between biomarkers

The amounts of exhaled active α-amylase, used as a biomarker of URT secretions, were tested for correlation with that of exhaled SP-A and albumin, used as biomarkers of LRT secretions.

If detected α-amylase was exclusively originated or marking the URT and detected SP-A and albumin were done so for the LRT, one should expect negative correlations between their quantities.

However, no statistically significant negative correlation was found between α-amylase and SP-A, or between the former and albumin. By contrast, a statistically significant positive correlation was found between α-amylase and albumin exhaled by NB over 30 min (r=0.66, p= 0.04) and by IC over 15, 30 and 60 min (r= 0.88, p=0.002; r= 0.87, p= 0.002; r=0.94, p= 0.0002; respectively). The full correlation results are presented in Table A.30.

3.3.5. Background *16S* rDNA bacterial signals of gelatine filters

Four unexposed filters were analysed for bacterial *16S* gene signals and four phyla with sub-classes: Firmicutes, Actinobacteria, Bacteroidetes, Beta- and Gamma-proteobacteria. Up to 10^6 copies per µL were found for the *16S* and Firmicutes assays, while signals for the other targets were below LLQ of 10^3 copies per µL (Figure 3.10).
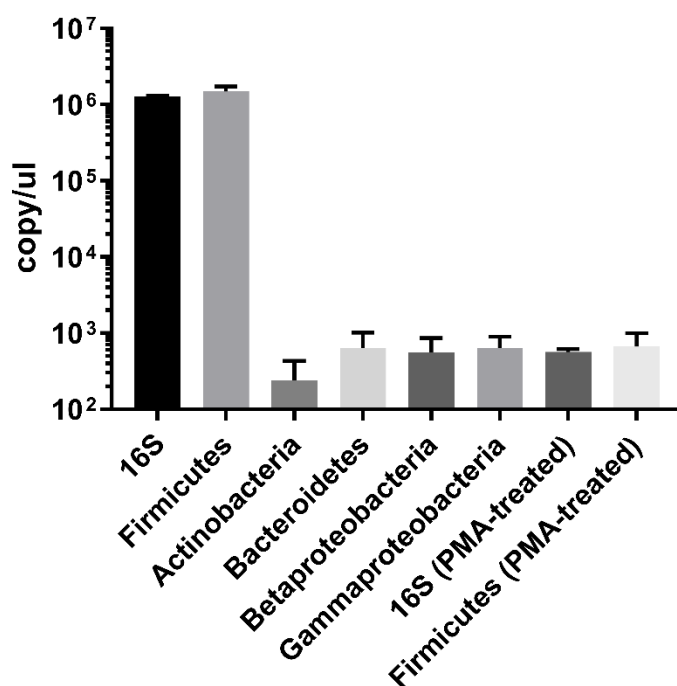
Figure 3.10 Background bacterial signals of blank gelatine filters
(Error Bars= SD, n= 4)

DNase treatment (10 µg/mL) was applied to the solubilised blank filters prior to extraction but resulted in only 1 log-fold reduction in the background signals. However, PMA treatment prior to extraction reduced the background to below the LLQ (Figure 3.10). Accordingly, all mask pellets were separately assayed following PMA for *16S* and Firmicutes and directly for the other four targets.

3.3.6. Bacterial signals produced by different respiratory activities

Mask sample DNA extracts were assayed for phylum level targets chosen for their known abundance in respiratory tract samples (Wang *et al*., 2016; Dickson *et al*., 2013; Pragman *et al*., 2012). This level of analysis was dictated by available resources. The exhaled bacteriome is examined further in Chapter Five.

Beta- and Gamma-proteobacteria were detected in all samples, while Bacteroidetes, Actinobacteria and Firmicutes detected in 105, 49 and 43 samples, respectively. Background controls were negative at the LLQ of 10^3 copies/reaction and the results are presented in Table A.9 to Table A.14.

When detectable, the signals fell within the range 10^5 to 10^8 copies per mask at all tested time periods. This was equivalent to 10^4 to 10^7 copies per min for masks obtained over 15 minutes, and to 10^3 to 10^6 copies per min for 30- and 60-minute sampling (Figure 3.11).

Mixed linear regression analysis of the signals failed to reveal any significant difference in the exhaled amounts of any phylum between any two time periods of any respiratory pattern (Figure 3.11). Notably, the overall pattern indicated no significant additional signal accumulation occurred above that obtained at 15 min. However, there were some significant differences in outputs between the breathing efforts. These included: Actinobacteria in IB over NB at 15 min but not over longer periods, Bacteroidetes exhaled by RL over both NB and IB at 15 and 30 min and Gamma-protobacteria exhaled by IC over IB at 60 min. The full analysis is presented in Table 3.3.

Signals from Actinobacteria, Bacteroidetes, Beta-proteobacteria and Gamma-proteobacteria were clustered using Euclidean distance. Interestingly, the exhaled quantities were separated into three major clusters based on abundance of the quantified phyla rather than by the breathing activity or time of sampling (Figure 3.12).
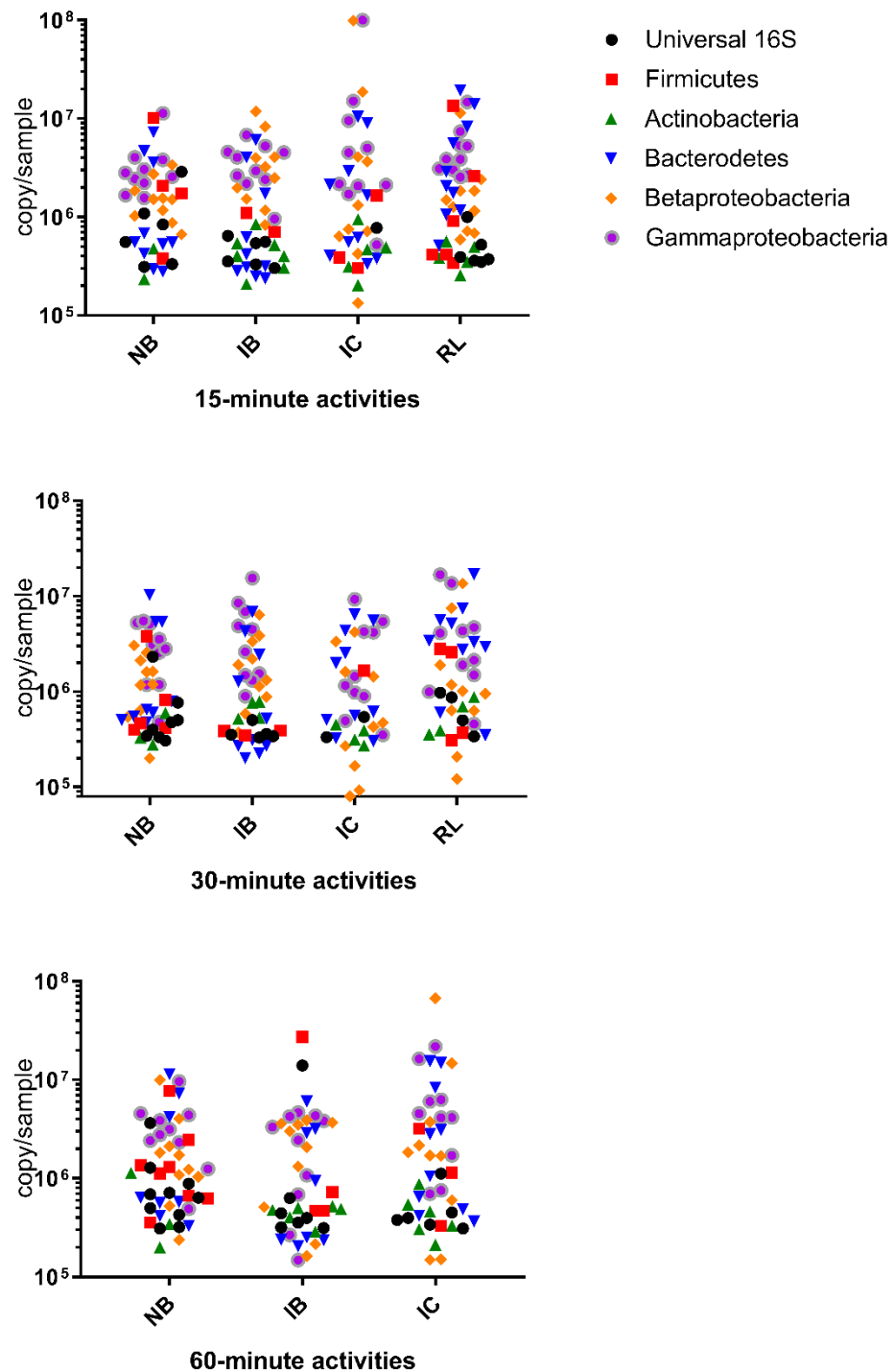
Figure 3.11 The quantity of exhaled phyla does not change between different time periods
67, 74 and 41 data points were below the lower limit of detection for 15-, 30- and 60-min period of sampling, respectively. No statistically significant difference was found between any two time periods of any respiratory pattern using mixed linear regression.

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Table 3.3 Mixed linear regression results showing the P value of comparing bacterial signals obtained by different breathing patterns for the same sampling periods

	Target	NBIIB		NBIIC		NBIRL		IBIIC		IBIRL		ICIRL	
		Med	p	Med	p	Med	p	Med	p	Med	p	Med	p
15 min	*16S*	3.22I3.16	0.12	3.22I0.01	0.01	3.22I3.55	0.16	3.16I0.01	0.09	3.16I3.55	0.82	0.01I3.55	0.06
	Firm	0.01I0.01	0.21	0.01I0.01	0.22	0.01I3.77	0.71	0.01I0.01	0.96	0.01I3.77	0.11	0.01I3.77	0.21
	Actin	0.01I3.52	0.02	0.01I1.02	0.11	0.01I1.28	0.21	3.52I1.02	0.50	3.52I1.28	0.32	1.02I1.28	0.75
	Bact	5.55I3.69	0.91	5.55I11.4	0.56	5.55I24.8	<0.01	3.69I11.4	0.64	3.69I24.8	0.01	11.4I24.8	0.10
	Beta	15.2I28.6	0.72	15.2I10.3	0.09	1.52I13.8	0.91	28.6I10.3	0.24	28.6I13.8	0.83	10.3I13.8	0.25
	Gamma	26.9I34.9	0.99	26.9I33.4	0.10	26.9I38.6	0.80	34.9I33.4	0.16	34.9I38.6	0.84	33.4I38.6	0.32
30 min	*16S*	3.71I1.64	0.07	3.71I0.01	0.01	3.71I0.01	0.27	1.64I0.01	0.35	1.64I0.01	0.46	0.01I0.01	0.10
	Firm	1.99I0.01	0.20	1.99I0.01	0.29	1.99I0.01	0.71	0.01I0.01	0.85	0.01I0.01	0.07	0.01I0.01	0.09
	Actin	0.01I0.01	0.23	0.01I0.01	0.85	0.01I0.01	0.34	0.01I0.01	0.35	0.01I0.01	0.82	0.01I0.01	0.44
	Bact	6.23I4.15	0.77	6.23I13.0	0.74	6.23I33.5	<0.01	4.15I13.0	0.89	4.15I33.5	0.02	13.0I33.5	0.08
	Beta	14.0I16.1	0.48	14.0I4.49	0.81	14.0I9.80	0.22	16.1I4.49	0.38	16.1I9.80	0.65	4.49I9.80	0.26
	Gamma	29.5I35.4	0.30	29.5I13.0	0.90	29.5I31.1	0.23	35.4I13.0	0.30	35.4I31.1	0.90	13.0I31.1	0.24
60 min	*16S*	6.63I3.38	0.02	6.63I3.25	0.02	NA		3.38I3.25	0.98	NA		NA	
	Firm	8.93I0.01	0.06	8.93I0.01	0.12	NA		0.01I0.01	0.43	NA		NA	
	Actin	0.01I3.46	0.40	0.01I2.58	0.37	NA		3.46I2.58	0.95	NA		NA	
	Bact	5.69I2.46	0.90	5.69I19.3	0.07	NA		2.46I19.3	0.07	NA		NA	
	Beta	14.8I25.3	0.97	14.8I17.7	0.17	NA		25.3I17.7	0.23	NA		NA	
	Gamma	29.6I28.8	0.63	29.6I43.6	0.06	NA		28.8I43.6	0.03	NA		NA	

Definition of abbreviation: Med= Median quoted as three significant figures of E+03 value; p= Probability value; NA= No data available; *16S* = Universal *16S*rDNA; Firm= Firmicutes; Actin= Actinobacteria; Bact= Bacteroidetes; Beta= Beta-proteobacteria; Gamma= Gamma-proteobacteria; Green-highlighted= p-value >0.05 – <0.10; Yellow-highlighted= p-value >0.01 – <0.05; Red-highlighted= p-value <0.01.
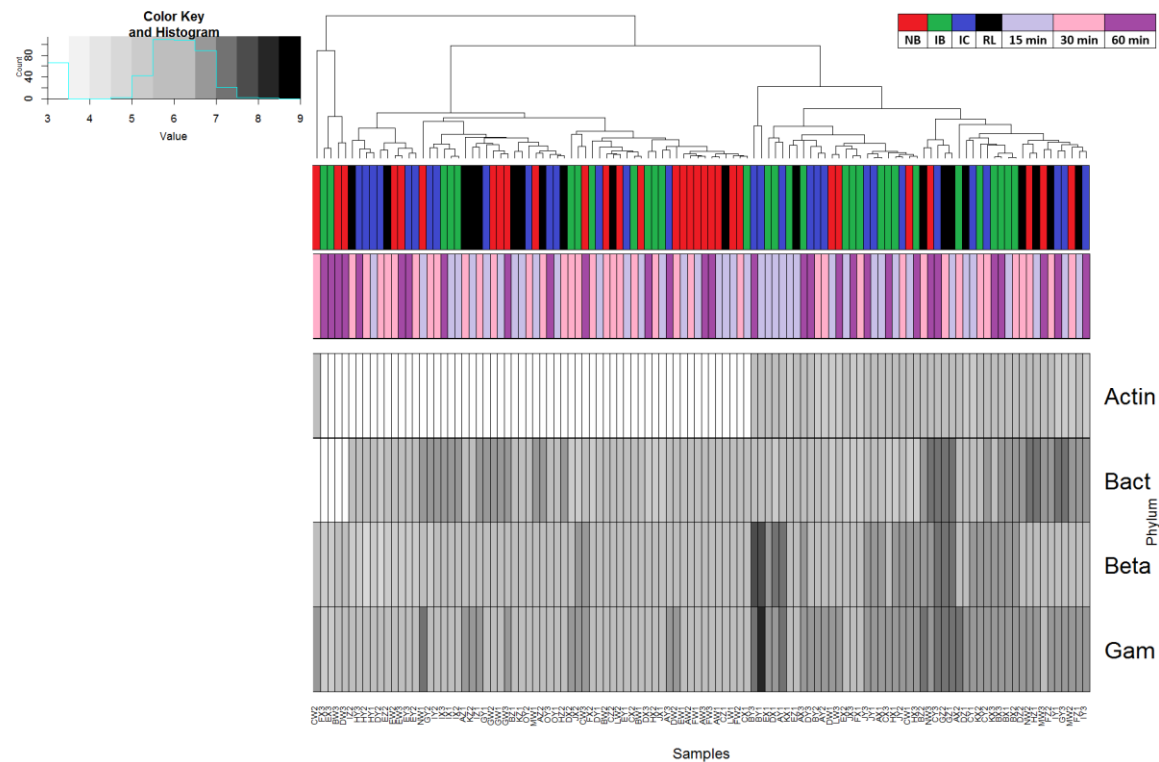
Figure 3.12 Face-mask samples form three major clusters based on abundance of four quantified phyla rather the breathing activity or time of sampling

Clustering was done using Euclidean distance. The two rows above the heatmap represent breathing patterns and time periods with red colour indicates normal breathing (NB), green- instructed breathing (IB), blue- intermittent coughing (IC), black- reading out loud (RL), dark purple- 60 min, pink- 30 min, light purple- 15 min. Each row represents a phylum in a mask sample and each column represents a mask sample. The heatmap is based on the absolute quantities of microbial constituents at phylum level. Actin represents Actinobacteria, Bact- Bacteroidetes, Beta- Beta-proteobacteria, Gam- Gamma-proteobacteria. "R" version 3.4.3 (R Development Core Team; http://www.R-project.org) was used to draw the heatmap and perform a hierarchical clustering.

3.3.7. Association between time of sampling and bacterial signals

Linear regression analysis revealed a general tendency for the bacterial signals to decline after 15 min (Figure 3.13) although this analysis did not reach significance (Table A.26).
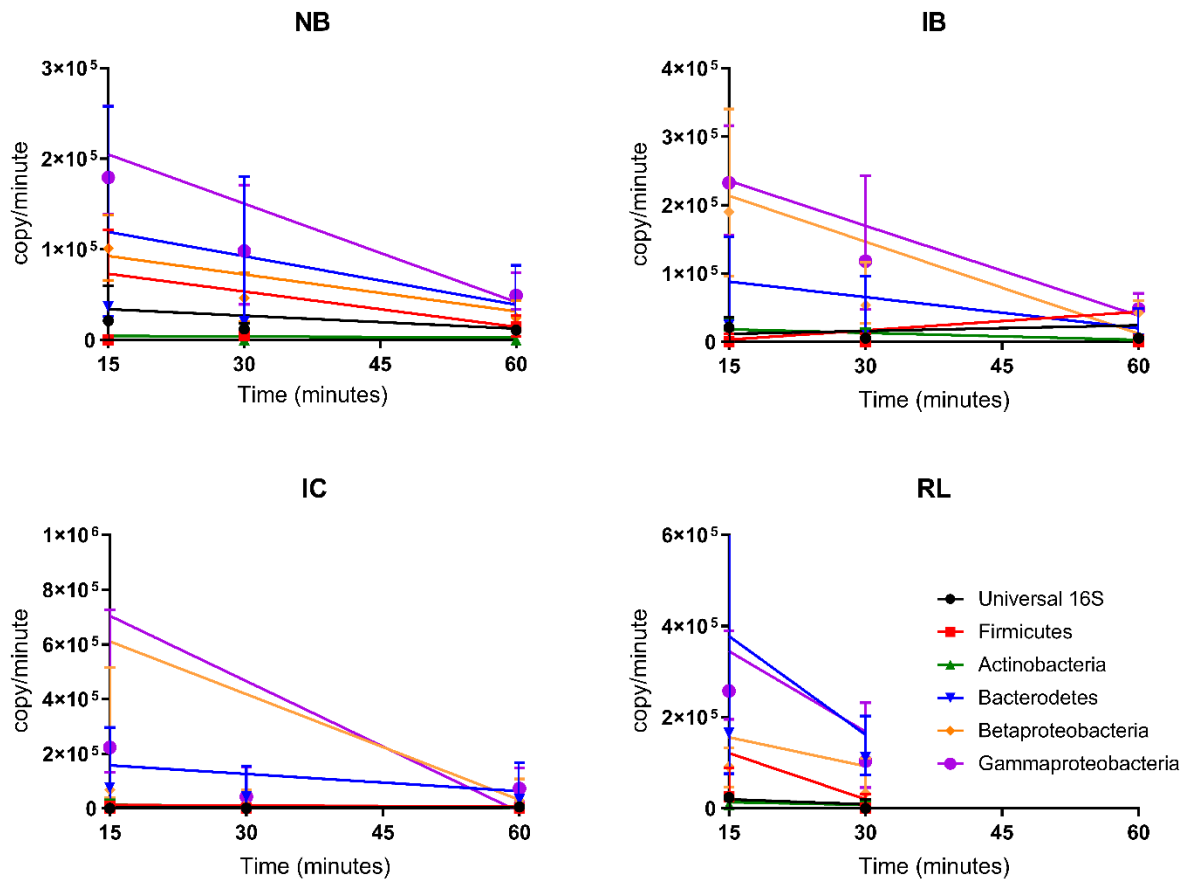
Figure 3.13 Linear regression of exhaled bacteria over time
(Error bars= IQR; n=10).

3.3.8. Potential contributions of lysozyme and DNase

In view of the evidence for lower signals obtained at times above 15 min, the potential contributions of lysozyme and DNase were investigated.

Ten samples collected by RL for 30 min were tested for DNase. All were negative for human DNase with LLD of 0.156ng per mL. In addition, all 110 samples were analysed for DNase activity with the real-time fluorogenic assay. Again, all the samples were negative with LLD of 0.002 units (~4pg) of activity per reaction.

All samples were also assayed for lysozyme activity using fluorescein-labelled *Micrococcus lysodeikticus* cell wall as the substrate. Activity was detected in 54 of 110 samples (Figure 3.14 and Table A.15). Background controls were negative.
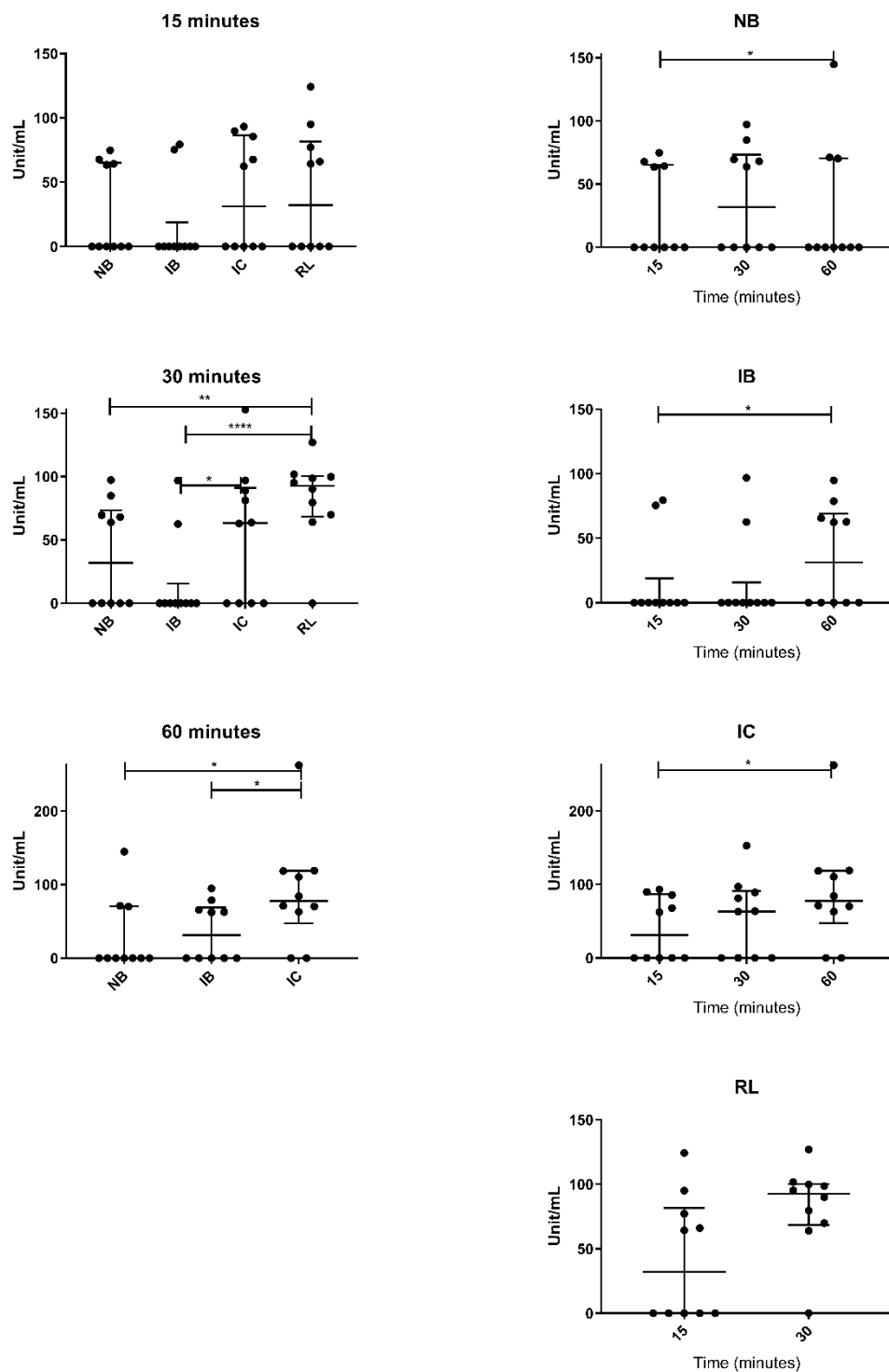
Figure 3.14 The quantity of exhaled active lysozyme of different respiratory activities and time periods
Mixed linear regression was applied. Error bars= IQR; n=10.

Regression analysis again showed positive trends that did not attain statistical significance (Figure 3.15 and Table A.27).
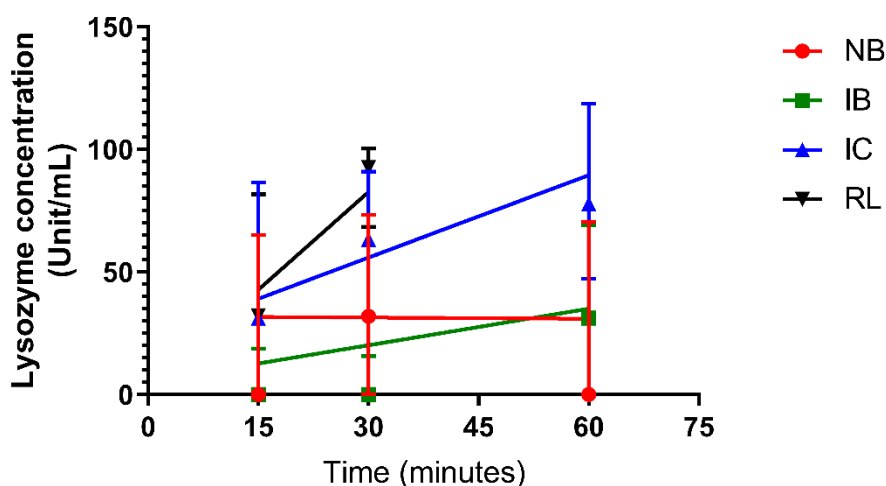
Figure 3.15 Linear regression of exhaled active lysozyme over time
(Error bars= IQR; n=10).

Correlation analysis showed moderate to very weak evidences of associations between lysozyme and PMA-untreated bacterial signals (Table 3.4). Interestingly, the correlations were more frequently negative over 15 min than over 30 and 60 min. The full results are presented in Table A.31.

Table 3.4 Associations between lysozyme and bacterial signals					
Lysozyme vs.		**NB**		**RL**	
		r	P	r	P
15 min	Actinobacteria			0.56	**0.10**
	β-proteobacteria	-0.74	**0.02**		
	γ-proteobacteria	-0.62	**0.06**	0.67	**0.04**
30 min	Actinbacteria			0.62	**0.06**
	Bacteroidetes			0.59	**0.08**
	γ-proteobacteria	0.61	**0.07**	0.58	**0.09**

3.3.9. Relative abundance of bacterial signals and relationships with lysozyme

The relative abundance of four bacterial components of exhaled aerosols was computed for four phyla. Actinobacteria, Bacteroidetes, Beta-proteobacteria and Gamma-proteobacteria were included in figuring the percentage while Firmicutes was excluded to avoid processing-related bias. The calculated percentages are presented in Figure 3.16.

Actinobacteria was more relatively abundant in aerosols produced through IB than other tested patterns. This behaviour was clearly shown over 15 min and with lower confidence at 60 min.

Similarly, Beta-proteobacteria were more relatively abundant in aerosols produced through IB than other tested patterns. This was shown over 15, 30 and 60 min with variable degrees of statistical confidence (Table 3.5).

Gamma-proteobacteria did not show a statistically significant behaviour in its relative abundance between any two respiratory patterns.

Bacteroidetes was more relatively abundant in IC and RL than in IB at 30 min.

Overall, the most interesting pattern was shown by IB and exhaled Actinobacteria and Beta-proteobacteria where the relative abundances of these were greater than with any other breathing pattern, particularly at 15 and 60 min (Table 3.5).
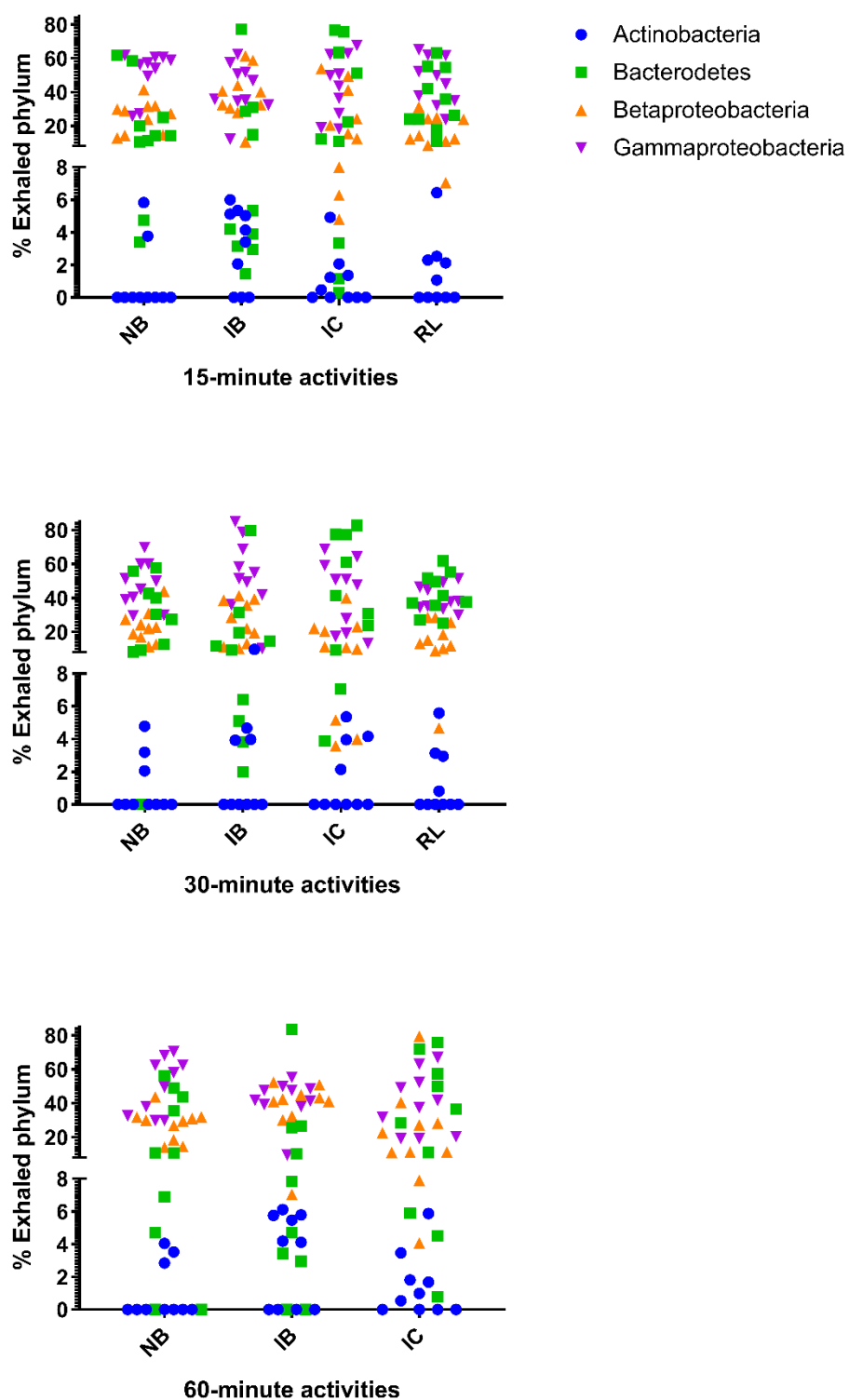
Figure 3.16 The relative abundance of exhaled phyla to each other
The percentage was normalized by adding the quantities of non-treated phyla to each other for making a total of 100%. The ratios were obtained from Table A.16 to Table A.19

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Table 3.5 Mixed linear regression results showing the P value of analysing the ratios of exhaled bacteria to each other (the relative abundance) in different breathing patterns

	Target	NBIIB		NBIIC		NBIRL		IBIIC		IBIRL		ICIRL	
		Med	p	Med	p	Med	p	Med	p	Med	p	Med	p
15 min	Actin%	**0.00l3.77**	0.01	0.00l0.24	0.96	0.00l0.54	0.58	**3.77l0.24**	0.02	**3.77l0.54**	0.05	0.24l0.54	0.57
	Bact%	14.2l4.76	0.53	14.2l17.3	0.39	14.2l31.2	0.19	4.76l17.3	0.12	4.76l31.2	0.06	17.3l31.2	0.83
	Beta%	**28.1l36.2**	0.02	28.1l17.8	0.78	28.1l13.2	0.13	**36.2l17.8**	0.02	**36.2l13.2**	<0.01	17.8l13.2	0.26
	Gamma%	56.7l41.1	0.06	56.7l46.8	0.26	56.7l47.3	0.60	41.1l46.8	0.56	41.1l47.3	0.22	46.8l47.3	0.35
30 min	Actin%	0.00l0.00	0.22	0.00l0.00	0.58	0.00l0.00	0.81	0.00l0.00	0.53	0.00l0.00	0.36	0.00l0.00	0.72
	Bact%	28.8l10.6	0.28	28.8l36.1	0.15	28.8l39.6	0.12	10.6l36.1	0.01	10.6l39.6	<0.01	36.1l39.6	>0.99
	Beta%	22.5l25.3	0.65	22.5l11.1	0.03	22.5l14.1	0.10	**25.3l11.1**	<0.01	**25.3l14.1**	0.03	11.1l14.1	0.14
	Gamma%	47.5l53.2	0.33	47.5l49.3	0.59	47.5l38.0	0.32	53.2l49.3	0.13	53.2l38.0	0.06	49.3l38.0	0.71
60 min	Actin%	**0.00l4.15**	0.02	0.00l0.77	0.66	NA		**4.15l0.77**	0.09	NA		NA	
	Bact%	10.7l6.27	0.61	10.7l32.4	0.30	NA		6.27l32.4	0.12	NA		NA	
	Beta%	**29.8l41.8**	0.08	29.8l16.9	0.74	NA		**41.8l16.9**	0.07	NA		NA	
	Gamma%	53.6l44.7	0.17	53.6l39.6	0.13	NA		44.7l39.6	0.82	NA		NA	

Definition of abbreviation: Med= Median quoted as three significant figures of perecntage value; p= p-value; NA= No data available; Actin%= Actinobacteria to other phyla ratio; Bact%= Bacteroidetes to other phyla ratio; Beta%= Beta-proteobacteria to other phyla ratio; Gamma%= Gamma-proteobacteria to other phyla ratio; Green-highlighted= p-value >0.05 – <0.10; Yellow-highlighted= p-value >0.01 – <0.05; Red-highlighted= p-value <0.01; Numbers made in **bold** show a pattern.

Regression analysis revealed significant negative correlation between the ratio of exhaled Beta-proteobacteria to other phyla and active lysozyme exhaled by NB over 15 min (r= -0.70, p= 0.03) while there was weak evidence of this relation during IB over 60 min (r= -0.58, p= 0.09). The former activity showed a weak evidence of positive correlation for Bacteroidetes (r= 0.62, p= 0.06). A significant positive correlation was found for the Actinobacteria ratio to other phyla and lysozyme exhaled by RL over 30 min (r= 0.70, p= 0.03). The full results are presented in Table A.32.

3.3.10. Associations between exhaled protein biomarkers and bacterial signals

The most significant correlations between exhaled protein biomarkers and bacterial signals are summarised in Table 3.6.

Table 3.6 Significant correlations[*] between protein biomarkers and bacterial signals

min		SP-A	Albumin	Amylase
15	**Firmicutes**		RL (r=**-0.70**, p= 0.03)	
	Actinobacteria	IC (r= **-0.73**, p= 0.02)		
	Bacteroidetes	RL (r=0.71, p= 0.03)	NB (r=0.66, p= 0.04)	
30	**Actinobacteria**		NB (r=0.63, p= 0.05)	
	Bacteroidetes		RL (r=0.75, p= 0.02)	NB (r= 0.64, p= 0.05); IB (r= 0.70, p= 0.03)
	β-proteobacteria	RL (r=0.65, p= 0.05)	RL (r=0.71, p= 0.03)	
	γ-proteobacteria		RL (r=0.76, p= 0.01)	
60	**Bacteroidetes**	IB (r= 0.68, p= 0.04)		
	γ-proteobacteria	IB (r= 0.67, p= 0.04)		

* Presented as breathing pattern (Spearman's rank correlation coefficient, Two-tailed p value). The full correlation results are presented in Table A.33 and Table A.34.

Yellow shaded cells represent negative correlations

Overall, Bacteroidetes signals were the most frequent phylum in showing associations with SP-A and albumin (LRT biomarkers) and with salivary α-amylase (URT biomarker) (six statistically significant correlations over 15, 30 and 60 min). RL was the most frequent pattern (six statistically significant correlations over 15 and 30 min) and 30 min was the most frequent time (seven statistically significant correlations over different breathing patterns) in showing associations with SP-A, albumin and α-amylase.

The full results are presented in Table A.33 and Table A.34.

3.3.11. Relationships between different breathing patterns and the ratios between captured proteins

The ratios of concentration of exhaled albumin to exhaled SP-A were computed after the quantification units were normalized. Arbitrary ratios of concentrations of exhaled active α-amylase, lysozyme and protease to SP-A were each calculated. These were then examined to investigate whether the ratios are constant over time and between different breathing patterns. This would further assist in conceptualizing the behaviour of aerosols in terms of the biological content. The computed ratios are presented in Table A.20 to Table A.23.

The ratios of albumin to SP-A and α-amylase to SP-A were found more stable over time while they were varying between the four tested breathing patterns. By contrast, the ratios of lysozyme to SP-A and protease to SP-A were found stable over time and between the four tested breathing patterns (Figure 3.17).

The detected amounts of exhaled active protease were tested for a possible correlation with the ratios of albumin to SP-A, α-amylase to SP-A and lysozyme to SP-A. There were no statistically significant correlations (Table A.35 to Table A.37) apart from a positive one found with the ratio of amylase to SP-A exhaled in NB over 30 min (r=0.69, p= 0.03).
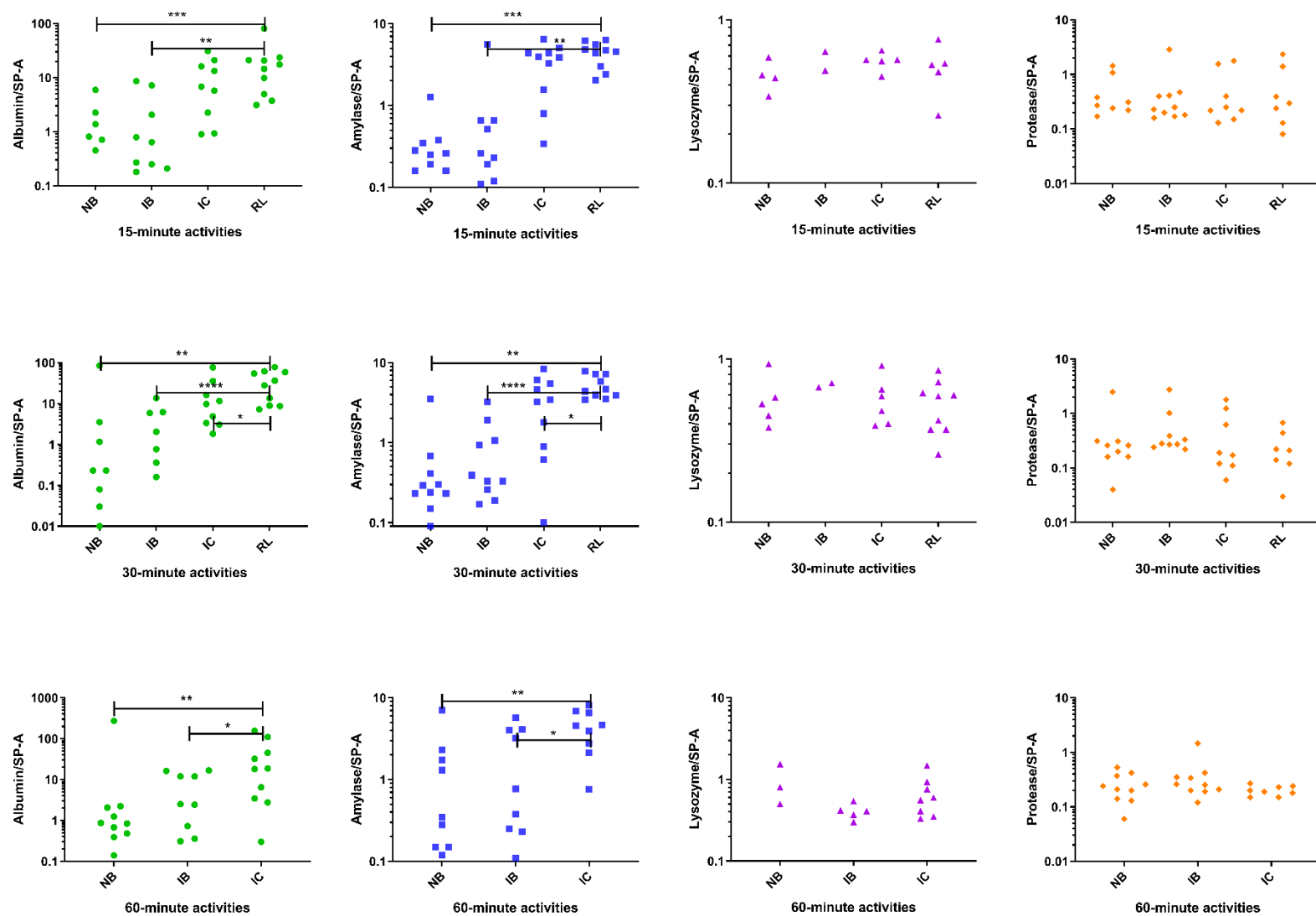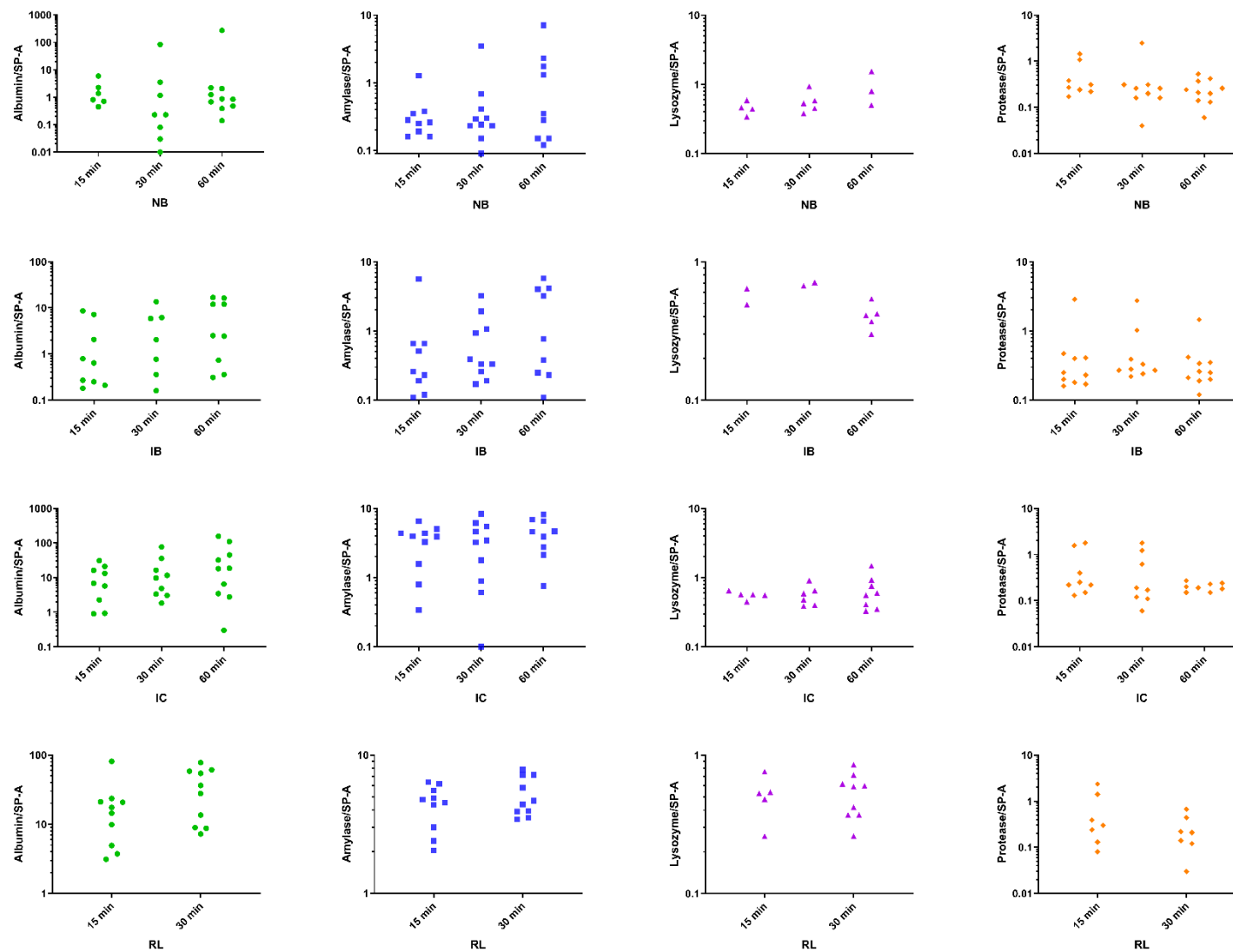
Figure 3.17 Ratios of exhaled proteins between different breathing patterns and over time
Mixed linear regression was done to determine statistically significant differences

3.4. Discussion

3.4.1. SP-A and albumin collected with different respiratory activities

It was hypothesised that a respiratory activity most productive of proteins localised in the lungs could be the most efficient for sampling the LRT using the mask system.

SP-A and albumin were selected as biomarkers of lung proteins based on what is known histologically alongside what has been previously found, particularly in PEx studies (Almstrand *et al.*, 2009 and 2010; Larsson *et al.*, 2012 and 2015; Bredberg *et al.*, 2012; Larstad *et al.*, 2015) as appearing to be the most relevant to this work.

The amounts of SP-A and albumin were compared in four respiratory activities feasible in different clinical settings. SP-A was detectable in all face-masks while albumin was detected in 88% of them (97 of 110 masks). This is in agreement with the findings of Larsson *et al.* (2012) showing 100% SP-A detection rate in PEx samples. The detectability of SP-A in all our face-mask samples and all PEx samples indicates that SP-A is a main component of exhaled aerosols.

RL was the most productive of exhaled SP-A and albumin. This indicates either performing this activity could improve mask sampling efficiency in capturing aerosols generated from the LRT (assuming SP-A is a specific biomarker of LRT) or the ability of this pattern to produce largest amounts of aerosols regardless whether SP-A was expressed in the URT or LRT (since SP-A was found a main component). In fact, RL can lead to a higher minute volume compared to NB, IB and IC.

RL was a simulation to talking. These findings could be supportive to the generally accepted argument that air during phonation is forcibly exhaled from

the lungs and rushes upwards through the gaps between the true and false vocal cords.

Several studies, however, have reported pulmonary surfactant phospholipids and SP-A in extrapulmonary sites. Schicht *et al.* (2013) described the expression of mRNA for SP-A in sinonasal mucosa in physiological and pathological conditions. The pathophysiological expression was reported even earlier by Wootten *et al.* (2006) and Woodworth *et al.* (2006). Nevertheless, SP-A has been known a recognised biomarker of pulmonary tissue taking its abundance in the alveolar compartment into account. Moreover, one could argue that if SP-A detected in this study was of nasal origin, its quantity then was more likely to be higher in samples obtained during NB in which nasal breathing often dominates, but this was not the case.

Vascular permeability is believed to result in leakage of plasma proteins into the airways and thus responsible for the abundance of albumin in RTLF. Albumin as an LRT biomarker lacks specificity but has been investigated by Larsson *et al*. (2012) and other PEx studies as a component of aerosols supposedly generated from LRT.

However, utility of albumin as a marker of increased alveolar capillary permeability in asthmatics was not supported by Larsson *et al*. (2015). In contrast, a trend for both exhaled albumin and SP-A to decrease with inflammation was observed. These authors suggest that during inflammation albumin content of RTLF is actively down-regulated (P Larsson, personal communication).

Contribution of nasal SP-A to mask samples could be minimized with applying nasal clips. Prolonged clipping, however, can stimulate nasal secretions draining into nasopharynx and provide an alternate pathway to contaminate exhaled material. This problem might be better overcome by targeting biomarkers exclusive to the alveolar epithelium such as: alveolar epithelial

cells type-I (AEC-I) antigens, Clara cell Protein 16 (CC16, CC10), Clara Cell Phospholipid-Binding Protein (SCGB1A1), alapa-1 antitrypsin or mucin-associated antigens (KL-6, 17-Q2, 17-B1). In a small trial (three subjects), it was possible to detect CC16, and this was in agreement with the PEx proteomic analysis of Bredberg *et al.* (2012).

Jones and Brosseau (2015) in their review claim that "*aerosols generated from infectious people are subject to the same transport processes that govern other aerosols*". If our findings were validated on TB patients, one could postulate an association between infectiousness and abundance of SP-A, with some allowance for highly infectious but rare laryngeal TB.

If it is agreed that RL is the most productive of LRT aerosols carrying pathogens, this potentially implies that talking, the simulator activity, is a more transmissible behaviour of airborne lung infections than other tested activities. Coughing was reviewed by Turner and Bothamley (2015) as a more important "infectious" behaviour in TB than talking. It could be argued that the latter received less attention since the general status of depleted "infectious" patients results in making sustained talking (15 min at least) unpracticable for them. Furthermore, we revealed in a recent study a dissociation between the frequency of normal coughs of TB patients over a prolonged period (24 hrs) and the positivity of their face-masks (Williams *et al.*, 2018).

Indeed, RL activity was not conducted for periods longer than 30 min because it is impracticable in clinical settings. If it was done, however, it was likely to result in similar findings. Compared to other respiratory patterns involving instructions, this activity is more controllable intra- and inter-subjects, particularly in poor-resource settings.

The IB pattern used here did not closely follow that used in PEx studies. While there is no definitive proof that airway reopening generates particles in small

airways (<2mm in diameter), this hypothesis is generally accepted (P Larsson, personal communication). Further studies investigating the aerosol output of other breathing patterns to induce frequent re-openings of the small airways would seem warranted.

Turning to the impact of different sampling times, these were used to investigate changes in exhaled material over time and to evaluate a period that could effectively sample LRT-generated aerosols in practice with minimal inconvenience.

There were no differences in the amounts of exhaled lung proteins, represented by SP-A and albumin, when time of sampling was increased from 15 to 30 min or from 30 to 60 min. This indicates that a 15 min duration suffices to sample the airways using the face-mask system. This should not be interpreted, however, as increasing time of sampling would not increase the captured amounts of exhaled proteins. Indeed, there was a trend of linearity between time of sampling and the amounts of captured proteins. The small sample size might have been behind that this clear trend was not confirmed statistically. Presence of protein-degrading factors might have also contributed, warranting further investigation (3.3.2 and 3.4.2). In addition, it might also be worth studying the impact of shorting the sampling time further (less than 15 min). On the other hand, this linearity was confirmed statistically only for SP-A indicating further that SP-A is a main component of exhaled aerosols.

The shown behaviour can also indicate that the exhaled burden does not statistically differ between 15 and 30 min. One could ask, if this was validated on the microbial component in airborne infections, whether an exposure time of 15 min to an infectious subject would not differ from being exposed to that source for 30 min.

Valway *et al*. (1998) reported extensive transmission of some strains of Mtb after time as short as 2 hrs of exposure to an index case. On the other hand, Dollner *et al*. (2012) reported only two cases developed latent TB after short-term exposure (4–12 hrs).

Indeed, the detectability of lung proteins (and bacteria as shown later) in aerosols produced in 15 min in all tested breathing patterns suggests that brief exposures are sufficient to be considered a risk factor for susceptible hosts, taking into account the agent virulence factor. The minimal time sufficient to transmit an airborne infection remains unknown and requires extensive studies.

3.4.2.   Protease assays

It is known that almost all proteins can be degraded through either protease- or proteasome-mediated pathways.

It was suggested that protease activity could have limited the abundance of exhaled proteins in the mask samples. Such activity was detected in 86% of the face-masks while in none of the background controls. This indicates that active protease was exhaled and could have contributed to the lack of time-dependent accumulation of other protein signals.

The detectability of protease in the majority of face-masks may indicate that protease is a component of aerosols, irrespective of whether these were generated from the upper or lower airways. For that reason, probably, the amounts were found stable among different breathing patterns and over time.

Regarding the origin and the expression site of protease, despite respiratory epithelial cells are known to secrete soluble factors such as cytokines, chemokines and host defense mediators including proteases and antiproteases, protease could also be secreted from bacteria inhabiting the respiratory tract. The used kit was not designed to differentiate in detection between human and bacterial protease activity.

The detectability of protease was in partial agreement with the PEx proteomic profiling (Bredberg *et al.*, 2012) where the identifiability rather the activity was tested. It appears that this is the first study where exhaled active protease was directly detectable and quantifiable.

Correlation studies, however, did not provide a quantitative explanation for our results. In contrast, there was a positive correlation between protease activity and SP-A levels. Thus, it can only be concluded that protease may have contributed and that there may have been different susceptibilities to degradation amongst the proteins assayed.

At a general level, proteins are sensitive toward pH, temperature and different enzymatic activities. The storage conditions and the processing method could have played a role. Whether or not this was the case, the impact can be consistent for all obtained samples.

One could suggest an alternative approach to investigate protein-degradation by assaying the mask extracts with Western blot after tagging SP-A or albumin. In case of degradation, this would show no band or multiple bands having lower molecular weight than that of protein of concern. However, the low concentration of exhaled proteins even after concentrating the extracts precluded going through this approach.

The detection of, at least, three proteins in the face-masks indicates that each sample was a pool of proteins (which were not purified in this study). Therefore, assaying protease activity could have been a subject for competitive inhibitors. For instance, α-1 antitrypsin was detected in PEx samples (Bredberg *et al.*, 2012) and its attendance in the face-masks should not be surprising. This could be devised, however, to prevent degradation through the protease-mediated pathway. We are working on developing PVA sampling matrix as a modifiable capturing surface which can contain high concentrations of these inhibitors. Similarly, inducing ubiquitylation or adding proteasome-inhibitors like peptide aldehydes (carbobenzoxyl-leucinyl-leucinyl-norvalinal MG115 or carbobenzoxyl-leucinyl-leucinyl-leucinal MG132) could prevent degradation via the proteasome-mediated pathway. These manipulations are worth being applied when detecting lung proteins in face-masks is sought for quality control.

3.4.3. Salivary α-amylase assays

Salivary amylase was measured to assess contributions from the oral cavity to the mask samples.

α-amylase was detected in 95.5% of the samples and this indicates that the collected material was not solely LRT in origin. RL which was the most productive of LRT biomarkers (SP-A and albumin) was also found, unsurprisingly, the most productive of salivary α-amylase. A number of possibilities arise.

First, the exhaled material was contaminated with salivary secretions. Bredberg *et al.* (2012) claimed no amylase was detected using liquid chromatography combined with mass spectrometry in their PEx samples pooled from more than 3,000 liters of exhaled air. Selecting exhaled particles of a specific size (0.3–20μm), being collected over a very short period and the undisclosed limit of detection of their assays could have been behind their inability to reveal these particles' contamination with URT secretions. Furthermore, their ruling out of PEx-technique contamination with saliva was overconfidently based on detectability of α-amylase which has a molecular weight of 62.0 KDa (Ito *et al.*, 1992). However, many salivary proteins with lower molecular weight were detected in the PEx samples. For instance, Ghafouri *et al.* (2003) identified zinc-α2-glycoprotein (34.5 KDa), apolipoprotein A-I (30.8 KDa), lipocalin 1 (19.4 KDa), prolactin-inducible protein (16.9 KDa), cystatin S (16.5 KDa), fatty acid-binding protein (14.8 KDa), β2-microglobulin (13.8 KDa) and calgranulin A (10.9 KDa) in human saliva. Similarly, α1 acid glycoprotein 1 (23.7 KDa) was also identified in salivary specimens of smokers and non-smokers by Jessie *et al.* (2010). All these were among the PEx proteomic profiling of the Bredberg group.

Although α-amylase is the most abundant protein in saliva constituting more than 50% of total salivary proteins (Vitorino *et al.*, 2004), its absence in a

sample does not imply the absence of other salivary proteins, particularly those of lower molecular weight. Given that aerosols and droplets are exhaled through the oronasal cavity, it is almost impossible for a collected sample to escape gross or microscopic contamination of oral and/or nasal secretions.

The contamination could be minimized, however, through applying a saliva trap or a nasal clip. The question would be then what the desired target on a mask sample is. Applying a salivary trap can be useful in getting rid of heavy contamination with saliva, but it might do so at the expense of the collection yield of the microbial component. For example, Jain *et al.* (2007) and Kawada *et al.* (2008) failed to detect Mtb by PCR and culture in EBC samples of patients with active TB. While they did not clearly mention use of a salivary trap for collecting EBC, this recommendation was already made by the American Thoracic Society and European Respiratory Society Task Force on EBC (Horvath *et al.*, 2005). On the other hand, Zheng *et al.* (2018) succeeded in detecting many bacteria using loop-mediated isothermal amplification with an EBC design which did not integrate a salivary trap. Furthermore, even after their more careful exclusion of simple saliva contamination of EBC by applying a salivary trap and not detecting α-amylase or salivary phosphorus, Griese *et al.* (2002) found that the majority of EBC proteins were present in saliva.

The **second** possibility is that the material captured on the face-mask was a mixture of aerosols and droplets produced in the upper and lower respiratory tract. It was acknowledged earlier that this system was not designed to differentiate in collection between aerosols and droplets. Effros *et al.* (2005) concluded that saliva is the origin of around 10% of droplets generated from the epithelial lining fluid of the airways. Fabian *et al.* (2011) concluded that both upper and lower airways contribute to the production of exhaled particles. The contribution of the URT is demonstrated by the significant amounts of α-amylase detected in all tested breathing patterns.

The distances between the face-mask and the nostril and mouth openings were between 1.5–6 cm and 2 cm, respectively (Figure 3.18). Thus, all exhalable material and capturable at these distances should be assumed collected. If PEx samples were truly negative for α-amylase, this alongside our findings could indicate that small exhaled particles (<20µm) preferentially include low molecular weight salivary proteins while larger droplets (20–1500µm; Xie *et al.*, 2009) include both high and low molecular weight proteins.
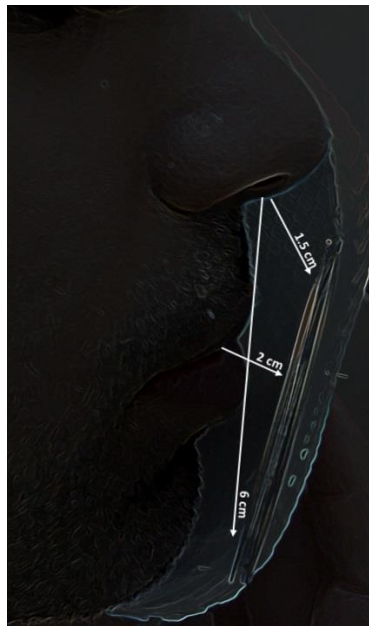
Figure 3.18 Longitudinal section of the face-mask showing the distances between the capturing surface and the openings of oronasal cavity

A **third** possibility is that the LRT is contaminated with oral cavity secretions associated with microaspiration, a recognised feature in lung microbiome studies (Segal and Blaser, 2014; Bassis *et al.,* 2015; Dickson and Huffnagle, 2015; Dickson *et al.*, 2016a and 2016b and 2017).

Quinn and Meyer were among the first who demonstrated aspiration in healthy subjects after showing on bronchograms intranasally-administrated lipiodol

during sleep (Quinn and Meyer, 1929 cited in Clarke *et al.*, 1981). "*Silent*" aspiration was described in healthy people to occur mainly during sleep, and a physiological clearance of aspirates without complications was suggested (Amberson, 1937; Hamelberg and Bosomworth, 1968 both cited in Bartlett *et al.*, 1974). Huxley *et al.* (1978) and Gleeson *et al.* (1997) showed, devising a radioactive tracer, aspiration of pharyngeal secretions in normal subjects during sleep. While the former group stressed on deep sleep for aspiration to take place, the latter did not associate that with the sleep quality. Clarke *et al.* (1981) showed salivary amylase activity in bronchial secretions obtained through percutaneous transtracheal aspiration from patients who were not fully comatose. Filloux *et al.* (2013) studied tracheal to oropharyngeal amylase ratio as a marker for microaspiration in mechanically-ventilated patients, detecting amylase in 75% of tracheal samples obtained during bronchoscopy in their control group (patients from their respiratory department). More recently, Almeida *et al.* (2015a) demonstrated microaspiration of saliva in healthy subjects.

On the other hand, some suggested that detection of α-amylase in BALs is a marker to screen for aspiration pneumonia or other lung disease in different age groups (Tripathi *et al.*, 2011; Bai *et al.,* 2013; Weiss *et al.*, 2013; Abu-Hasan *et al.*, 2014; Samanta *et al.*, 2018). In fact, this suggestion indirectly indicates that they found, or claimed, BALs under normal conditions were free of salivary amylase, and can to some extent limit the applicability of microaspiration for healthy subjects. However, Dewavrin *et al.* (2014) concluded that using α-amylase as a marker in tracheal aspirates is a worthless test for diagnosing oropharyngeal microaspiration in critically ill patients based on the area under the receiver operating curve.

Confirming or refuting microaspiration in a more rigorous way could be achieved by detecting pepsin as a biomarker of microaspirating gastric contents (Jaoude *et al.*, 2010), which is not known so far to be physiologically

expressed in respiratory epithelium. However, one of the main limitations is its narrow limit for detection (as short as few hrs) in tracheal aspirates (Metheny *et al.*, 2006).

The **forth** possibility is that amylase is secreted in the LRT. Ende (1961) noted "*a fair amount of amylase-like substance in normal lungs*". Skude (1975) reported salivary amylase activity in extracts of pulmonary tissues without clarifying whether these were from healthy subjects. Otsuki *et al.* (1977, two reports) and Maeda *et al.* (1982) demonstrated this activity in normal pulmonary tissue extracts. Berk *et al.* (1978) reported this in extracts obtained from three normal lungs without commenting whether its presence was due to pulmonary secretion or contamination with the salivary one. Sano *et al.* (1986) detected this activity in tracheobronchial fluid and normal lung tissues of all cases from a range of respiratory diseases. However, this alone does not confirm that α-amylase was secreted from the LRT epithelium. In fact, Hayashi *et al.* (1986) reported amylase activity in the bronchial rather than the alveolar epithelium. Seyama *et al.* (1994) reported co-expression of *AMY1* gene (salivary type) and *AMY2B* gene (pancreatic type) in normal and tumorous pulmonary tissue. Tsukawaki *et al.* (1992) reported a case of a small cell carcinoma in which the level of amylase was higher in specimens taken from the neoplastic tissue than in those taken from the case's normal lung tissue. Many further reports described secretion of amylase, mostly the salivary type, from lung cancer cells including adenocarcinomas and others (Weiss *et al.* and Luhr, 1951; Ende, 1960; Harada and Kitamura, 1971; Sudo and Kanno, 1976; Morohoshi *et al.*, 1980; Martin and Sarma, 1982; Tomita *et al.*, 1988; Kamio *et al.* and Nakamura *et al.*, 1989; Kitazawa *et al.* and Takano *et al.*, 1993; Lenler-Petersen *et al.*, 1994; Nakao *et al.*, 1996; Benedetti *et al.*, 2004; Sakai *et al.*, 2005; Yokouchi *et al.*, 2006; Yanagitani *et al.*, 2007; Nakatsuji, 2008; Ko *et al.*, 2008; Zhang *et al.*, 2013; Minami *et al.*, 2014; Wang and Wu, 2016). Furthermore, Nandapalan *et al.* (1995) proved the

presence of α-amylase and discussed its activity in tracheobronchial secretions of laryngectomized patients with a normal lung.

Whichever of the above four mechanisms was relevant to our samples, given the aerosols are exhaled through the oronasal cavity, the oronasal secretions must have contributed to the results.

3.4.4. Correlations between biomarkers

SP-A and albumin and α-amylase were selected as LRT biomarkers and an URT biomarker, respectively. As mentioned earlier, this was based on what is known histologically (Figure 3.1) alongside what has been previously found in the PEx studies.

The strong correlations found between α-amylase and albumin indicate that the latter was of an URT source. However, if this assumption holds true, a similar correlation between SP-A and albumin should indicate the latter is also of LRT source. This was tested, and the quantities of exhaled SP-A and albumin were correlated ($r=0.26$, $p=0.005$).

In fact, albumin lacks the specificity criterion more than SP-A which is also expressible in sinonasal mucosa. Similarly, α-amylase is also detectable in LRT as discussed earlier. Therefore, it cannot be stated with a definitive certainty that SP-A and albumin are specific to the LRT and α-amylase is a specific biomarker of the URT.

At least two aspects should be discriminated based on the question being asked. The specificity of the used biomarker in terms of origin from different parts of the respiratory tract, and the informativity of the collected material in terms of yield or "productive exhalation". In other words, to determine whether the aim is to collect solely LRT in origin material, or to collect a material rich in exhalable particles. In terms of the former, more specific LRT biomarkers are required, such as AEC-I antigens, CC16, CC10, SCGB1A1, alapa-1 antitrypsin or KL-6, 17-Q2, 17-B1 antigens, taking into account the microaspiration impact on the specificity. In terms of the latter, α-amylase as well as SP-A and albumin seem sufficient indicators.

The challenge lies in collecting a yieldful LRT material. It is noteworthy that for diagnostic purposes based on microbial detection, a material solely of LRT origin is not generally considered and does not outweigh a pathogen detection.

3.4.5. Background *16S* rDNA bacterial signals of gelatine filters

The face-mask system integrates a gelatine filter to capture exhaled air particles. These filters have been extensively used or recommended to detect airborne viruses and bacteria in human and non-human primates (Jaschhof, 1992; Neve *et al.*, 2003; Landman *et al.*, 2004; Tseng and Li, 2005; Burton *et al.*, 2005 and 2007; Yao and Mainelis, 2006; Verreault *et al.*, 2008; Rule *et al.*, 2008; Fabian *et al.*, 2009; Yao *et al.*, 2009a; Eninger *et al.*, 2009; Wu *et al.*, 2010; Zhao *et al.*, 2011 and 2014a; Prussin *et al.*, 2014; Zuo *et al.*, 2014).

Most of these studies and reviews describe culturing as a downstream assay and the gelatine is guaranteed "sterile" for this purpose. For using molecular assays, however, filters should be free from the molecules to be detected.

Gelatine is a mixture of peptides and proteins derived through partial hydrolysis of animal tissues containing collagen such as skin, bones and connective tissues. The tissues involved in manufacturing are obviously a subject to heavy contamination with different microorganisms during husbandry and in abattoirs.

Gelatine filters used in this work are commercially sterilized by gamma irradiation which is effective for killing microorganisms but not for eliminating their nucleic acids. Deragon *et al.* (1990) show plasmid DNA remained amplifiable in samples having more than 10^5 copies after being irradiated with gamma (200 KR). However, Trampuz *et al.* (2006) demonstrated that gamma irradiation had less effect on amplifiable DNA in viable bacterial cells than on free DNA.

The dose of gamma irradiation applied to the filters used here was not disclosed. Trampuz and colleagues found that irradiation was not sufficient to eliminate amplifiable DNA at their highest dose (12 KGy), indicating that this exposure would not degrade intracellular DNA.

The filters used here were significantly contaminated with bacterial *16S* and Firmicutes DNA. This precludes using them to assay these targets.

It may have been inappropriate to using collagenase derived from *Clostridium histolyticum* (a Firmicutes member) although this reagent was molecularly decontaminated (2.2.1). However, comparable quantities of *16S* and Firmicutes signals ($\sim 10^6$ copies) were present in filters hydrolysed with NaOH (collagenase-free) and these were less than 10^3 copies in the blank reagents (gelatine-free). Furthermore, we found elsewhere evidence that these filters were also contaminated with six antimicrobial resistance genes (*AcrA-05*, *AmpC*, *CfxA*, *FOX-5*, *PBP2X*, *tetA*) (Kennedy *et al.*, 2018).

Nonetheless, gelatine filters seem not to be significantly contaminated with nucleic acids of many bacteria, archaea and viruses (Van Droogenbroeck *et al.*, 2009; Yao *et al.*, 2009b; Fabian *et al.*, 2011; Spekreijse *et al.*, 2013; Hatagishi *et al.*, 2014; Ladhani *et al.*, 2017; and Nehme *et al.*, 2009). Of course, studies on Mtb from this laboratory can also be included (Williams *et al.*, 2014).

Sarkar and Sommer (1990) and Furrer *et al.* (1990) proposed applying UV irradiation and DNase treatment for PCR samples to eliminate DNA contamination. When this was trialed on gelatine, the background was reluctant to high UV dosage ($\sim$1 Joule) and DNase (10 µg/mL) treatment, indicating heavy and/or intracellular DNA contamination.

Shaw *et al.* (2008) compared four sterilization methods (UV, gamma and beta irradiation and ethylene oxide treatment) to assess their ability in eliminating contaminating DNA for DNA profiling. They found that ethylene oxide treatment (4hr exposure) was the most efficient technique to eradicate DNA. Another version of these filters was offered by the manufacturer disclosing that the raw material of the new version had to be gassed with ethylene oxide (Sartorius, personal communication). This was claimed "DNA-free" and

individually packaged. When tested, however, the new version showed only one log fold reduction of contaminating DNA, was more fragile and prohibitively expensive.

The background contamination of gelatine filters was significantly reduced when they were treated with PMA. PMA is a is a cell membrane-impermeable dye that covalently intercalates accessible nucleic acids. This renders DNA strands unelongatable with polymerase and subsequently non-amplifiable.

There are several limitations for using PMA to discriminate between viable and non-viable cells discussed elsewhere (Taylor *et al.*, 2014; Seinige *et al.*, 2014; Li *et al.*, 2017). The aim of applying PMA in this study, however, was merely to reduce the background contamination rather to identify viable and non-viable targets. The response shown to PMA suggests that the gelatine harbours extracellular bacterial DNA and/or PMA-permeable bacterial cells.

Nevertheless, using PMA to quantify only Firmicutes can bias the results if comparison with non-treated signals is sought. One could suggest quantifying all targets post-PMA treatment to avoid such bias. In addition to unavoidable loss of signals that this suggestion would result in, the aim was not to quantify PMA-accessible bacteria, taking all limitations of PMA treatment into account.

To overcome this, with colleagues in the Engineering department we are working, as introduced earlier, on PVA sampling matrix as a capturing surface that is sterilisable and structurally modifiable.

3.4.6. Bacterial signals produced by different respiratory activities

*16S*rDNA is present as multiple copies in genomes of most bacterial kingdom species (Case *et al.*, 2007). However, the quantities of exhaled bacterial *16S* signals were artificially lower than that of each quantified phylum. This is because of PMA-treatment applied to DNA extracts analysed for *16S* quantification. It demonstrates how using gelatine-based material can undermine, even after treatment, its serviceability in studying targets the material harbours. Nevertheless, the detected *16S* signals could reflect the exhaled quantities of viable or PMA-inaccessible bacteria. Interestingly, the quantities of such bacteria were higher in NB than in IC. This suggests possible presence of factors exhalable more abundantly during coughing, degrading exhaled bacterial signals.

Abundance of Firmicutes is well known in the respiratory tract (1.5), however it was detectable only in 39% of all obtained masks (43 of 110 samples), very likely due to the unavoidable PMA-treatment.

Actinobacteria was detectable in 44% of all face-masks (49 of 110 samples) and was the least abundant among all tested phyla. This might either reflect paucity of Actinobacteria harboured in the respiratory tract or might imply that members of this phylum exhibit characteristics rendering them less aerosolisable or exhalable. Cell hydrophobicity has been discussed as a basis for some mycobacterial strains being more aerosolisable (Jankute *et al*., 2017). Additional cellular features of aerosolisable microorganisms were discussed earlier (2.1.1.1). A third possibility could be differential effects of co-exhaled bacterial signal-degrading factors (see below).

Bacteroidetes and Proteobacteria are well known to inhabit the oral cavity (Aas *et al*., 2005; Dewhirst *et al*., 2010) and more recently known to shape the LRT microbiome (Dickson *et al*., 2013) based on the microaspiration hypothesis (Venkataraman *et al*., 2015). For this reason, irrespective of whether the

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aerosols were originated from the upper or lower airways, these phyla were the most abundant in the vast majority (95% and 100%, respectively) of samples.

In fact, if the microaspiration hypothesis was proven true, it would at least complicate differentiating the URT microbiome from the LRT one in terms of the identity, if that remains possible.

Some significant differences were found between the quantities of phyla exhaled by different breathing patterns when these were compared based on the type of breathing and the phylum identity (Table 3.3).

Actinobacteria was more exhalable through IB than in NB pattern. This indicates the former can be devised as a more efficient manoeuvre in sampling exhaled Actinobacteria. Bacteroidetes was more abundantly exhalable through RL than NB and IB, indicating the former is more efficient for sampling this phylum.

The quantities of Beta-proteobacteria showed a trend to be more exhalable through NB than IC, while Gamma-proteobacteria showed a trend to be more exhalable through IC than IB.

On the other hand, the quantities of exhaled Firmicutes did not show a difference between any two breathing patterns. Not surprisingly, PMA-treatment could have biased this finding.

Nevertheless, it could be concluded that bacterial communities inhabiting the respiratory tracts of healthy individuals are aerosolized in fixed proportions. Their quantities differed according to the breathing pattern and the bacterial identity, at least.

It is unknown whether these findings hold for all species or members belonging to the phyla studied. It is also unknown whether these are applicable in disease and further studies remain warranted.

3.4.7. Association between time of sampling and bacterial signals

The healthy volunteers studied here exhaled between 10^4 to 10^7 copies of *16S*rDNA representing different bacterial phyla per min in the first 15 min of sampling. This rate of collection appeared to decrease when the sampling was extended to 30 and 60 min. This indicates that 15 min may be optimal to sample exhaled bacteria with the gelatine filters. In fact, we succeeded in validating that informative results can be yielded with this period in a group of COPD patients (Kennedy *et al*., 2018).

It could be argued that the capturing surface had been saturated with what was exhaled in the first place. A number of these masks, however, were analysed by sequencing for characterizing the exhaled microbiome and a higher biodiversity was found with longer periods, invalidating such argument (5.3.3).

Moreover, it was also suggested that the gelatine surface became saturated with DNA material over time, undermining its capturability. Upon testing this through covering the gelatine surface with DNA (salmon sperm) and asking the volunteers to put the masks on for 15 and 30 min during similar breathing activities, the findings were not supportive to this hypothesis (data not shown).

Burton *et al.* (2007) investigated the physical collection efficiency of different filters by exposing them to a mixture of monodisperse polystyrene latex particles of different sizes (0.35–5.05μm). They found a 4-hr exposure did not undermine the collection efficiency of 0.3μm pore size polytetrafluoroethylene filters loaded by these particles. Burton and colleagues, however, did neither tested that on gelatine despite they used the same pore size nor tested on bacteria despite they used particles with sizes mimicking the bacterial ones. In addition, they tested that on ambient-generated aerosols which features might differ from our case.

Gelatine filters are a fragile material. This renders them more suitable for sampling smaller amounts of moist material like aerosols, which are logically capturable in shorter periods. This is in agreement with Fabian *et al.* (2009)'s findings that such filters are usable for sampling aerosols for less than 15 min because they readily desiccate and crack.

For diagnostic purposes, the ideal capturing surface is the one that becomes rapidly saturated with a desired target. However, it is a challenge for a surface to be selectively saturated by a microbe and not by others. One could ask about the point at which the capturing surface reaches its saturation level, and this requires further investigations.

Whether or not the saturation has played a role, the amounts of bacteria captured in the first 15 min were worthy of attention.

3.4.8.   Potential contributions of lysozyme and DNase

Since bacterial signals captured with sampling times longer than 15 min did not generally increase the yield and in some individuals actually gave lower yields, it was suggested that co-exhalation of factors able to degrade the signals may have contributed; this may also be a reflection of related host-microbe interaction.

As the used application was DNA-based, DNase whether of human or bacterial origin could have been responsible for these patterns. The employed analyses, however, did not reveal any human DNase or net DNase activity, ruling out this possibility within the detection limit and the assumption that DNA abundance did not inhibit the assay competitively.

Fleming of the last century reported the antimicrobial activity of nasal secretions through what he later named lysozyme (muramidase or N-acetylmuramide glycanhydrolase). In addition to poorly-characterized non-enzymatic bactericidal activities highlighted in Ibrahim *et al.* (2001)'s study, lysozyme is well known to enzymatically act on peptidoglycans of bacterial cell walls by hydrolysing β1–4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine residues in such mucopolysaccharide walls. Greater effects can be expected on Gram-positive rather than Gram-negative bacteria where access to peptidoglycan is limited by the outer membrane and affected further by ionic concentration, osmolarity and synergistic cofactors (Ellison III and Giehl 1991; Travis *et al.*, 1999).

Thompson *et al.* (1990) suggested that respiratory lysozyme is generated from the LRT where its abundance in healthy BALs was noted. More recently, Ganz (2002) described lysozyme as the most abundant antimicrobial polypeptide component of respiratory tract secretions.

It was hypothesized that degradation of exhaled bacteria was a result of lysozyme produced from the respiratory epithelium. This was supported by detecting active lysozyme in 49% of the obtained masks.

The quantitative results showed a trend of linearity for active lysozyme over time. Interestingly, NB which did not produce higher quantities of any phylum signals than other patterns showed relatively-stable amounts of lysozyme over time (Figure 3.15).

Positive correlation trends shown between exhaled lysozyme and Bacteroidetes, the most abundant phylum in LRT (Hilty *et al*., 2010; Dickson, 2016b), supports Thompson *et al.* (1990)'s claim that this zone is the source of respiratory lysozyme. However, Bacteroidetes are also the most abundant in URT.

In fact, lysozyme antibacterial role is known on Gram-positive bacteria and to less extent on Gram-negative ones. This explains the negative correlation found between Beta-proteobacteria and lysozyme in NB, while the positive correlation found with Gamma-proteobacteria in RL samples may reflect relative insusceptibility of this Gram-negative group. Paradoxically, PMA-inaccessible Firmicutes signals which are supposed to be the most-degraded by lysozyme activity showed no correlation. Actinobacteria also showed no correlation, possibly due to their exhalable paucity alongside their susceptibility to the enzyme. In addition, these results might have been influenced by correlating the quantities of phylum signals instead of that of individual members.

The site at which lysozyme had its effects remains unknown. This could have started on the respiratory epithelium, resulting in exhaling DNA and other intracellular components. However, it is unknown whether local conditions on the gelatine filters were able to support lysozyme activity. There might have been a cooperation between exhaled albumin and lysozyme based on protein-

protein interaction, with a possible more complicated role played by other attending proteins (Morsky, 1983; Rosch *et al.*, 2017).

Although lysozyme acts on peptidoglycans and not on nucleic acids, it could be that because the bacterial analyses were performed on centrifuged cell deposits, released nucleic acids were lost in supernatants. This could be classified as an artificial anti-nucleic acid role and could be investigated through quantifying free DNA in the mask supernatants.

While lysozyme was studied here, there are many other antimicrobial and potential bacterial signal-degrading factors, such as lactoferrin, neutrophil and epithelial defensins, secretory leukoprotease inhibitors, cathelicidin LL-37, myeloperoxidase, hypothiocyanite and immunoglobulins (IgA, IgG, and IgM), that were not investigated. A number of these factors were detected in the proteomic profiling of PEx samples (Bredberg *et al.*, 2012) and their attendance in the masks must not be surprising.

3.4.9. Relative abundance of bacterial signals and relationships with lysozyme

It was hypothesised that different breathing patterns generate aerosols from different anatomical zones of the respiratory tract and the generated aerosols harbour a microbiological component mirroring that of the source zone.

Actinobacteria and β-Proteobacteria showed an interesting pattern where they were more relatively abundant in IB than in other breathing patterns. This pattern was statistically supported at 15 min and to less extent at 60 min. At least two possible reasons can be discussed for this not been shown at 30 min. First, the sample size was small to result in a statistical significance. However, one could argue that the same sample size was studied for 15 and 30 min, supporting the second possibility. This might be the impact of simultaneous production of bacterial signal-degrading factors which seem to have exhibited their maximal impact after being built-up over 30 min.

Relative abundance of Actinobacteria in IB raised again the question of the source of aerosols generated from this activity. Going back to SP-A (as a biomarker of LRT), this type of breathing exhaled comparable amounts to that of NB. It is likely that the source for both activities has a significant contribution from the URT.

Bassis *et al.* (2014) found that the nasal cavity is dominated by Actinobacteria and Proteobacteria which were also present in the oral cavity but at a lower abundance. Moreover, they found that the overall level of β-Proteobacteria was statistically comparable between oral and nasal cavity communities.

In contrast, Bacteroidetes was more relatively abundant in IC and RL at 30 min than in IB. It seems that members of this phylum are more resistant to degradative factors. The former two activities exhaled larger amounts of LRT biomarkers than the latter, suggesting that their generated aerosols harbour a significant component from the LRT.

It is difficult to claim with a definitive certainty that the LRT is dominated with Bacteroidetes based on these results, supporting the microaspiration hypothesis, because this phylum is also known to dominate the oral cavity, the URT entrance zone.

Charlson *et al.* (2011) studied the microbiota from different anatomical sites of the respiratory tract (oral wash, nasopharyngeal swab, orally-introduced-bronchoscopy-assisted glottis aspirates, BAL and LRT brush). They concluded that the respiratory microbiota is largely comparable between the upper and lower airways, but its biomass decreases with going deeply. On the other hand, Man *et al.* (2017) in their review stated that the LRT microbiome harbours a distinguishable set from the upper one, yet they largely resemble each other.

Unfortunately, the capturing surface background of the face-mask limits its serviceability in characterizing the captured microbiome conclusively as will be detailed later. Nevertheless, it was possible to reveal the relative abundance of four from main exhaled phyla known to shape the respiratory tract microbiome.

3.4.10. Associations between exhaled protein biomarkers and bacterial signals

Most recent culture-independent studies have provided convincing evidence for a resident lung microbiota, challenging conventional views of lung sterility. The main common limitation of these studies is their failure to isolate cultivable bacteria, where culturing "*appears fundamental for successful genome assemblies and for downstream functional experiments*" as Moffatt and Cookson (2017) reviewed. Similar comments have been made based on non-human primate studies (Scheiermann and Klinman, 2017; Morris *et al.*, 2016). However, such failure could be merely a misuse of culturing conditions. For example, Venkataraman *et al.* (2015) revealed that 61% of taxonomic members detected by sequencing bacterial DNA of pulmonary specimens from healthy subjects were identifiable by culturing when different conditions were manipulated.

Hilty *et al.* (2010)'s study, the first report in characterizing the lung microbiome, showed Bacteroidetes in pulmonary samples (upper left lobe brushings) more frequently detected in healthy controls than in COPD patients.

Many studies demonstrated based on microaspiration that the lung microbiome is closely comparable to the oral microbiome despite the former does not derive entirely from the latter (Charlson *et al.*, 2011; Pragman *et al.*; 2012; Morris *et al.*, 2013; Segal *et al.*, 2013; Venkataraman *et al.*, 2015; Bassis *et al.*, 2015).

Several reviewers and investigators describe Firmicutes and Bacteroidetes as predominant phyla of lung microbiome (Shukla *et al.*, 2017; Marsland *et al.*, 2015; Dickson and Huffnagl, 2015; Dickson *et al.*, 2013; Erb-Downward *et al.*, 2011; Hilty *et al.*, 2010). Firmicutes signals in this study showed negative correlations with albumin, the non-specific LRT biomarker used. This could be

due to several factors including the degrading factors co-exhaled with albumin as well as PMA treatment. However, Actinobacteria which signals were not treated with PMA also showed a negative correlation, supporting the first possibility. Furthermore, Actinobacteria signals showed a borderline positive correlation with albumin in NB which exhaled relatively low amounts of lysozyme.

The positive correlation in RL found between the used LRT biomarkers (SP-A and albumin) and Proteobacteria classes and Bacteroidetes supports previous studies describing these phyla as major components of the lung microbiome. However, Bacteroidetes also showed positive correlation with α-amylase exhaled by NB and IB.

While it must be acknowledged that the sample size is relatively small to reach a definitive conclusion, several possibilities can be discussed. The first is that the source of captured material from NB and IB was the URT (including the oral cavity) where the abundance of Bacteroidetes and α-amylase is well known. However, if this was the case, RL was more likely to show at least stronger correlation but it did not even show a statistically significant one, supporting that the main source of the material captured from this activity was the LRT. The second is that the source of captured material from NB and IB was the LRT where Bacteroidetes and α-amylase attendance is supported by microaspiration. The third is that the captured material was sourced from both the upper and lower respiratory tract, supporting Fabian *et al.* (2011)'s conclusion, and this seems the most probable.

Interestingly, Bacteroidetes exhaled by NB and RL over 15 min and by IB over 60 min showed a positive correlation with SP-A and albumin, while when exhaled by NB and IB over 30 min showed that with amylase. This suggests that 15 min and 60 min can be used to sample LRT Bacteroidetes while 30 min to sample URT one per the corresponding activity.

Firmicutes showed a negative correlation with albumin exhaled by RL over 15 min, suggesting their signals are sourced from URT.

Actinobacteria showed a negative correlation with SP-A exhaled by IC over 15 min whereas a positive one with albumin exhaled by NB over 30 min, suggesting longer periods are required to sample LRT Actinobacteria.

β-proteobacteria showed a positive correlation with SP-A and albumin exhaled by RL over 30 min, suggesting their signals at 30 min are from LRT.

γ-proteobacteria showed positive correlation with albumin exhaled by RL over 30 min and with SP-A exhaled by IB over 60 min, suggesting their signals at 60 min are sourced from LRT.

In summary, these results provide further evidence for different microbial outputs with different respiratory activities, however the weakness presented by the small sample size remains to be addressed in future studies.

3.4.11. Relationships between different breathing patterns and the ratios between captured proteins

Ratios of exhaled albumin to SP-A and α-amylase to SP-A were relatively stable over time however the values varied between different breathing patterns. In contrast, the ratios of lysozyme to SP-A and protease to SP-A were relatively stable among different sampling times and breathing patterns.

These ratios were calculated in relation to SP-A since SP-A was found a main component of the captured material.

Exhaled albumin to SP-A and α-amylase to SP-A were noted always above 1 in all RL samples. Since this activity was found the most productive of lower airway aerosols (assuming SP-A is a specific biomarker), obtaining high ratios (>1) of these in a mask sample might be devised as an indicator for determining the quality of collected samples in terms of a subject's producibility. However, it could be argued that the higher α-amylase to SP-A is the lower the sample quality with regards to an LRT sourcing.

Albumin is known as an inflammatory biomarker. It is worth noting therefore that albumin to SP-A ratio could be high in inflammatory conditions, potentially limiting its usefulness as a quality control in patients with respiratory infections.

Exhaled protease was not found to correlate with any of the ratios with one exception (α-amylase to SP-A during NB for 30 min). Indeed, this might further support the validity of these ratios determined in the manner described to be used as indicators in health.

Assuming the sampling matrix makes no contribution and the quantitative effect of signal competitions is minimal, one could speculate that different anatomical zones of the respiratory tract are covered by RTLF in different compositional ratios of SP-A, albumin and amylase, while in comparable ones for lysozyme and protease according to protein expression sites (at least).

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During performing a certain respiratory effort, aerosols are expected to be generated from a zone that dominates the effort, holding the compositional signature of the generating zone. When the particles pass through up, exposed to different anatomical surfaces, their compositional ratios may not be maintained. Such hypothetical dynamics might explain the significant changes among different efforts with a relative stability over the time axis of a particular effort. However, deliberate performing a certain effort at a certain time point does not exclude co-performing other efforts involuntarily. This, alongside the impact of attended degradative factors, could explain the smaller changes shown by these ratios over time.

While the findings presented here appear unprecedented, their implications in terms of the underlying physiological mechanism(s) are currently unknown and validation with a larger sample size and comparison with other respiratory specimens could well be warranted.

3.5. Concluding remarks

It must be acknowledged first that the following conclusions are limited by the relatively small sample size of this study

- SP-A was a consistently-detectable protein component of exhaled aerosols
- Exhalation of particles containing largest amounts of SP-A and albumin was associated with that of salivary α-amylase
- RL for 15 min was sufficient to sample the LRT should SP-A considered a specific biomarker of LRT
- The quantity of captured bacteria did not increase after 15 min and fell within the range of $10^5 - 10^8$ copies per mask
- Bacteria belonging to different phyla and harboured in the respiratory tract were not exhaled in fixed proportions. The quantities differed, at least, according to the breathing pattern and the bacterial identity
- Exhaled particles were composed of, at least, two components: biochemical and microbiological, and there was a degree of interaction between them. Increasing the sampling time resulted in greater quantities of the former but not necessarily of the latter
- Use of gelatine filters limited the range of characterizable microbes because of significant background DNA contamination
- The face-mask system might be used as a non-invasive tool to sample the LRT in health
- The exhaled particles (aerosols and droplets) are a clinical specimen received a relatively negligible attention and it seems they are physiologically regulated like other human biological samples
- Future work should investigate:
  - Mask detectability of more specific biomarkers to LRT such as AEC-I antigens, CC16, CC10, SCGB1A1, alapa-1 antitrypsin or KL-6, 17-Q2, 17-B1 antigens
  - Use of a sampling matrix free from molecular contamination

- Effectiveness of other IB patterns in exhaling aerosols from different anatomical zones of the respiratory tract
- The value of using nasal clips and saliva traps during sampling
- The impact of shorting the sampling time further on the yield of sampled aerosols

Chapter Four: Face-mask sampling in suspected *Pneumocystis jirovecii* pneumonia

4.1. Introduction

As noted in previous Chapters, mask sampling offers potential advances in the diagnosis of lung infections where invasive methods such as BAL are part of the standard approach. In discussion with colleagues in the local haematological oncology unit, the use of mask sampling in the diagnosis of pneumocystis pneumonia was explored.

Pneumocystis jirovecii, as an aetiological agent, was selected as a challenging paradigm. Biologically, its potential detection by the mask can provide evidence that air is one route of this infection and technically, such detection can reflect the developments applied for mask sampling, where the challenge lies in detecting exhalations from sources with low fungal burden. Clinically, a potential detection can provide insights into the mask value as a non-ivasive tool in approaching PJP diagnosis in terms of the microbiological detection.

While pneumocystis pneumonia has been widely abbreviated as 'PCP', PJP will be adopted here to reflect the agent's classification more precisely.

4.1.1. General background on PJP

Pneumocystosis is an opportunistic, life-threatening, extracellular-invasive pulmonary infection in immunocompromised patients (Mills, 1986; Thomas and Limper, 2004 and 2007; Brown *et al.*, 2012; Sax, 2016). It was common in human immunodeficiency virus (HIV)-infected individuals before introduction of the highly active antiretroviral treatment (HAART) and anti-pneumocystis prophylaxis (CDC, 2017); and was also a frequent defining manifestation of acquired immune deficiency syndrome (AIDS) in Europe (Blaxhult *et al.*, 2000). Pneumocystosis has also been reported in non-immunocompromised (Jacobs, 1991; Cano *et al.*, 1993; Niedermaier *et al.*, 1997; Harris *et al.*, 2012; Koshy *et al.*, 2015; Okahisa and Tobino, 2017).

The aetiological agent specific to humans was named *Pneumocystis jirovecii* in 1999 to be distinguished from *P. carinii*, the infective species of rats, and the name originally assigned to human infection. The original descriptions of Chagas (1909), Carini (1910) and Delanoe and Delanoe (1912) are outlined in recent reviews (Stringer *et al.*, 2002; Wakefield, 2002).

In fact, *Pneumocystis* species are host-specific (indicating a possible "*co-evolution*"; Walzer, 2013) with no proven cross-species infections: *P. carinii* and *P. wakefieldiae* in rats, *P. murina* in mice, *P. oryctolagi* in rabbits and *P. jirovecii* in humans (Redhead *et al.*, 2006; Aliouat-Denis *et al.*, 2008; Stringer *et al.*, 2001 and 2002; Stringer, 2002). *Pneumocystis* spp. have also been detected in other mammalian hosts including ferrets, shrews, sheep, monkeys and cetaceans (Laakkonen, 1998; Gigliotti *et al.*, 2014).

Microbiology

P. jirovecii is a eukaryotic, non-motile, obligate parasitic (requiring thiamine and lacking nitrogen and sulphur assimilation; Spanu, 2012), unicellular ascomycetous fungus. It was initially mistaken for a trypanosome based on its morphological features until, through sequencing the small ribosomal RNA subunit, Edman and colleagues (1988) demonstrated its fungal nature, a conclusion supported by subsequent studies of Colthurst *et al.* (1991), Ypma-Wong *et al.* (1992) and Belfield *et al.* (1995) (Stringer *et al.*, 2002; Thomas and Limper, 2004; Hauser, 2014; Cisse *et al.*, 2012 and 2014; Alanio and Bretagne, 2017). Lack of response to amphotericin B (Bartlett *et al.*, 1994; Porollo *et al.*, 2012) and other antifungals (Masur, 1992) has maintained some of the confusion over classification (Georgopapadakou and Walsh, 1996). PJP was also misclassified in the 10th Revision of the International Classification of Diseases (ICD-10) under the code B59+ (Pneumonia in parasitic diseases) and is planned to be re-classified in the ICD-11 under the code CA40.2 (Fungal

pneumonia). The 8.1 Mb genome of *P. jirovecii* has a reduced guanine-cytosine content and lacks well-characterized virulence genes, mycotoxins and most enzymes required for amino acid biosynthesis (Cisse *et al.*, 2012; Helweg-Larsen *et al.*, 1999; Gigliotti *et al.*, 2014). *P. jirovecii* is not cultivable axenically in-vitro (excluding cell cultures; Schildgen *et al.*, 2014), making its life cycle poorly understood, and is poorly maintained in repeated cell cultures (Thomas and Limper, 2004).

Pathogenesis of PJP

Three major stages have been described in the putative life cycle of *Pneumocystis* (Figure 4.1): the trophic form stage (1-5μm in diameter, plasma-membraned, mononucleated, pleomorphic, frequently seen in a cluster pattern) and the cyst stage (5-8μm in diameter, rigidly-walled) which contains several (4-8) spores (1-2μm in diameter, thinly-walled) (Gigliotti *et al.*, 2014; Wyder *et al.*, 1994). The infectious stage remains unknown, but some evidence supports that this may be the cyst. Using a rat model, Martinez *et al*. (2013) demonstrated occurrence of cysts by rats and mice following 12-hr-contact with a "*seeder*" rat infected by cystic rather than by trophic forms. The latter forms appear resistant to the (1,3)-Beta-D-glucan synthetase inhibitor, anidulafungin (a semisynthetic echinocandin), while the cysts are sensitive. Thus, cessation of anidulafungin administration to rats and mice with only trophic forms in their lungs leads to the occurrence of cysts (Cushion *et al*., 2010). Most interestingly, these authors further showed that anidulafungin reduced the number of cysts, left the trophic form number intact and prevented transmission. Recent evidence that cyst (ascospore) production is dependent on mating type (*MAT*) genes (Richard *et al*., 2018; Almeida *et al*., 2015b) provides further insight into potential transmission mechanisms (P Hauser, personal communication).
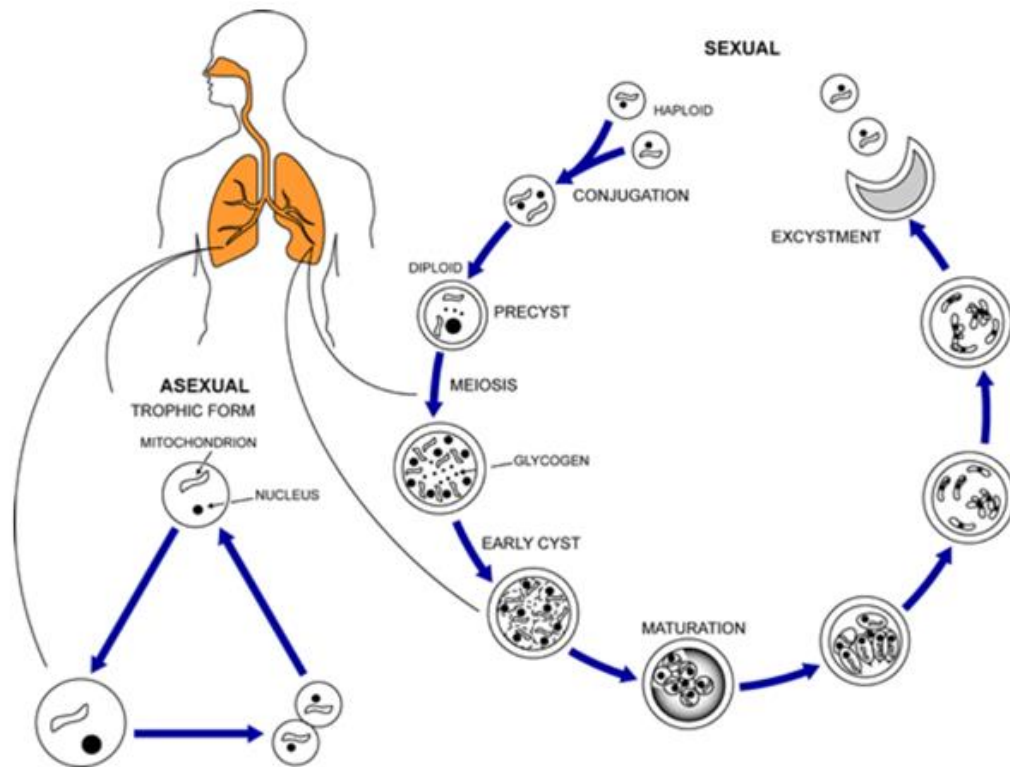
Figure 4.1 Putative life cycle of *Pneumocystis*
This was reproduced from a drawing by Dr. John J. Ruffolo, South Dakota State University, USA published in Cushion M. *Pneumocystis carinii*. In: Collier L., Balows A., Sussman M., editors. Topley and Wilson's Microbiology and Microbial Infections: Volume 4 Medical Mycology, 9th ed. New York: Arnold Publishing; 1998. p. 674. Copyright held by Arnold Publishing and reproduced here with the permission of John Willy & Sons Limited through PLSclear.

It is clear that the presence of *Pneumocystis* in a host can represent multiple relationships including colonization and infection (Cushion, 2010). PJP may reflect endogenous or exogenous infection (Morris and Norris, 2012). Data from human epidemiological studies and from animal models indicate that the incubation period for exogenous PJP ranges from a few weeks to several months (Manoloff *et al*., 2003; Schmoldt *et al*., 2008; Yazaki *et al*., 2009; Le Gal *et al*., 2012; Kim *et al*., 2015).

Pneumocystis establishes itself through contact with alveolar epithelium (Kottom *et al*., 2003 and 2011; Kottom and Limper, 2013) with attachment potentially mediated by glycoproteins, fibronectin, vitronectin, laminin, mannose receptors and pneumocystis cytoskeletal components (Limper and

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Martin 1990; Limper, 1991a; Dei-Cas, 2000; Thomas and Limper, 2004). Secretion of proteolytic enzymes like chymase and release of reactive oxygen species may assist establishment (Patel and Koziel, 2004) and impair cellular lung function (Limper and Martin, 1989), while a surface glycoprotein may trigger surfactant lipids abnormalities (Lipschik *et al.*, 1998).

Attachment occurs mainly, or selectively, to type-I pneumocytes (Lanken *et al.*, 1980; Yoshida *et al.*, 1984; Shiota *et al.*, 1986; Long *et al.*, 1986; Millard *et al.*, 1990; Limper *et al.*, 1993; Yoneda and Walzer, 1980; Pottratz and Weir, 1995) which constitute 95% of alveolar cell population but only 40% of alveolar surface area. Sueishi *et al.* (1977) and Murphy *et al.* (1977) demonstrated apposition and interdigitation between the membranes of the organism and the target cell.

Immunity to *Pneumocystis* has both protective and detrimental consequences on the host (Walzer, 1999). Clearly the association of PJP with AIDS implies that normal function of T lymphocytes is beneficial (Kelly and Shellito, 2010), while the value of glucocorticoids in therapy indicates suppression of harm (Gigliotti and Wright, 2005; Thomas and Limper, 2018; Sax, 2018). The importance of sufficient number of functional T-cell lymphocytes (CD4+) has been established. Both T helper type-1 and type-2 cytokines are associated with effective clearance of *Pneumocystis* (Patel and Koziel, 2004).

PJP has been reported in patients with B-lymphocyte defects but intact cell-mediated immunity, suggesting a role for humoral immunity (Rao and Gelfand, 1983; Esolen *et al.*, 1992). Moreover, a therapeutic or protective role of antibodies against pneumocystis antigens has been suggested (Roths and Sidman, 1993; Gigliotti and Hughes, 1988; Harmsen *et al.*, 1995; Marcotte *et al.*, 1996; Garvy and Harmsen, 1996; Garvy *et al.*, 1997; Bartlett *et al.*, 1998).

Wang *et al*. (2005) hypothesized that the interaction of *P. carinii* with alveolar epithelium induces the expression of proinflammatory genes that promote the immune response and showed that this interaction activate NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) protein complex signalling pathway in alveolar type-II cells in female mice, suggesting that these cells recognize *Pneumocystis* through distinct receptor-ligand interactions but excluding the need for a firm attachment.

The pathological features of PJP include exudates filling the alveoli, hypertrophy or hyperplasia of type-II alveolar cells (and possibly type-I), interstitial infiltrates with mononuclear cells and increased permeability of the alveolar capillary membrane.

Extrapulmonary infection is rare (2-5% in HIV-infected cases; Kasper and Buzoni-Gatel, 1998) but has been reported (e.g. thyroid, lymph nodes, spleen, liver, bone marrow and cerebral cortex). Direct invasion, haematogenous and/or lymphatic routes have been implicated (Northfelt, 1989; Dyner *et al*., 1989; Mariuz *et al*., 1991; Peiro-Cabrera *et al*., 1994; Kaplanski *et al*., 1996; Raviglione, 1990; Cohen and Stoeckle, 1991; Guttler *et al*., 1993; Ng *et al*., 1997; Bartlett and Hulette, 1997; O'Neal and Ball, 2008; Valdebenito *et al*., 2015).

4.1.2. Epidemiology and transmission of *P. jirovecii*

An environmental reservoir or ecological niche for *P. jirovecii* has not been determined (Cushion, 2004) although some studies identified pneumocystis nucleic acids in rural ambient air (Wakefield, 1994 and 1996) and pond water (Casanova-Cardiel and Leibowitz, 1997). No exosaprophytic form has been identified (Nevez *et al.*, 2003).

Vargas *et al.* (2001) showed seropositivity to *P. jirovecii* antigens in 85% of 79 healthy infants, suggesting that they could play an important "infectious reservoir" role in the community. These authors further suggested an association between pregnancy and nasal carriage, by detecting *P. jirovecii* DNA in nasal swabs of >15% of 33 healthy women in the third-trimester versus none of 28 nonpregnant controls (Vargas *et al.,* 2003). Similarly, Medrano *et al.* (2005) found that 20% of 50 healthy adults had *P. jirovecii* DNA in their oropharyngeal washes. While these observations support a potential role for healthy individuals as an "infectious" reservoir, the association of both populations with healthcare exclude such environments as the major reservoir.

PJP transmission and acquisition are poorly defined. Nonetheless, most authorities support airborne transmission route as the primary route with significant person to person spread (Thomas and Limper, 2017; CDC, 2017). Several routes have been discussed and, following the work of Gajdusek (1957), perinatal and even vertical transmission have been suggested (Pavlica, 1962; Bazaz *et al.,* 1970; Mortier *et al.,* 1995; Miller *et al.,* 2002; Montes-Cano *et al.,* 2009).

The potential importance of endogenous reactivation has been discussed (Hughes 1977 and 1987) but evidence for latency was not found in two studies

(Chen *et al.,* 1993; Vargas *et al.*, 1995). In contrast, there is evidence for clearance of infection shown in human and animal studies (O'Donnell *et al.*, 1998; Chen *et al.*, 1993) and exogenous re-infection. Genotype change between episodes has been reported by Helweg-Larsen *et al.* (2001) and Kim *et al.* (2012). Furthermore, multiple clustering studies on PJP cases support exogenous dissemination rather than endogenous reactivation (Watanabe *et al.*, 1965; Ruskin and Remington, 1967; Brazinsky and Phillips, 1969; Singer *et al.*, 1975; Haron *et al.*, 1988; Bensousan *et al.* and Goesch *et al.*, 1990; Dohn *et al.* and Morris *et al.*, 2000; Choukri *et al.*, 2010*;* Mori *et al.*, 2010a; Phipps *et al.*, 2011; Le Gal *et al.*, 2012).

Multiple studies have reported evidence for *Pneumocystis* in air samples from clinical and non-clinical environments (Wakefield, 1996; Olsson *et al.,* 1996a, Bartlett *et al.*, 1997; Olsson *et al.*, 1998; Sing *et al.*, 1999b). Choukri *et al.* (2010) studied air diffusion of *P. jirovecii* in the environment of hospitalized patients with PJP. They found the fungal burden was detectable in low levels in air samples collected at up to 8m distance from patients. They provided the first quantitative data of air levels of *P. jirovecii* in the vicinity of PJP patients. They were not able, however, to detect DNA in the close proximity (1m) of 21% of their 40 studied patients. More recently, Le Gal *et al.* (2015) were able to detect *P. jirovecii* DNA in around 50% of air samples collected at 5m and 1m distance from the head of 4 PJP patients and 10 colonized hosts, with quantification medians of 2.20E+03 and 1.70E+04, and 7.80E+02 and 1.00E+03 copy/m^3, respectively. It is clear, however, that the burden in environmental samples does not necessarily reflect the natural burden directly exhaled from PJP patients.

4.1.3. Detection of *P. jirovecii* in asymptomatic individuals

There is extensive evidence of exposure to and colonisation by *P. jirovecii* in individuals expressing no related clinical manifestations.

Based on serology, Meuwissen *et al.* (1977) suggested that almost 100% of children are pneumocystis-colonised in the first two years after birth. Pifer *et al.* (1978) stated that by 4 years of age two thirds of healthy children had seroconverted to pneumocystis antigens. Using immunofluorescence staining and PCR, Contini *et al.* (1998) identified *P. jirovecii*-carriers by detecting the agent in nasopharyngeal aspirates in ~20% of 28 immunocompetent children with chronic lung disorders versus none of 30 healthy children; they suggested underlying pathology might predispose to colonization and this might contribute to exacerbations. Medrano *et al.* (2003) showed that >70% of a non-selected sample of 230 school-age children were seropositive, observing an increase with age from 6 to 13 years and suggesting an infantile exposure.

Similar findings have been reported in adults and the rates were varying mainly based on the type of specimen, the method of detection and the medical state (Calderon *et al.,* 1996; Armbruster *et al.*, 1997; Sing *et al* 1999a; Ponce *et al.,* 2010).

4.1.4. PJP diagnosis

Provisional diagnoses of PJP are usually made in patients presenting with pneumonia, suggestive radiographic findings (4.1.5.1), reduced blood oxygen saturation or increases in the alveolar-arterial oxygen tension gradient and one or more predisposing factor particularly associated with reduced cell mediated immunity (Thomas and Limper, 2017). Interestingly, Maini and colleagues (2013) have related increasing incidence of PJP in England from 2000 to 2010 to pre-existing pulmonary disease.

In traditional clinical practice, a "definitive" diagnosis requires a laboratory detection (4.1.5.2) of *P. jirovecii* in a respiratory specimen obtained either invasively (BAL, nasopharyngeal and endotracheal aspirates, transthoracic needle-biopsy, transbronchial lung biopsy) or semi-invasively (induced sputum). Spontaneously-expectorated sputa have been suggested to be of less diagnostic valuable than induced ones (Mirdha and Guleria, 2000; Matos *et al.*, 2001; Alanio *et al.*, 2016; Fishman *et al.*, 1994; Bartlett *et al.*, 2000; Bigby *et al.*, 1986; Metersky and Catanzaro, 1991) although there is disagreement on this point (Metersky *et al.*, 1998; Aderaye *et al.*, 2008; Helweg-Larsen *et al.*, 1998a; Samuel *et al.*, 2011; Bandyopadhyay *et al.*, 2000). The inter-studies disagreements might be due to different downstream analyses and their related prerequisites, and different patient characteristics.

Differential diagnosis of PJP

Several conditions (including opportunistic respiratory infections in immunocompromised groups) should be considered when approaching the diagnosis of PJP. These include I. pneumonias and respiratory tract infections with another aetiological agent (Table A.45), II. non-infectious interstitial lung disease like sarcoidosis and alveolar proteinosis, III. pulmonary embolism, IV. acute respiratory distress syndrome and V. lymphocytic interstitial pneumonitis, organizing pneumonia, eosinophilic pneumonia, exogenous lipoid pneumonia and pulmonary haemorrhage syndromes (Thomas and Limper, 2017).

Since molecular-based applications are being increasingly employed, verifying molecular specificity through excluding possible cross-reactions with other microbial genomes (Table A.45) is a prerequisite.

4.1.5.   Investigations

4.1.5.1.   Radiographic findings

Overall, the most suggestive radiological features are bilateral ground glass opacities in the upper lobes and an interlobar septal thickening (Thomas and Limper, 2017).

Chest X-ray (CXR) and high-resolution computed tomography (CT) are most frequently used. Neither investigation can exclude the diagnosis particularly in AIDS patients, although absence of certain features may reduce the diagnostic likelihood (Sax, 2016).

In HIV-uninfected patients, the typical abnormalities are diffuse, bilateral interstitial infiltrates (butterfly pattern). Apical distribution may be prominent in those receiving inhaled pentamidine (Conces *et al.*, 1989; Chaffey *et al.*, 1990; Jules-Elysee *et al.*, 1990), while lobar or segmental infiltrates, solitary or multiple nodules with potential cavitation, pneumatoceles, pneumothoraces and pleural effusion are all possible but less frequent patterns. A CT scan may reveal ground-glass opacities or cystic features against normal, equivocal or nonspecific findings on CXR (Thomas and Limper, 2017). In HIV-infected patients, alveolar or nodular infiltrates may be found (Sax, 2016), while patchy or nodular ground-glass attenuation was found to give a high diagnostic sensitivity and specificity in one study (Hartman *et al.*, 1994).

Gallium-67 citrate scintigraphy can also be used as a screening test in high-risk groups to demonstrate an intense, diffuse bilateral uptake; but despite of its high sensitivity (94%) it lacks specificity (74%), time- and cost-effectiveness (Barron *et al.*, 1985; Sax, 2016).

4.1.5.2. Laboratory detection

Microscopy

As *P. jirovecii* cannot be isolated in axenic culture, microscopical visualization in stained respiratory specimens remains a widely used approach (Thomas and Limper, 2017). The thin-walled trophic forms, which are usually numerous, have been known to be more challenging for conventional microscopic detection than the non-abundant thick-walled cysts (Meuwissen *et al.*, 1977). Gram-Weigert, Wright-Giemsa or modified Papanicolaou stains have been used to visualize the former while calcofluor white, cresyl echt violet, Grocott-Gomori's methenamine silver or toluidine blue-O have been used for staining the wall of the latter. Recent authors recommend direct immunofluorescence staining with monoclonal antibodies (Thomas and Limper, 2017; Sax, 2016; Alanio *et al.*, 2016).

Beta-D-glucan assay

(1,3)-Beta-D-glucan (BDG) is a natural polysaccharide non-specific component of the cell wall of *P. jirovecii* and other fungi (Figure 4.12). Its detection has been utilized as a screening test of disseminated or invasive fungal infections, including PJP (Thomas and Limper, 2017).

A number of studies have reported the presence of BDG in some bacterial species (*S. pneumoniae*, *P. aeruginosa* and *Alcaligenes faecalis*) highlighting significant potential cross-reactivity and false positivity that has also been associated with intravenous administration of amoxicillin-clavulanic acid and exposure materials containing BDG such as some haemodialysis filters and surgical gauze (Mennink-Kersten *et al.*, 2008; Mennink-Kersten and Verweij, 2006; Marty and Koo, 2009).

A negative BDG assay appears to have good negative predictive value for PJP (Alanio *et al.*, 2016). Some data of the performance of BDG test (in serum, plasma or blood) in approaching PJP diagnosis are presented in Table 4.1.

Table 4.1 Some diagnostic data on the performance of BDG assay

Investigator study	Sample size (pts)	HIV-status	Sensitivity	Specificity	PPV	NPV	Reference positive value
Tasaka *et al.*, 2007	279	6%+ive	92	86	61	98	≥ 31 pg/mL
Sax *et al.*, 2011	252	+ive	92	65	85	80	≥ 80 pg/mL
Matsumura *et al.*, 2012	128	4%+ive	81	81	75	86	≥ 6.0 pg/mL
Wood *et al.*, 2013	159	+ive	93	75	96	60	≥ 80 pg/mL

Definition of abbreviation: pts= Patients; PPV= Positive predictive value; NPV= negative predictive values

Polymerase Chain Reaction (PCR)

A comprehensive literature search using the PubMed database was carried out for the present study. The search commands used to identify papers from 1990 onwards were: PCR or polymerase chain reaction and pneumocystis or pneumocystosis. Results were limited to English language journals with good impact factor and to human subjects. After identifying suitable articles from the abstract, a further filtration was applied looking for 1) study population criteria comparable to this study, 2) PCRs validated on a large sample size and 3) on samples with low fungal loads.

A total 64 reports on the use of PCR in the diagnosis of PJP have been reviewed. The diagnostic value in terms of sensitivity, specificity, positive and negative predictive values has been found to vary widely (Table A.38). This variation was mainly based on the gold standard and there were other contributing factors.

Ideally, the most rigorous criteria considered for a gold standard were either post-mortem histological confirmation based on suggestive history, or clinical findings confirmed by microscopic examination (direct immunofluorescence staining with monoclonal antibodies) and response to anti-pneumocystis (clinically and radiologically) with absence of alternative diagnoses.

However, the former was rarely practicable, revealing only one study (Robert-Gangneux *et al.*, 2014) and the latter was not strictly followed. Assays meeting some rigorous criteria in this regard are summarized in Table 4.2 (further details in Table A.38)

Table 4.2 Some diagnostic data on the performance of PCR in PJP

Investigator study	Sensitivity	Specificity	PPV	NPV
Robert-Gangneux *et al.,* 2014	100	92	69	100
Alanio *et al.*, 2011	100	88	28	100
Larsen *et al.*, 2002	100	67	65	100
Orsi *et al.*, 2015	100	94	80	100
Sing *et al.*, 2000	100	96	60	100
Tia *et al.,* 2012	100	88	77	100

Definition of abbreviation: PPV= Positive predictive value; NPV= negative predictive values

Some investigators have clearly categorized the diagnostic value according to the HIV-status (4, 9, 31, 35, 38, 52, 63 in Table A.38) where HIV-positivity resulted in a better value. This has been accounted for the higher organism burden in this group. In contrast, others did not consider HIV-status of their studied subjects (12, 26, 39, 44, 62). It could be argued that the latter was a consequence of the implication of the HAART and PJP prophylaxis.

Other investigators have correlated the PCR diagnostic value with the type of specimen used (3, 4, 7, 9, 13, 54, 63). Not surprisingly, the BAL, lung biopsy and induced sputum have been considered in many studies a "gold standard" comparator when evaluating the value of other specimen types (7, 8, 10, 12, 13, 16, 30, 34, 36) though these are either invasive or semi-invasive procedures beside the time and cost factors.

Other investigators have differentiated the PCR diagnostic value based on the type of PCR or the cycling conditions used (3, 7, 9, 13, 15, 18, 21, 22, 38, 51,

54). Nested PCRs and qPCRs with a touchdown protocol have been shown to have a convincing diagnostic value. In general, conventional PCR techniques used in nested PCRs are liable to a considerable risk of cross-contamination responsible for a high false-positivity rate in addition of being time-consuming and low cost-efficient thus less practicable in clinical laboratories. On the other hand, qPCR ability in aiding the differentiation between colonization and overt clinical disease has been discussed, however without a consistent discriminating cut-off point and with a wide grey zone (13, 16, 20, 22, 23, 29, 31, 40, 41, 42, 45, 51, 52, 55, 56, 59). Some have clearly attributed the PCR diagnostic value to the molecular target (12, 17, 19, 23, 38). This value has been varied, solely based on the gold standard comparator as mentioned earlier (33, 42, 62).

Comparing PCR with microscopical detection, the former is of a particular use in HIV-uninfected patients in whom the sensitivity of microscopical visualisation is lower (Thomas and Limper, 2017) due to the expected lower fungal load than in HIV-infected individuals. However, some studies have showed microscopical detection to guide delivering PJP diagnoses in HIV-uninfected cases where PCR failed (13% of 39 PJP-diagnosed patients) and the success of the latter where the former failed (>66% of 14 HIV-uninfected cases diagnosed with probable or definitive PJP) (Azoulay *et al.*, 2009).

It must be acknowledged that all these laboratory approaches are investigative tools that have limitations and thus should be interpreted within the wider clinical context on a case-by-case basis.

4.1.6. Hypotheses and objectives

We hypothesized here that:

1. The developed face-mask protocol can detect *P. jirovecii* in HIV-uninfected patients with PJP and provide a further evidence that *P. jirovecii* is an airborne infection
2. Detection of *P. jirovecii* by the face-mask can provide a non-invasive means of approaching the microbiological diagnosis on PJP

Objectives:

1. To detect and quantify exhaled *P. jirovecii* from HIV-uninfected patients with suspected PJP
2. To relate the face-mask results to other conventional features used in approaching PJP diagnosis including:
   - clinical presentation
   - laboratory findings (mainly BDG and BAL results)
   - radiological findings (CXR and CT)

4.2. Materials and methods

4.2.1. Collection of face-masks

Following initial discussions with haematology and microbiology consultants at the Leicester Royal Infirmary (Principally Prof Hunter and Dr Perera), it was agreed that mask samples could be evaluated as part of a service quality improvement study.

Suitable patients were identified by their healthcare consultants who deemed that no additional ethical approval beyond patients' verbal assent was required. In addition to the physical suitability assessed by their consultants, the selected patients were deemed suitable if, with a high suspicion of PJP, anti-PJP agents were not commenced >48 hrs prior to mask sampling.

For approaching PJP diagnosis, the EORTC/MSG definitions and the ECIL guidelines highlighted in De Pauw *et al*. (2008) and Alanio *et al*. (2016) were adopted by the local healthcare provider. A flow chart is shown in Figure 4.2.

The selected patients were asked to wear the face-masks for up to one hr. No instruction on breathing patterns during sampling was given. The patients had been asked to remove the mask if there was any inconvenience, but none did so. The masks were collected in the morning to mid-afternoon hrs (9am to 3pm). Following sampling, the face-masks were placed back in their resealable plastic bags and transported to the laboratory for processing. If not processed immediately, the samples were stored at -20°C.

Metadata on the demographics (gender and age), HIV-status, immune background and immunosuppression factors, history of PJP, symptoms and signs at presentation of the current episode, laboratory findings (WBC, differential WBC, current or last known CD4+ count, BDG, BAL or other

respiratory samples examination results), radiological findings (CXR and CT), receipt of anti-pneumocystis prophylaxis (as a preventive measure taken during the episode after sampling), receipt of anti-pneumocystis treatment, response to anti-pneumocystis treatment, receipt of other antimicrobial reagents, any documented respiratory coinfection or any non-infectious aetiology that could result in similar clinical or radiological findings, and the final clinical diagnosis (with regards to PJP) were collected and anonymised for downstream analysis. The diagnosis made by an advisory team was not physically accessible. On the basis of the views expressed in the case notes and discharge letters, the individual diagnoses were assigned "likely" and "unlikely" PJP.
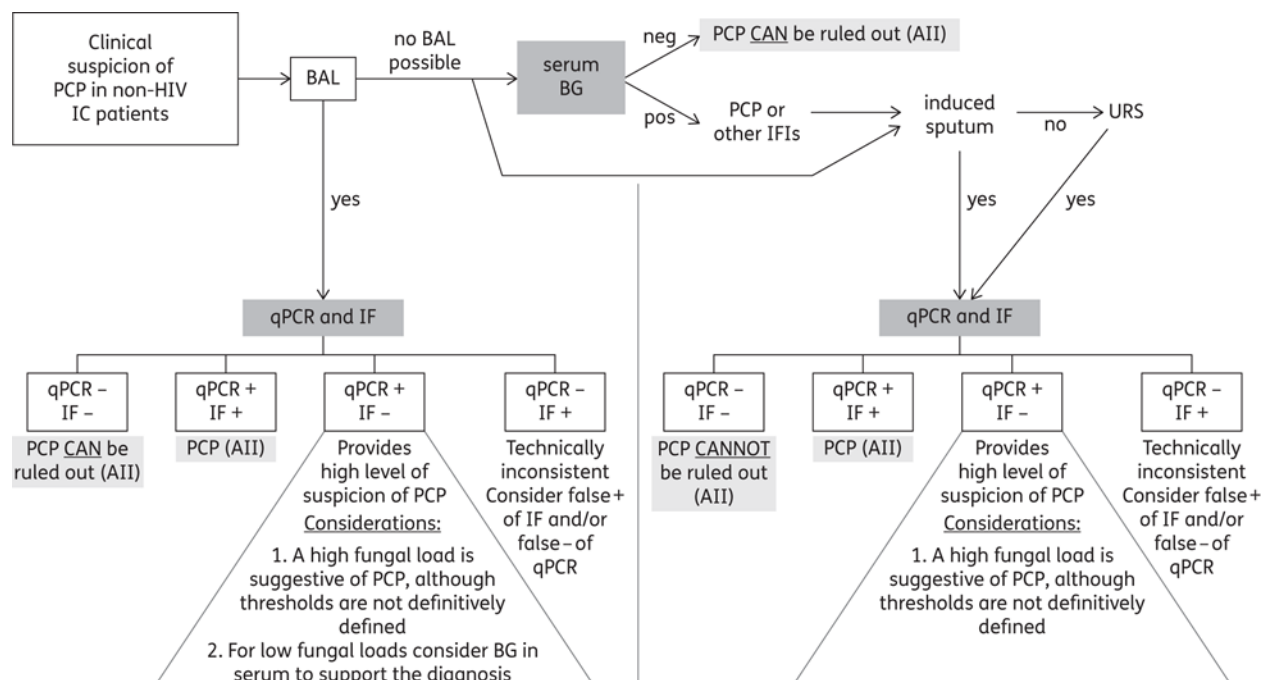
Figure 4.2 Flow chart for PJP diagnosis in HIV-uninfected immunocompromised patients
PCP= PJP; IC=Immunocompromised; BG= β-d-glucan; IFI= Invasive fungal infection; URS= Upper respiratory specimen; IF= Immunofluorescence; Biological tests are highlighted in dark grey and recommendations in light grey; A-II= level of recommendation of the European Conference on Infections in Leukaemia (see Alanio *et al*., 2016 for details)
This was reproduced from Alanio *et al*. (2016). Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy and reproduced here with the permission of Oxford University Press.

4.2.2. The used mask designs

Two versions of the face-mask design were used:

1) Gelatine-filter face-masks assembled as detailed in 3.2.2.

2) PVA sampling matrix face-masks. These were assembled with gloved hands and sterile forceps inside the laminar flow cabinet from either FFP1 masks (specified before) or Duckbill masks (Integrity 600-3004 Duckbill Facemask with Pouch, Rapid electronics, UK) fitted with PVA strips (3D-printed by Mrs Al-Taie and Prof Pan of the University Engineering Department) (Figure 4.3).

Figure 4.3 Duckbill mask integrated with four 3D printed 9x1cm PVA strips

The assembled face-masks were treated with UV exposure (2.2.1) and individually packed in resealable plastic bags.

4.2.3. Air sampling as a background control

Indoor air was sampled using automatic multi-vial cyclone air sampler (C90Ma, Burkard Manufacturing Co. Ltd., England) run up to 48hrs in different locations. 100µL of 0.05% w/v Tween 80 was added to samples collected into 1.5mL Eppendorf tubes. The samples were vortexed, briefly centrifuged and the lysates transferred to 2mL O-ring seal screw cap tubes. These were processed as detailed for the mask samples 4.2.3.

4.2.4. Processing of face-mask samples

4.2.4.1. Extraction of exhaled material

The samples were processed blindly (i.e. without knowledge of the final diagnosis or relevant clinical or laboratory findings).

For gelatine-filter face-masks, the masks were left in a biological safety cabinet II (Walker Safety Cabinets Ltd., UK) until the gelatine discs were completely dried. The gelatine-filters were then released from their metal holders using sterile forceps and processed per 2.2.6.3 inside the cabinet. The resultant lysates were centrifuged at 15,000xg for 10min. The supernatants and pellets were separately collected into 2mL O-ring seal screw cap tubes. The extracts were stored at -20°C if not processed on the same day.

For PVA face-masks, the PVA filters or strips were released from the masks using sterile forceps and placed in a stomacher bag (Seward Stomacher 80 Bag 5-80ml with closure, Scientific Laboratory Supplies Limited, UK). 4mL of molecular grade water were added then the bag was closed with clips, placed in a sealed box mounted on a vortexer and vortexed at maximum speed for 5–7min. The dissolved material was transferred to 5mL centrifuge tubes (SuperClear centrifuge tubes, VWR International, UK) and centrifuged at 21,000xg for 20min. The supernatants and pellets were separately collected into 5mL tubes and 2mL O-ring seal screw cap tubes, respectively. The extracts were stored at -20°C if not processed on the same day.

4.2.4.2. DNA extraction

DNA was extracted per 2.2.7.4 from resultant pellets of 4.2.4.1.

4.2.4.3. qPCR analysis

Target genes, primers and probes

Oligonucleotide primers and probes used are presented in Table 4.3. The specificity of primer pairs was verified by performing BLAST analysis at the

GenBank using Primer BLAST tool with the mRNA, genomes and nr Databases. In addition, *in silico* PCR was done individually for microorganisms listed in Table A.45.

Table 4.3 Oligonucleotide primers and probes used in Chapter Four

Designation	Target	Oligonucleotide sequence	Amplicon size	Reference
MtLSU	The large subunit mitochondrial ribosomal RNA gene (*MtLSU*) of *P. jirovecii* genome	Forward: 5' CTTAAAATAAATAATCAGACTATGTGCGATAAG 3'	79 bp	Meliani *et al.,* 2003
		Reverse: 5' GGAGCTTTAATTACTGTTCTGGGC 3'		
		Probe: 5' 6-FAM AGATAGTCGAAAGGGAAC 3' MGBNFQ		
pH207	*MtLSU*	Forward: 5' ACAAATCGGACTAGGATATAGCTGGT 3'	301 bp	Chabe *et al.,* 2014
pAZ102-E		Reverse: 5' TGGGCTTGGAAACAGCCATC 3'		
PJLSUF0	*MtLSU*	Forward: 5' TGGCAAATTGTTTATTCCTCT 3'	171 bp	Tia *et al.,* 2012
PJLSUR0		Reverse: 5' AGGGAAACAGCCCAGAACAGT 3'		
PJLSUF1	*MtLSU*	Forward: 5' AAATAGTAGGTATAGCACTG 3'	113 bp	Tia *et al.,* 2012
PJLSUR1		Reverse: 5' CAGACTATGTGCGATAAG 3'		
pAZ102-H	*MtLSU*	Forward: 5' GTGTACGTCGCAAAGTACTC 3'	347 bp	Wakefield *et al.,* 1990
pAZ102-E		Reverse: 5' TGGGCTTGGAAACAGCCATC 3'		
pAZ102-X	*MtLSU*	Forward: 5' GTGAAATACAAATCGGACTAGG 3'	252 bp	Wakefield, 1996
pAZ102-Y		Reverse: 5' GCTCCCCAATTAATATTAAGTGA 3'		
HβG	Human β-globin gene	Forward: 5' GGTTCTTTGAGTCCTTTGGGGATC 3'	164 bp	El Bali *et al.,* 2014
		Reverse: 5' GTCACAGTGCAGCTCACTCAGTGTG 3'		
Duplex Oligo	Standard	Sense: GGAGCTTTAATTACTGTTCTGGGCTGTTTCCCTTTCG ACTATCTACCTTATCGCACATAGTCTGATTATTTATTT TAAG	79 bp	GenBank Accession number: JX499143
		Antisense: CTTAAAATAAATAATCAGACTATGTGCGATAAGGTAG ATAGTCGAAAGGGAAACAGCCCAGAACAGTAATTAAA GCTCC		

In-house *MtLSU* qPCR TaqMan Assay (modified from Meliani *et al.*, 2003)

25µL PCR mixture of each reaction tube was prepared in 0.1mL Rotor-Gene PCR tubes containing 12.5µL 2X TaqMan universal PCR master mix +AmpErase UNG (specified before), 1µL of each 10µM *MtLSU*-forward and 10µM *MtLSU*-reverse primers, 0.5µL of 10µM TaqMan *MtLSU*-probe and 10µL of DNA template. Cycling conditions were one holding cycle at 50°C for 2min and a further one at 95°C for 10min, 40 amplification cycles at 95°C for 15sec, 60°C for 60sec with acquisition on FAM/Green channel (470nm) and one holding cycle at 40°C for 20sec. Duplex oligonucleotide of *MtLSU* sequence (Integrated DNA Technologies, UK) was used to prepare standards for absolute quantification.

In-house *MtLSU* qPCR SYBR-Green Assay

25µL PCR mixture of each reaction tube was prepared in 0.1mL Rotor-Gene PCR tubes containing 12.5µL 2X SensiFAST SYBR No-ROX (specified before), 1µL of each 10µM *MtLSU*-forward and 10µM *MtLSU*-reverse primers, 5µL of DNA template and the volume was completed to 25µL with molecular grade water. The cycling conditions were one holding cycle at 95°C for 15min for polymerase activation, 40 amplification cycles at 95°C for 15sec for denaturing, 60°C for 30sec for annealing, 72°C for 20sec for extension with acquisition at cycling A and 82°C for 20sec for extension with acquisition on FAM/Green channel (470nm) at cycling B. Melting condition was set at 60°C to 95°C rising 1°C per cycle. Duplex oligonucleotide of *MtLSU* sequence was used as specified earlier.

In-house *MtLSU*2 qPCR SYBR-Green Assay (modified from Chabe *et al.*, 2014)

15µL PCR mixture of each reaction tube was prepared in 0.1mL Rotor-Gene PCR tubes containing 7.5µL 2X SensiFAST SYBR No-ROX (specified before),

1µL of each 7.5µM pAZ102-E (forward) and 7.5µM pH207 (reverse) primers, 2µL of DNA template and the volume was completed to 15µL with molecular grade water. The cycling conditions were one holding cycle at 95°C for 15min, 50 amplification cycles at 95°C for 10sec, 59°C for 15sec, 72°C for 24sec for extension with acquisition at cycling A and 82°C for 24sec for extension with acquisition on FAM/Green channel (470nm) at cycling B. Melting condition was set at 59°C to 95°C rising 1°C per cycle. Duplex oligonucleotide of *MtLSU* sequence was used to prepare standards for relative quantification.

Human β-globin qPCR SYBR-Green Assay

The extracts were assayed for human β-globin gene as described by El Bali *et al.* (2014). Human genomic DNA (BIO-35025, Bioline Ltd, UK) was used to prepare standards for the qPCR assay.

qPCR standards preparation method was as described in 2.2.8.3 with changing the parameters for each genome accordingly.

Molecular grade water was used as negative control of amplification. Blank filters processed as above were used as negative control of extraction. Mask-samples collected from heathy volunteers (3.2.1) were used as negative control of sampling. All the assays were run in technical duplicates at least and analysed on Corbett Life Science Rotor-Gene 6000 real-time DNA analysis system. LLD was 100 copies per reaction. The analyses were accepted when the R^2 was ≥0.98, efficiency was ≥0.70 and negative control of amplification had <100 copy/reaction. The slope correct option was selected. The quantitative readings were accepted when the coefficient of variation of the replicates was <20%. Ct for all the assays was set at 0.0153 for uniformity and comparability. Copy numbers per sample were adjusted according to the total volume of DNA extract.

4.2.4.4.   Conventional nested PCR analysis

Conventional nested PCR was carried out using external primers (PJLSUF0, PJLSUR0) (pAZ102-H/ pAZ102-E) and internal primers (PJLSUF1, PJLSUR1) (pAZ-X, pAZ-Y), respectively, as described by Tia *et al.*, 2012. The PCR was run on Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, USA).

4.2.4.5.   Gel analysis of nested PCR amplicons

PCR amplicons were separated by electrophoresis on 1–2% w/v agarose gels contained 0.5μg/mL ethidium bromide, depending on the size of the DNA fragment to be separated. The gel was prepared by dissolving 1–2g of agarose in 100mL of Tris-acetate-EDTA (TAE) buffer supplemented later with 5μL of 10mg/mL ethidium bromide stock. 1/6 volume of 6X gel loading dye (Thermo Fisher Scientific, UK) was added to the samples before loading. The samples were analysed on the gel alongside with a 100bp DNA ladder (New England biolabs, UK). Gel electrophoresis was performed on Bio-Rad PowerPac 3000 (Bio Rad) at 80–100V for 45–120min, depending on the gel size. Following electrophoresis, the amplicons were visualised under a gel documenting system (Bio Rad). The images were recorded, and the band sizes confirmed.

4.2.4.6.   Purification of PCR amplicons

PCR amplicons were purified using the Genomic DNA Clean and Concentrator Kit-10 (Zymo Research, USA) per the manufacturer's instructions.

4.2.4.7.   DNA sequencing and analysis

Purified PCR amplicons from selected samples were sent to GATC Biotech for DNA sequencing. Resulting DNA sequences were analyzed by BLAST database. DNA alignment was performed using Clustal Omega 1.2.4 multiple sequence alignment online tool at The European Bioinformatics Institute (EMBL-EBI), UK. https://www.ebi.ac.uk/Tools/msa/clustalo/

4.2.4.8. α-Amylase activity detection in exhaled aerosols

Active α-amylase of the aerosol extracts was detected per 3.2.4.9.

4.2.4.9. Lower limit of detection

The same principles described in 3.2.4.10 were applied.

4.2.5. Statistical analysis

Data were analysed as described in 2.2.9.

This study was not designed as a formal diagnostic evaluation. Therefore, no statistical power calculation was required and no epidemiologic data on the local prevalence of PJP was available. The diagnostic parameters were arbitrarily calculated, using the standard method as indicated, to provide preliminary data paving the way for future studies.

4.3. Results

4.3.1. Patients and controls

45 patients with suspected PJP were sampled by the face-mask system between 1st May 2016 and 30th June 2018. One patient was sampled twice for two episodes separated by 5-months and one patient was sampled twice for the same episode. A total of 46 masks were collected (30 gelatine and 16 PVA). The metadata were not available for six patients; therefore, they were excluded from the clinical analysis, but their masks were analysable.

Of the 39 included patients, 26 were male and the median age was 71.5 years (range: 24 – 87). 35 patients were HIV-uninfected and four were with an unknown HIV-status. All had diagnosed or suspected malignancies, all but two of haematological origin and all were immunocompromised as a consequence of their condition and/or its management (Table 4.4). Only one patient had a history of PJP (patient 36 in Table 4.4).

Table 4.4 Demographic details, primary diagnosis and HIV status sorted in the scope of PJP diagnosis

	Likely PJP					Unlikely PJP			
Serial*	Age (Y)	Gender	Condition	HIV-status	Serial*	Age (Y)	Gender	Condition	HIV-status
1	66	M	CLL	-ive	2	59	M	NHL (DLBCL)	-ive
5	68	M	PCL	-ive	3	54	M	AML	-ive
7	69	M	CLL	-ive	4	83	M	NHL (DLBCL)	-ive
8	69	F	MM	-ive	6	86	M	PMR	-ive
12	74	M	NHL (DLBCL)	-ive	9	60	M	NHL (TC)	-ive
13	76	M	NHL (DLBCL)	-ive	10	52	M	NHL	-ive
19	69	M	MDS	-ive	11	66	M	CLL	-ive
24	73	M	MM	U	14	68	M	NHL (TC)	-ive
25	77	M	CLL	-ive	15	53	F	M. BrCa	U
26	87	M	NHL (MCL)	-ive	16	68	M	MDS	-ive
28	74	F	NHL (TC)	-ive	18	44	F	AML	U
29	71	F	NHL (TC)	-ive	21	75	F	AML	-ive
30	75	F	MM	-ive	22	42	M	HCL	-ive
31	59	F	NHL (FL)	-ive	23	81	M	CLL	-ive
32	73	M	NHL (DLBCL)	-ive	27	62	M	AML	-ive
33	80	M	NHL (DLBCL)	-ive	38	54	M	MM; IVDU	-ive
34	87	F	CLL	-ive	39	24	F	HL	-ive
35	61	F	BrCa	U	40	72	F	NHL (TFL)	-ive
36	66	M	CLL	-ive	41	77	F	NHL (BC)	-ive
37	27	M	NHL (BL)	-ive					

Definition of abbreviations: AML= Acute myeloid leukaemia; CLL= Chronic lymphocytic leukaemia; HCL= Hairy cell leukaemia; PCL= Plasma cell leukaemia; MM= Multiple myeloma; HL= Hodgkin lymphoma; NHL= Non-Hodgkin lymphoma; BC= B-cell NHL; TC= T-cell NHL; DLBCL= Diffuse large B-cell lymphoma; FL= Follicular lymphoma; TFL= Transformed follicular lymphoma; BL= Burkitt lymphoma; MDS= Myelodysplastic syndrome; BrCa= Breast cancer; M= Metastatic; PMR= Polymyalgia rheumatica; IVDU= Intravenous drug user; U= Unknown. Patients (17, 20, 42–45) were excluded. Rows shaded in yellow indicate positive masks.

* The sampled patients are listed in a numerical order concordant with that of the mask-samples

Ten patients were with normal CXR findings and two were with normal CT findings. The radiological findings are summarised in Table 4.5. 23 patients had positive serum BDG (>80 pg/mL), one had an equivocal result (60–80 pg/mL) and a further one was not tested (Table 4.5). Seven patients had *P. jirovecii* detected by PCR in BAL, and two in induced sputa. None had *P. jirovecii* visualised microscopically by calcofluor white staining (Table 4.5). 28 patients received anti-pneumocystis therapy; of these, four did not show a "favourable" outcome (two deceased, one had primary disease progression and one did not complete PJP therapy) (Table 4.6). 20 patients were classified as having "likely" PJP (Table 4.4).

Table 4.5 Laboratory and radiological data

		Likely PJP								Unlikely PJP					
Serial	BDG	BAL		ORS		CXR	CT	Serial	BDG	BAL		ORS		CXR	CT
		PCR	Stain	Type	PCR	grade¶	grade¶			PCR	Stain	Type	PCR	grade¶	grade¶
1	+ive	+ive (A)	-ive			0	3	2	+ive	-ive (A)	-ive			0	0
5	+ive	+ive (A)	-ive			1	2	3	-ive	-ive (A)	-ive			1	3
7	+ive	+ive (A)	-ive	LB	+ive (A)*	4	4	4	+ive	NA	NA			2	2
8	-ive	NA	NA			2	2	6	+ive	NA	NA			0	1
12	-ive	-ive (A)	NA			1	2	9	-ive	-ive (B)	-ive			1	1
13	+ive	NA	NA			0	2	10	-ive	-ive (A)	-ive			3	2
19	+ive	+ive (B)	-ive			0	2	11	-ive	NA	NA			1	2
24	BL	+ive (A)	-ive			1	1	14	+ive	NA	NA			1	2
25	+ive	+ive (A)	-ive			2	4	15	-ive	NA	NA			3	1
26	+ive	NA	NA			3	3	16	+ive	-ive (B)	NA			0	2
28	+ive	NA	NA			1	4	18	-ive	NA	NA	ETS	-ive (A)	3	2
29	+ive	NA	NA	IS	+ive (A)	3	3	21	-ive	NA	NA			0	0
30	+ive	NA	NA	IS	+ive (C)	0	2	22	-ive	-ive (A)	NA			1	2
31	-ive	NA	NA			2	3	23	+ive	-ive (A)	NA			0	4
32	+ive	-ive (A)	-ive			2	3	27	-ive	NA	NA			3	3
33	+ive	NA	NA			1	3	38	-ive	NA	NA			1	1
34	+ive	NA	NA			1	3	39	+ive	NA	NA			1	4
35	+ive	NA	NA			2	2	40	NA	NA	NA			2	3
36	+ive	+ive (A)	-ive			2	1	41	-ive	NA	NA			0	NA
37	+ive	NA	NA			0	0								

Definition of abbreviations: BDG= (1,3)-Beta-D-glucan assay (serum or BAL); BAL= Bronchioalveolar lavage; ORS= Other respiratory specimens; CXR= Chest X-ray; CT= Chest computed tomography; ¶= According to Table 4.9; BL= Borderline; NA= No data available; A, B, C= The procedure was made after (A), before (B) or on the contemporaneous day (C) of collecting the mask; LB= Lung biopsy; *= Panfungal PCR; ETS= Endotracheal secretions; IS= Induced sputum; Patients (17, 20, 42–45) were excluded. Rows shaded in yellow indicate positive masks.

Table 4.6 Antimicrobial management of the sampled patients

	Likely PJP						Unlikely PJP					
Serial	Receipt of anti-PJP PPx	Receipt of anti-PJP Tx	Type/duration of anti-PJP Tx in days	Receipt of other* agents	Response to anti-PJP Tx	Other excluded or treated aetiologies	Serial	Receipt of anti-PJP PPx	Receipt of anti-PJP Tx	Type/duration of anti-PJP Tx in days	Receipt of other* agents	Response to anti-PJP Tx	Other excluded or treated aetiologies
1	+	+	PEN21	–	+	PE; CHF; HHV6; CMV	2	–	–		+		PE; CMV
5	–	+	TMP-SMX05	+	+		3	+	–		–		
7	–	+	ATO15/CAS21	–	+		4	–	–		+		*Candida albicans*
8	–	+	TMP-SMX07	–	+	HV; PE	6	–	–		–		
12	–	+	TMP-SMX21	+	+		9	+	–		+		
13	–	+	TMP-SMX09	+	+	PE	10	+	–		–		MPV
19	–	+	TMP-SMX07/CLI05/PRI04	–	+	Influenza B	11	+	+	TMP-SMX04	–	+	Rhi-virus
24	–	+	TMP-SMX09/CAS24	+	+		14	+	+	CLI05/PRI02	+	+	*Pseudomonas spp.*
25	–	+	TMP-SMX18	+	+	*Facklamia hominis*	15	+	–		+		PEF
26	–	+	TMP-SMX07/PRI15	–	+	ASP; PEF	16	–	–		–		CMV; parvo virus
28	–	+	TMP-SMX06	–	–	PE; ASP	18	–	+	TMP-SMX05	–	–	
29	+	+	TMP-SMX16/CLI02/PRI01	–	+	*C. albicans*	21	–	–		–		
30	–	+	TMP-SMX14	+	+	*C. albicans*	22	–	+	TMP-SMX21	+	+	
31	+	+	TMP-SMX09	–	+	MPV; PE	23	+	+	TMP-SMX17	+	+	HAP; *C. albicans*
32	+	+	TMP-SMX18	–	+	PE	27	+	+	TMP-SMX08	+	+	PO; PE
33	–	+	TMP-SMX12/CAS04	+	–	PO; PE	38	+	+	TMP-SMX02	+	–	PE; PEF
34	+	+	TMP-SMX07	+	+	PE	39	–	–		–		
35	–	+	TMP-SMX04	–	+	PE	40	–	+	TMP-SMX21	+	+	PE; TB
36	–	+	PEN19/CAS08	+	+	HHV 6 & 7; *Escherichia coli*; *C. albicans*	41	+	–		–		VM; PO
37	–	+	TMP-SMX19	–	+	CMV; EBV; PE							

PPx= Prophylaxis; Tx= Treatment; *=mainly posaconazole, voriconazole, fluconazole or doxycycline; PEN= Pentamidine; TMP-SMX= Trimethoprim-sulfamethoxazole; ATO= Atovaquone; CAS= Caspofungin (Echinocandin); CLI= Clindamycin; PRI= Primaquine; PE= Pulmonary embolism; CHF= Congestive heart failure; HHV6= Human herpesvirus 6; CMV= Cytomegalovirus; HV= Hypervolemia; MPV= Metapneumovirus; PEF= Pleural effusion; HAP= Hospital-acquired pneumonia; ASP= Aspiration pneumonia; PO= Pulmonary oedema; EBV= Epstein–Barr virus; TB= Pulmonary tuberculosis; VM= Viral myocarditis; Patients (17, 20, 42–45) were excluded. Rows shaded in yellow indicate positive masks.

Controls

Four air samples were collected from patients' rooms and from the corridors leading to these rooms (Figure 4.4).
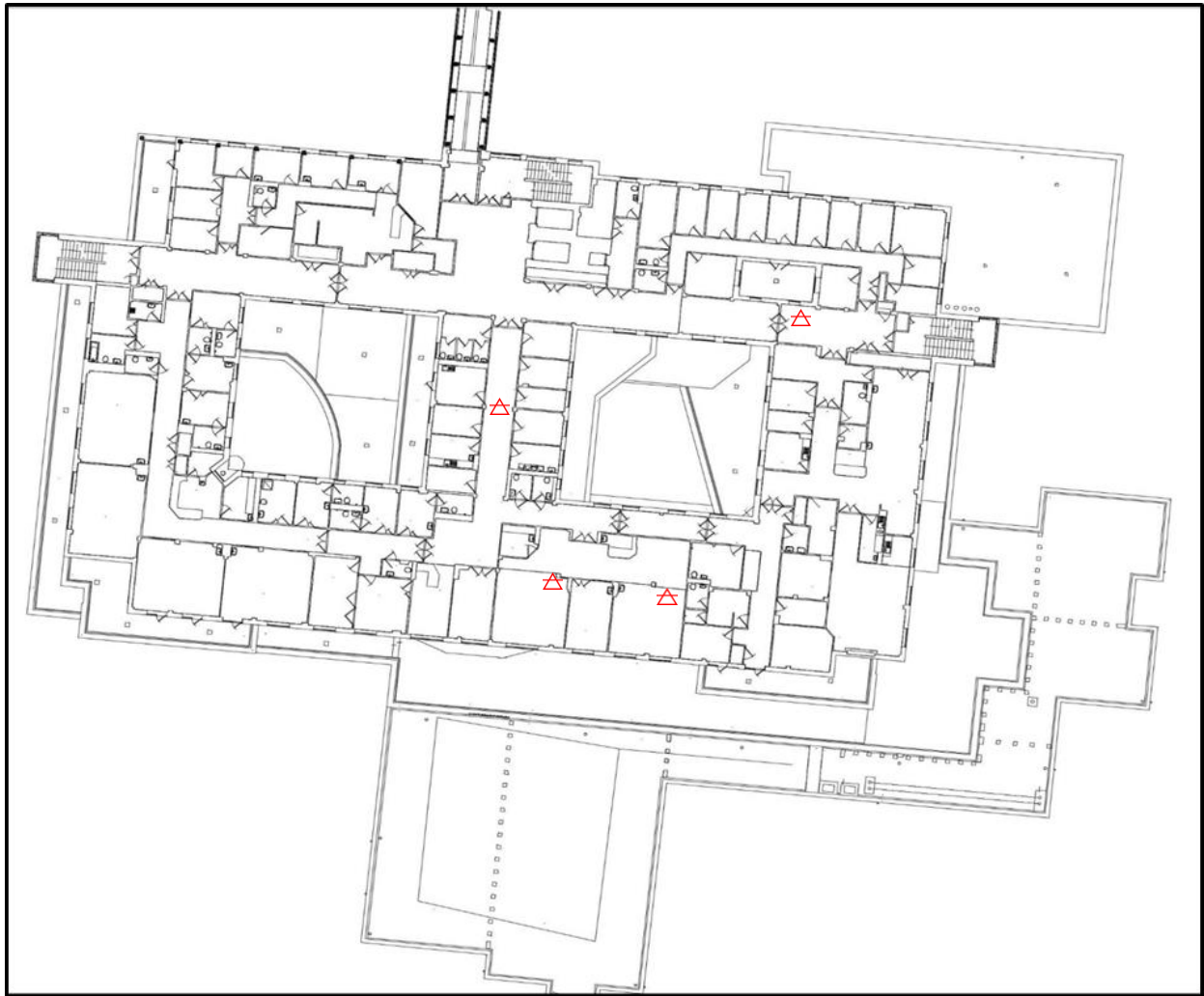
Figure 4.4 Structure map of sampled locations at LRI
△= sampled locations. This was reproduced with permission of NHS Estates and Facilities

Face-masks collected from healthy volunteers (3.2.1) were used as negative control.

4.3.2. Analysis of face-mask samples for *P. jirovecii*

Of the 45 patients who were sampled, *P. jirovecii* was detected in ten (22.2%) (seven males; median age: 70 years) using the in-house TaqMan assay. There were neither gender- nor age-related differences in distribution. The median captured signal was 8.59×10^4 (IQR= 3.01×10^5 – 1.81×10^4) *MtLSU* copies per mask.

Of the 20 patients who were classified with likely PJP, *P. jirovecii* was detected in seven (35.0%). The median captured signal was 2.27×10^4 (IQR=1.97×10^5 – 1.33×10^4) copies per mask.

Of the 19 patients how were classified with unlikely PJP, *P. jirovecii* was detected in three (15.8%). The median captured signal was 1.51×10^5 (IQR= 4.50×10^5 – 1.40×10^5) copies per mask.

The face-mask results are presented in Table 4.7.

When detected, there was no statistically significant difference between the quantity of exhaled *P. jirovecii* from patients who were classified with "likely" PJP and the quantity of those classified with "unlikely" PJP (p=0.27) (Figure 4.5). This finding was observable even after the outliers were excluded (p= 0.14).

None of the indoor-air samples or the face-masks collected from the healthy volunteers was positive for *P. jirovecii*.

Table 4.7 Quantification results of exhaled *P. jirovecii* presented as *MtLSU* gene copy/mask

Likely PJP		Unlikely PJP		Excluded	
Serial	**Result**	**Serial**	**Result**	**Serial**	**Result**
1	3.51E+05	**2**	1.28E+05	**17**	<LLD
5	4.34E+04	**3**	<LLD	**20**	<LLD
7a	1.86E+03	**4**	7.49E+05	**42**	<LLD
7b	<LLD	**6**	1.51E+05	**43**	<LLD
8	<LLD	**9**	<LLD	**44**	<LLD
12	<LLD	**10**	<LLD	**45**	<LLD
13	<LLD	**11**	<LLD		
19	2.27E+04	**14**	<LLD		
24	<LLD	**15**	<LLD		
25	<LLD	**16**	<LLD		
26	<LLD	**18**	<LLD		
28	1.66E+04	**21**	<LLD		
29	1.28E+07	**22**	<LLD		
30	1.01E+04	**23**	<LLD		
31	<LLD	**27**	<LLD		
32	<LLD	**38**	<LLD		
33	<LLD	**39**	<LLD		
34	<LLD	**40**	<LLD		
35	<LLD	**41**	<LLD		
36	<LLD				
37	<LLD				

Definition of abbreviation: LLD= Lower limit of detection. In-house *MtLSU* qPCR TaqMan assay was used for quantification.
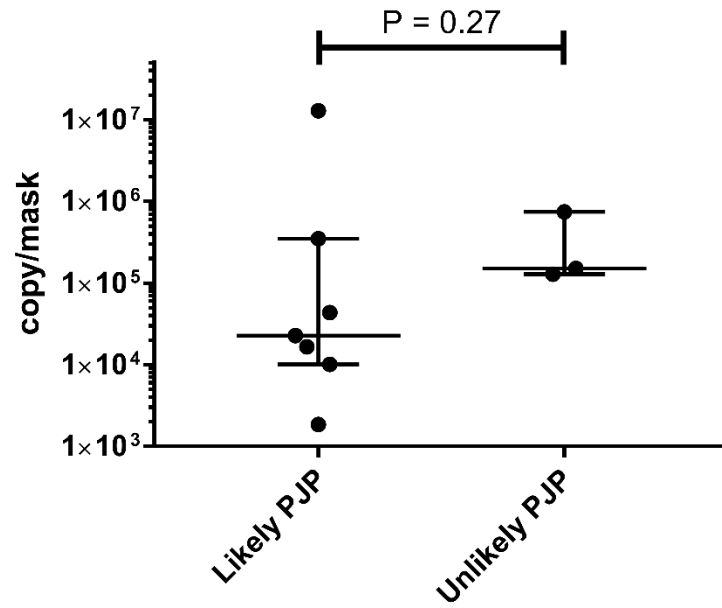
Figure 4.5 Comparison of the exhalation burden (copy/mask) between patients with likely and unlikely PJP

Mann-Whitney test was done to determine statistically significant difference at $p<0.05$. (Error Bar= IQR).

4.3.3. Correlation with the clinical picture and radiological findings

Of the 39 patients who were included for clinical correlation, *P. jirovecii* was detected in ten (25.6%) using the in-house TaqMan assay.

As described earlier, the clinical picture is not specific to PJP. The most common symptom among the patients with positive masks was pyrexia (>38°C) presented by seven patients (70.0%). Two patients (20.0%) were *P. jirovecii*-exhaler with low grade fever (37 to <38°C). One patient (10.0%) was exhaler with normal body temperature. Coughing as a symptom was presented by only five patients (50.0%), in four of whom it was a productive cough (40.0% of *P. jirovecii*-exhalers and 80.0% of coughers) and for one was a dry cough (10.0% of *P. jirovecii*-exhalers and 20.0% of coughers). Four exhalers (40.0%) were dyspnoeic, four (40.0%) were hypoxic and three (30.0%) had both features. Three exhalers (30.0%) were with neutropenic sepsis.

However, all these features were indistinguishably shared among the whole study cohort.

These figures for the 29 who yielded negative masks were 18 (62.1%), 1 (3.5%), 8 (27.6%), 15 (51.7%), 5 (17.2%; 33.3%), 10 (34.5%; 66.7%), 20 (69.0%), 22 (75.9%), 19 (65.5%) and 12 (41.4%), respectively.

There were no distinguishing features in symptoms between *P. jirovecii*-exhalers and those with negative masks (Table 4.8). Interestingly, patients 6 and 7 were presented with very few symptoms but were *P. jirovecii*-exhalers.

Table 4.8 Clinical picture of the sampled patients

Likely PJP

Serial	CP	WBC	N	L	M	E	B	CRP	CD4*
1	1, 1A, 3, 4	2.50	1.40	0.82	0.12	0.02	0.01	38	230
5	1, 3, 4	3.80	3.30	0.18	0.16	0.06	0.02	52	NA
7a	7, 8	138.90	6.95	131.96	0.00	0.00	0.00	20	7%
7b	7, 8	180.80	9.04	171.76	0.00	0.00	0.00	17	7%
8	1, 1A, 2, 3, 4, 5, 6B, 8	1.10	0.99	0.05	0.02	0.00	0.01	91	NA
12	3, 4, 6	6.00	3.90	0.69	0.90	0.05	0.05	104	3%
13	1, 1A, 6	7.50	6.28	0.19	0.63	0.02	0.07	45	320
19	1A, 6B, 8, 10	3.50	0.77	2.43	0.21	0.00	0.00	60	NA
24	3, 4	3.60	2.42	0.61	0.38	0.03	0.01	74	NA
25	1, 2B, 3, 4, 6	24.00	14.70	8.33	0.31	0.01	0.13	31	100
26	3, 4, 8, 10	4.50	4.17	0.17	0.07	0.03	0.00	144	150
28	1, 4, 6, 8	5.20	3.52	0.97	0.39	0.03	0.04	17	860
29	1, 2B, 8, 9	1.50	1.02	0.32	0.10	0.01	0.00	26	150
30	1, 3, 4, 6B, 8	2.30	1.09	0.52	0.29	0.40	0.01	54	100
31	1, 1A, 2, 3, 4, 6B, 10	2.50	1.75	0.43	0.16	0.11	0.01	56	2%
32	1, 1A, 1B, 3, 4, 5, 6, 8, 9	22.30	20.47	0.76	0.63	0.07	0.17	121	1%
33	1A, 3B, 4, 5, 6, 8	0.40	0.24	0.15	0.00	0.00	0.00	52	NA
34	1B, 3, 4, 6B	44.70	1.69	40.55	0.14	0.01	0.47	<5	710
35	1, 3, 4, 5, 6	8.40	5.87	1.06	1.05	0.23	0.06	67	NA
36	1, 3, 4, 6B	1.70	0.98	0.49	0.14	0.07	0.01	36	230
37	1, 3B, 4, 7B, 8	5.10	3.35	0.96	0.45	0.16	0.03	<5	280

Unlikely PJP

Serial	CP	WBC	N	L	M	E	B	CRP	CD4*
2	1, 2B, 3, 5, 9	5.70	4.98	0.21	0.27	0.09	0.02	150	110
3	1, 1A, 2, 6B	0.10	0.05	0.03	0.00	0.00	0.00	231	NA
4	1A, 6B, 10	2.80	1.81	0.51	0.32	0.03	0.01	44	2%
6	1, 8	3.70	2.92	0.25	0.21	0.09	0.02	71	NA
9	1, 1A	3.80	3.30	0.14	0.24	0.08	0.01	124	NA
10	1, 1A, 6, 10	1.60	1.05	0.12	0.08	0.18	0.04	199	NA
11	1, 2, 3, 4, 8	10.00	7.39	1.88	0.57	0.03	0.07	33	310
14	3, 4, 6	14.40	13.18	0.58	0.49	0.01	0.03	12	60
15	3, 4, 8	9.70	8.84	0.22	0.53	0.03	0.01	422	NA
16	1A, 3, 4, 8	0.60	0.32	0.15	0.11	0.03	0.00	12	NA
18	3, 4	8.60	5.93	0.32	0.42	1.77	0.02	103	NA
21	1, 2B, 8, 9, 10	58.8	49.73	4.01	2.64	0.04	4.92	260	NA
22	1, 1A, 10	1.10	0.61	0.43	0.01	0.01	0.01	50	220
23	1, 4	67.10	11.68	51.21	0.91	0.05	0.10	26	2%
27	1, 1A, 4, 6, 8	0.10	0.06	0.01	0.02	0.00	0.00	219	NA
38	1, 3, 4, 5	3.40	1.94	0.65	0.18	0.48	0.04	42	NA
39	7, 7B	2.30	1.06	0.96	0.04	0.05	0.02	<5	NA
40	1, 1A, 3, 4, 6, 7B, 8	1.90	0.91	0.40	0.34	0.23	0.00	<5	220
41	3, 4, 7B	7.20	3.31	3.53	0.07	0.00	0.00	115	7%

Definition of abbreviations: CP= Clinical presentation; WBC= White blood cell count (x109/L); N= Neutrophil count (x109/L); L= Absolute lymphocytes count (x109/L); M= Monocytes count (x109/L); E= Eosinophils count (x109/L); B= Basophils count (x109/L); LDH= Lactate dehydrogenase (IU/L); CRP= C-reactive protein (mg/L); CD4= Cluster of differentiation 4 (cell/mm3); % quoted results= Bone marrow result; *= The result was the one available within 3 months of the episode due to the nature of this test; NA= No data available; Ex= Excluded; 1= Pyrexia; 1A= Neutropenic sepsis; 1B= Low-grade fever; 2= Rigors; 2B= Night sweats; 3= Dyspnoea; 3B= Dyspnoea on exertion; 4= Hypoxia, decreased oxygen saturation (↓SaO2); 5= Tachycardia; 6= Dry cough; 6B= Productive cough; 7= Chest lesions (radiology finding); 7B= Chest pain; 8= Malaise; 9= Weight loss; 10= Others: Rash, Coryzal symptoms, Paleness, Collapse or Vomiting; Rows shaded in yellow indicate positive masks. Patients (17, 20, 42–45) were excluded and their masks were negative

Of the ten patients who produced positive masks, no significant difference was found between the *P. jirovecii* signals from those who did and those who did not receive anti-pneumocystis prophylaxis (as a measure taken by the clinician during the episode after the mask was collected) (p=0.27), even after outlier exclusion (p= 0.14). It is worth mentioning that those classified with likely PJP had received the prophylaxis while those with unlikely PJP had not.

The radiological findings were graded from 0 to 4 (Table 4.9). This was informed from the medical literature (4.1.5.1) and from Mu *et al.* (2016).

Table 4.9 Radiological findings grading scale	
Grade	**Definition**
0	The radiologist reported the findings within normal limits or excluded infection
1	Unilateral findings including unilateral parenchymal changes, atelectasis, hyperinflation, consolidation or increased interstitial markings
2	Bilateral infiltrates on CXR or bilateral diffuse GGO and patchy consolidations on CT
3	Bilateral consolidations on CXR or bilateral diffuse GGO and ITS or bilateral predominant consolidations on CT.
4	Cavitation (indicates chronicity)
Definition of abbreviations: CXR= Chest X-ray; CT= Computed tomography (chest); GGO= Ground glass opacities; IST= Interlobar septal thickening	

No statistically significant differences were found between *P. jirovecii*-exhalers (positive masks) and non-exhalers based on the CXR grade (p= 0.49) or the CT grade (p=0.77) (Figure 4.6).
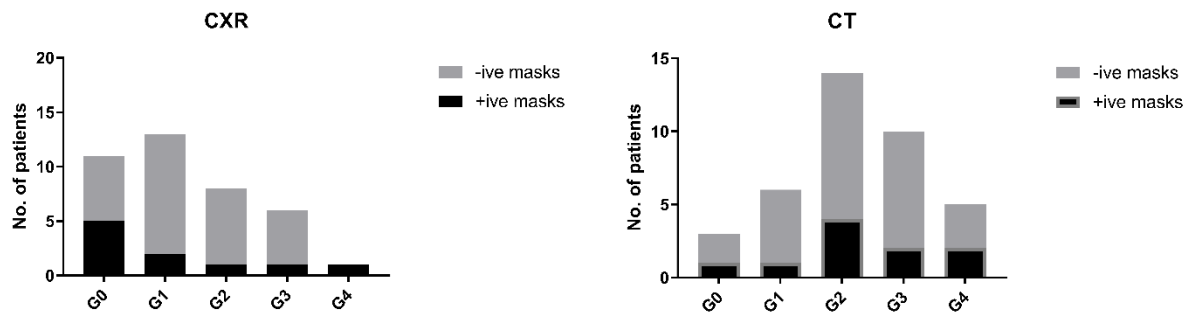
Figure 4.6 Comparison of positive and negative masks based on the radiological grade (G)
Chi-square test for trend was done to determine statistically significant difference at p<0.05. The included mask results were obtained by the in-house TaqMan assay.

Five of ten exhalers (50.0%) were with normal findings on CXR and their masks were positive with an exhalation burden (measured by captured signal) of 1.28×10^5 copies per mask (IQR=1.51×10^5 – 2.27×10^4). The radiologist excluded infection on CT for one patient producing a positive mask with an exhalation burden of 1.28×10^5 copies per mask. All other *P. jirovecii*-exhalers had a CT scan with abnormal findings (Table 4.5).

4.3.4. Comparison with invasive and semi-invasive diagnostic procedures

Of the 39 patients who were included in the clinical correlation, 16 (41.0%) were subjected to BAL, one (2.6%) to endotracheal aspiration and two (5.1%) to sputum induction.

In-situ localized *P. jirovecii* was PCR-detected in seven (43.8%), none (0.0%) and two (100%), respectively.

Exhaled *P. jirovecii* was detected in four (25.0%), none (0.0%) and two (100%), respectively, per the face-mask using the in-house TaqMan assay. Of these and with regards to the timing, *P. jirovecii* status was confirmed per the face-mask in three (75.0%), one (100%) and one (50.0%), respectively, before the diagnostic procedure was carried out. *P. jirovecii* was detected per the face-mask on the same day of conducting sputum induction for one patient (50.0%) and after the BAL was carried out for a further one (25.0%).

Of the 17 patients who were subjected to BAL or endotracheal aspiration, there was consistency with the face-mask (both negative) in nine patients (52.9%). Of these and with regards to the timing, *P. jirovecii* status was confirmed negative per the face-mask in seven (77.8%) before the BAL or the aspiration did and in two (22.2%) after (Figure 4.7).
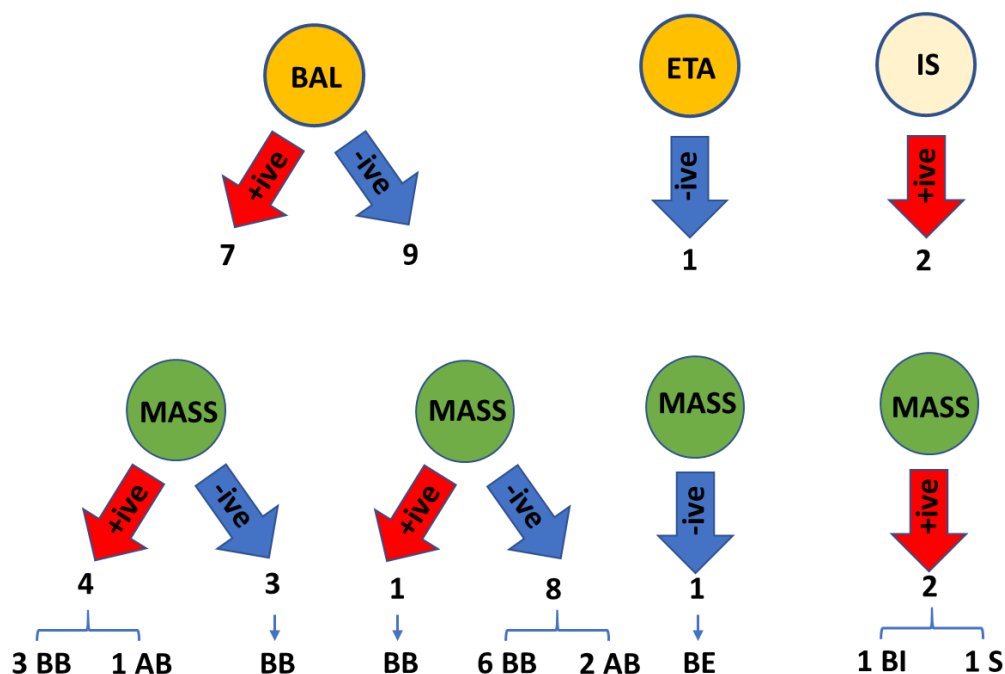
Figure 4.7 Results of invasive (BAL and ETA) and semi-invasive (Induced sputum; IS) procedures and non-invasive mask aerosol sampling system (MASS)
Timing abbreviation: BB= Before BAL; AB= After BAL; BE= Before ETA; BI= Before sputum induction; S= On the same day.

Overall and considering the BAL and endotracheal aspiration as a "gold standard", of the seven patients who were tested positive, *P. jirovecii* was detected in four (57.1%) per the face-mask. Of the 10 who were tested negative, 9 (90.0%) had negative masks. In other words, the true positive (TP), true negative (TN), false positive (FP) and false negative (FN) results of the face-mask using the in-house TaqMan assay were 4, 9, 1 and 3, respectively. This resulted in sensitivity, specificity, positive and negative predictive values of 57.1%, 90.0%, 80.0% and 75.0%, respectively (Table 4.10).

Considering the BAL, endotracheal aspiration and sputum induction as a "gold standard", the TP, TN, FP and FN of the face-mask were 6, 9, 1 and 3, respectively. This resulted in sensitivity, specificity, positive and negative

predictive values of 66.7%, 90.0%, 85.7% and 75.0%, respectively (Table 4.10).

Considering the final clinical diagnosis as a "gold standard", the TP, TN, FP and FN of the face-mask were 7, 16, 3 and 13, respectively. This resulted in sensitivity, specificity, positive and negative predictive values of 35.0%, 84.2%, 70.0% and 55.2%, respectively (Table 4.10).

For the 19 patients cohort and considering the final clinical diagnosis as a "gold standard", the TP, TN, FP and FN of the BAL, endotracheal aspiration and sputum induction together were 9, 8, 0, 2. This resulted in sensitivity, specificity, positive and negative predictive values of 81.8%, 100%, 100% and 80.0%, respectively (Table 4.10).

Table 4.10 Diagnostic values of the Mask, BAL, ETA, IS and BDG

Diagnostic value of	Mask										BAL, ETA and IS		BDG		BDG and Mask	
Gold standard	BAL and ETA		BAL, ETA and IS		BDG (any)		BDG (≤7 days)		Clinical Dx		Clinical Dx		BAL, ETA and IS			
	Value	95% CI	Value	95% CI	Value	95% CI	Value	95% CI	Value	95% CI	Value	95% CI	Value	95% CI	Value	95% CI
Sensitivity	57.14	18.41 – 90.10	41.67	22.11 – 63.36	35.00	15.39 – 59.22	40.91	20.71 – 63.65	35.00	15.39 – 59.22	81.82	48.22 – 97.72	100	66.37 – 100	66.67	29.93 – 92.51
Specificity	90.00	55.50 – 99.75	100.00	76.84 – 100.00	84.21	60.42 – 96.62	100.00	78.20 – 100.00	84.21	60.42 – 96.62	100	63.06 – 100	60.00	26.24 – 87.84	90.00	55.50 – 99.75
PLR	5.71	0.80 – 40.85			2.22	0.67 – 7.34			2.22	0.67 – 7.34			2.50	1.17 – 5.34	6.67	0.98 – 45.29
NLR	0.48	0.20 – 1.15	0.58	0.42 – 0.82	0.77	0.53 – 1.12	0.59	0.42 – 0.84	0.77	0.53 – 1.12	0.18	0.05 – 0.64	0.00		0.37	0.14 – 0.95
PPV	80.00	35.88 – 96.62	100.00		70.00	41.32 – 88.55	100.00		70.00	41.32 – 88.55	100		69.23	51.30 – 82.78	85.71	46.90 – 97.61
NPV	75.00	55.44 – 87.85	50.00	41.63 – 58.37	55.17	45.80 – 64.19	53.57	44.90 – 62.03	55.17	45.80 – 64.19	80.00	53.31 – 93.34	100		75.00	53.79 – 88.55
Accuracy	76.47	50.10 – 93.19	63.16	45.99 – 78.19	58.97	42.10 – 74.43	64.86	47.46 – 79.79	58.97	42.10 – 74.43	89.47	66.86 – 98.70	78.95	54.43 – 93.95	78.95	54.43 – 93.95

Definition of abbreviation: PLR= Positive Likelihood Ratio; NLR= Negative Likelihood Ratio; PPV= Positive Predictive Value; NPV= Negative Predictive Value; CI= Confidence Interval; BAL= Bronchioalveolar lavage; ETA= Endotracheal aspiration; IS= Induced sputum; Dx= Diagnosis; BDG= (1,3)-Beta-D-glucan; MASS= Mask aerosol sampling system. All listed values are presented as percentages except for PLR and NLR. Gray shaded cells represent indefinable or incalculable values.

4.3.5. Investigation of possible false-negative results

It was suggested based on published data on the environmental widespread of *P. jirovecii*, described as an "ubiquitous" fungus, and its colonization of the respiratory tract (4.1.3), that positive masks should have been encountered more frequently. However, this did not seem to be the case.

Two possible reasons for false negative masks were considered:

1) Technical factors. Related experiments are described below.

2) Biological factors. These will be discussed in 4.3.7.

The face-mask DNA extracts were submitted anonymously to an external commercial laboratory (Micropathology; the routine provider for PJP PCR assays for the local laboratory of the University Hospitals of Leicester NHS Trust). The results were completely concordant with those produced in this study. The external laboratory, however, declined to disclose their PCR protocol.

The face-mask extracts were tested for possible presence of PCR inhibitors. A known concentration of *P. jirovecii* DNA was added to a number of extracts (22 samples) which were not amplifiable with the in-house TaqMan assay. The same concentration was used to prepare a positive control of amplification. The artificially-contaminated clinical extracts showed around 1 log fold reduced PCR-quantification compared to the control. No full inhibition, however, was detected (Table A.40). A further manipulation was trialed through diluting a number of extracts with molecular grade water (1:4), and a further set was treated with a commercial PCR-inhibitor removal kit according to the manufacturer's instructions (OneStep PCR Inhibitor Removal kit, Zymo Research, USA). No improvement in the amplification, however, was noted using either way.

The purity of extracted DNA was estimated spectrophotometrically and found comparable in all extracts (Table A.41).

Radomski *et al.* (2013) found that merely a high DNA purity was not sufficient to achieve accurate mycobacterial quantification with qPCR when the samples contained >4log ng of host DNA. A further set of the extracts (21 samples) was analysed for human β-globin gene (*HβG*) to mimic an "internal control" function alongside to investigate the burden of host DNA. Of these, *HβG* was amplifiable and quantifiable in 17 (80.9%) of the tested extracts. The remaining 4 extracts showed amplification that was not differentiable from that of the negative control therefore they were interpreted as negative (Table A.42). Furthermore, it was suggested that the presence of *HβG* as a non-specific template could have undermined the amplification of the target of interest (*MtLSU*) due to template competition for reaction compounds (Kalle *et al.*, 2014). The quantity of exhaled *P. jirovecii* was tested for possible negative correlation with the quantity of exhaled *HβG*. However, neither a statistically significant nor a trend of negative correlation was found (r= 0.24, p= 0.29) (Table A.46).

It was also thought that the efficiency of the original TaqMan probe was possibly undermined by a prolonged storage. Therefore, the extracts were re-analysed with a new probe, however without a useful outcome.

Le Gal *et al.* (2017) reported a rare single-base polymorphism at position 210 of the *MtLSU* rRNA gene of *P. jirovecii* (Figure 4.8). This punctual mutation was located within the hybridization region of the TaqMan probe and led to a misleading false-negative result when Le Gal's group assayed their samples with a similar TaqMan assay (originally developed by Meliani *et al.*, 2003). Based on Le Gal *et al.* (2017)'s findings, a probe sequence with an N nucleobase (a mixed base from the 4 bases at an equimolar ration of 25:25:25:25) at the position 210 was designed. Using the "N" TaqMan probe,

one extract that was not previously amplifiable with the original probe had been successfully amplified (Sample 19 in Table A.39).
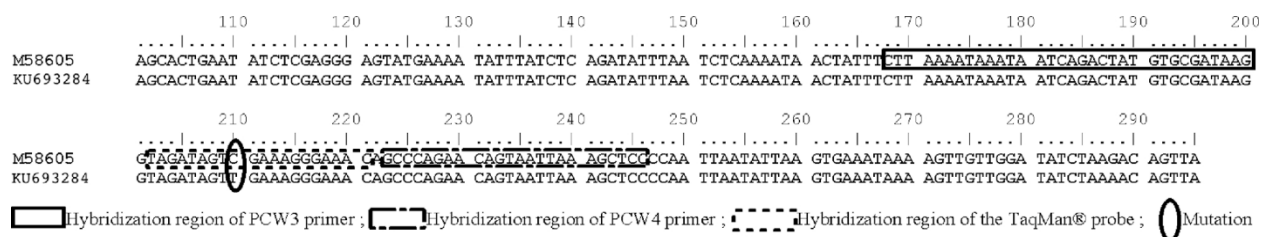
Figure 4.8 Alignment of the *MtLSU* rRNA gene reference sequence of *P. jirovecii* (Accession number M58605) with the sequence (Accession number KU693284) which led to false-negative qPCR result in the Le Gal *et al.* (2017)'s study. This was reproduced from: A misleading false-negative result of *Pneumocystis* real-time PCR assay due to a rare punctual mutation: A French multicenter study. Med Mycol. 2016;55(2):180-184. Published by Oxford University Press on behalf of The International Society for Human and Animal Mycology and reproduced here with the permission of Oxford University Press. PCW3 and PCW4 are the hybridization regions of the used primers. The reference amplicon (79 bp) is located between 168 and 246 nucleotide positions.

To address a possible mutation in the TaqMan probe, a selected set of the extracts were assayed with SYBR-Green chemistry using the same primers (*MtLSU*-F, *MtLSU*-R) used for amplifying the *MtLSU* rRNA gene with the TaqMan chemistry. Interestingly, a number of extracts which were negative with the TaqMan assay were positive with the in-house SYBR-Green assay (Table A.39). Since the amplicon size is relatively small to be run with SYBR-Green chemistry (79bp), a selected set was further assayed for a larger amplicon (301bp) covering the original target sequence using pH207, pAZ102-E primer pair (Figure 4.9). The results are presented in Table A.39.
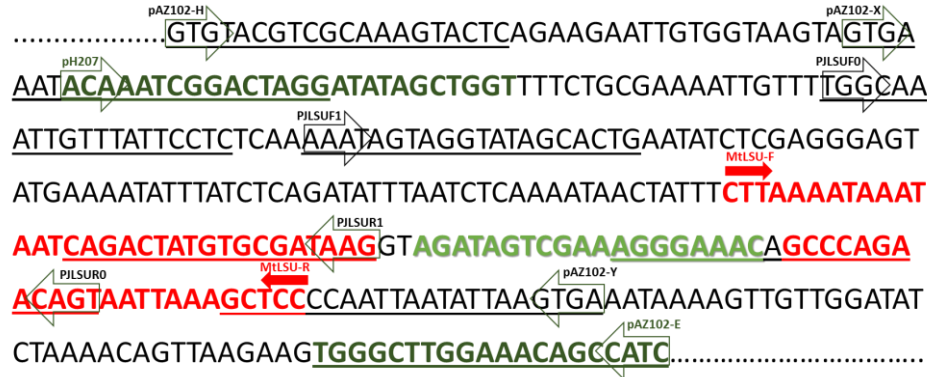
Figure 4.9 Schematic representation of position and sequence of primer sets used for targeting the mitochondrial large subunit (*MtLSU*) within the nucleotide sequence of *P. jirovecii* (Accession number: JX855937)

Arrows indicate the position of the target sequences. The shadowed green (between bold red) region indicates the hybridization region of the TaqMan probe. The bold red indicates the hybridization region of the primers *MtLSU*-F and *MtLSU*-R which can produce an amplicon size of 79bp. The bold green indicates the hybridization region of the primers pH207 and pAZ102-E which can produce an amplicon size of 301bp. The external primers (PJLSUF0, PJLSUR0) or (pAZ102-H, pAZ102-E) and internal primers (PJLSUF1, PJLSUR1) or (pAZ-X, pAZ-Y), respectively, were used for the nested PCR. The primers (pAZ102-X and pAZ102-Y) were used to produce an amplicon for sequencing.

These findings had led to re-assay a number of the extracts that were negative on the TaqMan assay with an in-house nested PCR developed by Tia *et al.* (2012) targeting the same gene (Figure 4.9). The nested PCR assay revealed three positive masks were negative with the TaqMan assay (Table A.43; Figure 4.10).
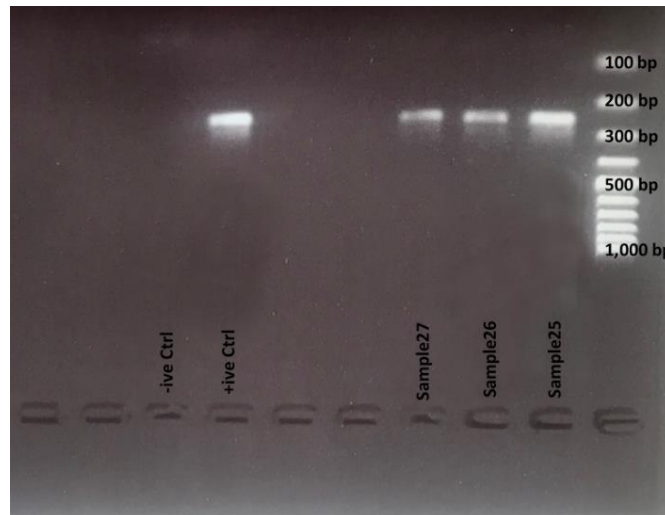
Figure 4.10 Gel analysis of nested PCR amplicons
The external primers (pAZ102-H and pAZ102-E) and internal primers (pAZ-X and pAZ-Y) were used. The amplicon size is 252 bp. 100-bp DNA ladder was used.

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Interestingly, upon submitting one of these extracts (Sample 25) to sequencing the *MtLSU* gene fragment including the hybridization region of the TaqMan assay, no mutation was detected, and the sequences were completely aligned (Figure 4.11).

```
JX855937    GTGAAATACAAATCGGACTAGGATATAGCTGGTTTTCTGCGAAAATTGTTTTGGCAAATT    60
Sample25    --------------------------AATCAAGCTTCTGCGAATTGTTTTGGCAATTG     32
                                           *   *   *************** *

JX855937    GTTTATTCCTCTCAAAAATAGTAGGTATAGCACTGAATATCTCGAGGGAGTATGAAAATA    120
Sample25    TTTATTCCTCTATAAAAATAGTAGGTATAGCACTGAATATCTCGAGGGAGTATGAAAATA     92
                **   *  *      **********************************************

JX855937    TTTATCTCAGATATTTAATCTCAAAATAACTATTTCTTAAAATAAATAATCAGACTATGT    180
Sample25    TTTATCTCAGATATTTAATCTCAAAATAACTATTTCTTAAAATAAATAATCAGACTATGT    152
                **************************************************************

JX855937    GCGATAAGGTAGATAGTCGAAAGGGAAACAGCCCAGAACAGTAATTAAAGCTCCCCAATT    240
Sample25    GCGATAAGGTAGATAGTCGAAAGGGAAACAGCCCAGAACAGTAATTAAAGCTCCCCAATT    212
                ****************************************************** ******

JX855937    AATATTAAGTGA---    252
Sample25    AATATTAAGTGAAAA    227
                ************
```

Figure 4.11 Alignment of the *MtLSU* gene reference sequence of *P. jirovecii* (Accession number JX855937) with the *MtLSU* gene sequence of the sample 25 which was negative with the TaqMan assay and positive with nested PCR
The squared area indicates the hybridization region of the primers and probe used for the TaqMan assay (79bp)

4.3.6.   Use of salivary amylase assays to assess sample quality

It was found earlier that the captured material on the face-mask contained a significant contribution of active salivary amylase exhaled by different respiratory activities in health (3.3.3). In order to validate this in disease and to determine whether predominantly URT samples might have contributed to false negative results (within the clinical context), a subgroup of the masks (39 samples) was analysed for active α-amylase

Activity was clearly detectable and quantifiable in 22 samples (56.4%) and at low levels in a further nine (23.1%) (Table A.44). No amylase activity was detected in the blank reagents.

These results were tested for negative correlations with the *P. jirovecii* signals. No such correlation was found, in contrast, there was a trend towards a positive correlation (r= 0.24, p= 0.14) (Table A.46).

4.3.7. Correlations between mask results and selected blood analyses

Correlations were sought between the face-mask results (TaqMan) and blood assays for BDG, leukocyte counts and CRP levels.

BDG is known to have a good negative predictive value in those at-risk for fungal infections (Alanio *et al.*, 2016).

In the 19 patient group (who was subjected to invasive or semi-invasive procedures) and considering BAL, ETA and induced sputa as a gold standard, the TP, TN, FP and FN results of the BDG result (in serum or BAL with its borderline value interpreted positive) were 9, 6, 4, 0. This resulted in sensitivity, specificity, positive and negative predictive values of 100%, 60.0%, 69.2%, 100%, respectively (Table 4.10).

In this group, adding the mask to BDG results, the TP, TN, FP and FN were 6, 9, 1, 3. This resulted in sensitivity, specificity, positive and negative predictive values of 66.7%, 90.0%, 85.7%, 75.0%, respectively, improving the specificity and diagnostic likelihood ratios (Table 4.10).

Of the included 39 patients, one was not tested for BDG in neither serum nor BAL (Patient 40 in Table 4.6). BDG results (wherever were done i.e. serum or BAL level) of 38 patients was tested for correlation with the face-mask results.

BDG levels were positively correlated with the exhaled *P. jirovecii* signals, showing a paired positivity and negativity (r= 0.65, p<0.0001) (Table A.46).

Considering BDG result (with its borderline value interpreted positive) as a "gold standard", the TP, TN, FP and FN results of the face-mask using the in-house TaqMan assay were 10, 14, 0 and 14, respectively. This resulted in sensitivity, specificity, positive and negative predictive values of 41.7%, 100%, 100% and 50.0%, respectively (Table 4.10).

Interestingly, considering BDG result within seven days of face-mask sampling as a "gold standard", the TP, TN, FP and FN results of the face-mask using the in-house TaqMan assay were 9, 15, 0 and 13, respectively. This resulted in sensitivity, specificity, positive and negative predictive values of 40.9%, 100%, 100% and 53.6%, respectively, improving the negative predictive value (Table 4.10).

The pathophysiologic role of lymphocytes and alveolar macrophages in *P. jirovecii* infection was detailed by Kelly and Shellito (2010) and Gigliotti and Wright (2005).

It was suggested that immune components might have played a role in controlling the amounts of exhaled *P. jirovecii*. If this was the case, one may expect a negative correlation between the leukocytes counts and the quantities of exhaled *P. jirovecii*. However, no statistically significant correlations were found (Table A.46)

Serum CRP is an inflammatory biomarker which elevated level can indicate PJP severity (Sage *et al.*, 2010).

Investigating whether there was an association between PJP severity and the exhaled burden, serum CRP was tested for possible correlation with the exhaled *P. jirovecii* signals. However, again, no statistically significant correlation was found (Table A.46).

It might be worth mentioning that all the tested values of leukocyte counts, CRP levels and exhaled *P. jirovecii* signals were obtained synchronously (within 48 hrs).

4.4. Discussion

4.4.1. Patients and controls

39 recruited patients were classified into "likely" and "unlikely" PJP groups on the basis of the views expressed in the case notes and discharge letters which in turn were based on the ECIL guidelines highlighted in De Pauw *et al*. (2008) and Alanio *et al*. (2016) studies. However, the made diagnoses should ideally have been approached by an advisory team involving a haematologist, a pulmonologist, an infectious disease specialist and a microbiologist blinded to the mask results. Although this approach would be ideal, a "proven" PJP in clinical terms should not be used as a synonym of a "definitive" aetiology in scientific terms.

Reaching a "definitive" diagnosis in the most rigorous manner might require in many cases a thorough examination of an autopsied lung. Contributions from all other potential pathogens must be ruled out. Moreover, even a reliance on a response to anti-pneumocystis agents might not suffice. The principal therapeutic agent, trimethoprim-sulfamethoxazole, is a broad-spectrum antimicrobial agent with activity against many bacterial pathogens as well as other fungi, notably *Aspergillus* species (Afeltra *et al.*, 2002). Therefore, although reaching a definitive diagnosis would be optimal, such definitivity is almost impracticable. This further justified classifying the clinical diagnoses into "likely" and "unlikely".

Most importantly, it was noted that isolation precautions were not applied for this study cohort.

4.4.2. Analysis of face-mask samples for *P. jirovecii*

It was hypothesized that the face-mask system can be devised to detect *P. jirovecii* exhaled from patients with PJP. It was possible to detect exhaled *P. jirovecii* in 35% of 20 patients with likely PJP and in 15.8% of 19 patients with unlikely PJP, without those HIV-uninfected patients being instructed to exert any specific respiratory effort.

Interestingly, there was no statistically significant difference in detectable quantities of exhaled *P. jirovecii* between the "likely" and "unlikely" groups. This is not in accordance with quantitative differences between colonized and clinically infected individuals reported (regardless of analytic confidence) in BAL, as a surrogate for respiratory fungal load, and other respiratory specimens (Larsen *et al.*, 2002 and 2004; Flori *et al.,* 2004; Fillaux *et al.,* 2008; Fujisawa *et al.,* 2009; Rohner *et al.*, 2009; Alanio *et al.,* 2011; Chumpitazi *et al.,* 2011; Botterel *et al.,* 2012; Matsumura *et al.,* 2012; Muhlethaler *et al.,* 2012; Maillet *et al.,* 2014; Louis *et al.,* 2015; Montesinos *et al.,* 2015; Fauchier *et al.,* 2016; Unnewehr *et al.,* 2016; Montesinos *et al.,* 2017; Rudramurthy *et al.,* 2018).

Le Gal and colleagues (2015) reported that pneumocystis DNA signals in air samples collected at 5m and at 1m distance from subjects' heads were higher in PJP patients than in colonized ones, although the quantitative differences were not statistically significant. Exhaled samples are different from environmental air. Therefore, they might have postulated incorrectly that colonized patients exhale "*low and potentially undetectable*" burden.

In contrast, here there was a non-significant trend towards higher outputs from patients with unlikely PJP (~1 log). This, however, does not imply that members of this group were more frequently exhalers since the fungus was detected in only 15.8% of them while this was at least doubled reaching 35% for patients with likely PJP.

Several possibilities can be discussed. First, the unlikely group was possibly misdiagnosed. The traditional clinical opinion is that making a "definitive" diagnosis on PJP requires detecting *P. jirovecii* in a respiratory specimen. This view is an oversimplification as discussed in 4.4.1. It requires ruling out of all other aetiologies, and, while this is often attempted, it is rarely achieved. DNA from some of the samples reported here were analysed for the presence of bacterial pathogens and the majority were positive for one or more agents (P Bird and M Barer, personal communications).

A second possibility is that exhaled material captured by the face-mask is different from other respiratory specimens. The concept agrees with Nardell *et al.* (2016) who concluded that aerosols produced by TB patients are different from sputa since each holds a distinguishable transcriptomic signature. Similarly, we observed somewhere else discrepancies in ratios of two antimicrobial resistance genes, *mefA* (macrolide efflux pump) and *tetM* (tetracycline ribosomal protection protein), between paired sputum and masks collected from COPD patients (Kennedy *et al.*, 2018). However, the small sample size (three subjects) limits the strength of this conclusion.

The third consideration takes into account that the patients' medical conditions may have resulted in a low volume of air movement through the lower airways. Thus, the volume of air sampled could be lower in sicker patients. This limitation would not apply to BAL samples.

However, the finding that the mask samples did not identify that patients designated with likely PJP as exhaling higher levels of *P. jirovecii* than those designated unlikely, could be considered a limitation of this sampling method.

On the other hand, detecting exhaled *P. jirovecii* in 25.6% of 39 immunocompromised patients of whom at least 89.7% were HIV-uninfected is appreciable within the known low fungal load of this group. Previous studies which detected *P. jirovecii* DNA in room air samples did so with either HIV-

infected patients or with AIDS patients (Bartlett *et al.,* 1997, Sing *et al.,* 1999b, Le Gal *et al.,* 2015 and Choukri *et al.,* 2010). Therefore, it could be argued that the face-mask yielded false negative results (at the level of clinical diagnosis) however these were true negative (at the level of PCR and the exhaled fungal load). It would nonetheless be surprising if the mask could not show higher detectability in HIV-infected patients.

It was acknowledged earlier that the mask system can capture both aerosols and droplets. Detection of *P. jirovecii* in the room air of HIV-infected subjects (Bartlett *et al.,* 1997, Sing *et al.,* 1999b, Le Gal *et al.,* 2015 and Choukri *et al.,* 2010) and in exhaled air from HIV-uninfected patients (to the best knowledge of the author, for the first time by this study) should provide convincing evidence that *P. jirovecii* is an airborne infection.

The positive *P. jirovecii* detections of this study at 1.5–6cm (Figure 3.18), Sing *et al* (1999b) at 30cm, Le Gal *et al* (2015) at 1m and 5m and Choukri *et al* (2010) at 8m from PJP patients should lead to recommending aerosol isolation precautions for selected immunocompromised patients for both protective and source control reasons. This contrasts with droplet isolation precautions recommended by other authors (de Boer *et al.*, 2018) and in this regard, it is noteworthy mentioning many of the patients sampled here were not isolated. Isolation applicability should be further addressed within the documented occurrence of outbreaks in different health care facilities.

It must be acknowledged that detecting DNA on the face-mask may not indicate the presence of intact cells despite this is very likely due to the devised processing method (pre-extraction centrifugation). This problem could be addressed by use of PMA as previously described, while use of a Wells-Riley system (Riley *et al.,* 1978) would provide a more definitive though costly alternative.

Since the detection method was molecular based, concluding definitive data on the airborne stage remains among the limitations.

Finally, one could argue that choosing healthy volunteers as negative controls was not appropriate (age mismatching and more importantly a microenvironment mismatching). Collecting, however, negative masks from matching patients reassures that there was no cross-contamination between cases, in addition to the strict measures followed throughout processing the samples separately (temporally and spatially). Furthermore, none of the indoor air samples was positive. While the healthcare staff (as potential exhaling carriers) were not sampled, if the air was contaminated, it was likely to obtain positive masks, or at least positive air samples, more frequently, but this was not the case.

4.4.3. Correlation with the clinical picture and radiological findings

The radiological findings are one of the cornerstones in approaching PJP diagnosis despite neither the clinical nor the radiological features are pathognomic to PJP. It is clearly desirable, however, to predict from the clinical or radiological picture whether a patient is *P. jirovecii* exhaler, particularly if taking isolation precautions is sought.

In the natural course of PJP, the radiological features continuously progress to reflect PJP severity (Hardak *et al*., 2010). It was suggested that *P. jirovecii* exhalers might show a different picture from those who were not. Unfortunately, no explicit difference was found. Furthermore, 50% of the exhalers were with CXRs interpreted by the radiologist within normal limits. This indicates that neither the clinical nor radiological features are reliable measures to predict the exhaled fungal burden.

Indeed, two patients were nearly asymptomatic, but they were exhalers. It is not sensible that a number of clinical experts postulate that "*symptomatic patients might be at higher risk of transmission because of apparent higher density of colonisation*" (Cooley *et al.*, 2014).

Radiologically speaking, there might be no findings in the initial phase of PJP (4.1.5.1). This can further indicate that "silent" exhalation can occur during this period.

Moreover, while collecting the datum of anti-pneumocystis prophylaxis receipt was a useful mark that a clinical suspicion was high toward PJP, it demonstrates how such suspicion was neither sufficient to enable a practical differentiation nor to recognize an exhalation burden.

Since identifying all groups at risk is increasingly impracticable, the current orientation that prophylaxis is being selectively applied (de Boer *et al.*, 2018) renders isolation precautions a more efficacious approach. This is particularly true to eschew side effects like myelosuppression and hepatotoxicity of

chemoprophylaxis in at-risk groups with low incidence rate. In addition, chemoprophylaxis-related hypersensitivities leading to regime alterations have been documented to result in higher rates of PJP breakthroughs (Stern *et al.*, 2014 cited in de Boer *et al.*, 2018).

While one of the limitations is that the data were retrospectively collected, the obtained clinical picture could be further characterised. For example, the Visual Analogue Scale (VAS) could be applied to estimate patients' perception of cough severity. However, such scale is subjective, and must cover the wide spectrum of possible psychometric responses obtained, usually, verbally. Moreover, this as an approach in depleted patients does not seem judicious. Vernon *et al.* (2009) identified three domains: frequency, intensity and disruptiveness from daily activities to measure cough severity in chronic coughers, involving also the VAS. More objective assessors include cough flow, electromyography, Leicester cough monitor system and computerized cough acquisition system. Similarly, further dyspnoea features (Williams, 2017; Stenton, 2008; Fletcher *et al.*, 1959) and the Baseline Dyspnoea Index might be applied. In addition, scoring with APACHE II (Acute Physiology and Chronic Health Evaluation II) might have revealed different data. The last's potential value, however, would be limited to those at intensive care units, while the included patients were not. The radiological findings are a more objective approach that was not useful in identifying exhalers. Nevertheless, the applied system of grading radiographic severity might be improved by simulating that of TB (Ralph *et al.*, 2010) although the current results do not support such hypothesis.

4.4.4. Comparison with invasive and semi-invasive diagnostic procedures

It was hypothesized that detection of *P. jirovecii* using the face-mask could provide data on utilizing this tool in approaching PJP diagnosis.

As noted earlier, detection of the organism in a respiratory specimen is a clinical key for approaching a microbiological diagnosis on PJP. It is obvious that the detection itself does not suffice to claim that the organism was the aetiological agent. Beside a carrier, subclinical or colonized status, predisposing, concurrent and superimposed infections should be investigated thoroughly.

Among the invasive investigative tools are lung biopsies and BALs obtained through diagnostic bronchoscopies. Similarly, ETA is not less invasive. Sputum induction could be described as a semi-invasive procedure that has been reported to cost around 40% of what bronchoscopy does (Glenny and Pierson, 1992).

It was possible to detect exhaled *P. jirovecii* before it was detected or excluded in BAL, ETA and induced sputa, in 75%, 100% and 50% of 19 patients subjected to these procedures, respectively. These rates are appreciable within the low fungal load known in this study cohort. However, they indicate that the mask in its current version cannot replace BAL for microbiological detection. Furthermore, this tool was not able to differentiate in quantitative terms between PJP and *P. jirovecii* colonized patients. In addition, one could argue the mask cannot replace "diagnostic" bronchoscopies in biopsying the respiratory tract, despite this is beyond our current scope.

For obtaining a microbiological evidence, the mask showed a great potential to be an intermediate step after a high clinical suspicion necessitating proceeding to a diagnostic invasive procedure. This justifies evaluating the mask in a formal diagnostic study. Apart from eschewing contraindications of

invasive and semi-invasive procedures and cost- and time-effectiveness offerable by the mask system, the ethics of exposing patients on palliative as well as on curative management to suffering and risks associated with invasive procedures could be debated.

However, compared to invasive procedures in obtaining respiratory specimens, the mask sensitivity and specificity were ~57% and 90% (Table 4.10). Despite these values remain appreciable in this study cohort known to have a low pulmonary fungal load, the sensitivity was poor indicating that the mask in its current design cannot replace BAL or ETA in confirming a microbiological diagnosis on PJP. The specificity was more acceptable indicating that a negative mask can predict a negative BAL or ETA. Nonetheless, the sample size was relatively small, and a formal statistically powered diagnostic study remains required.

There were few dissociations in terms of timing. Three patients had negative masks before a positive BAL, and one had a positive mask before a negative BAL. Several scenarios can explain this. First, the negative mask was a false negative at the technical level (i.e. related to sample processing) and this will be later discussed (4.4.5 and 4.4.6). Second, the negative was a false negative at the techno-clinical level (i.e. the patient was exhaler but at low, undetectable load). Third, the negative was a true negative at the clinical level (i.e. the patient was not exhaler, possibly due low "intra-corpus" fungal burden) resulting in a true negative at the technical level. Any of these can lead to false negativity at the diagnostic level. Similar scenarios could be discussed for the case showing a positive mask before a negative BAL, however in terms of false and true positivity. First, one should exclude cross-contamination which was already ruled out. Second, the PCR used for BAL is less sensitive than that used for masks. Such possibility, however, can be excluded based on the lung fungal burden, particularly for cases with likely PJP. For cases with unlikely PJP, the mask positivity could represent a

colonisation status (4.1.3). Since this case was classified with unlikely PJP, this scenario is particularly true if one assumed the mask sampled the URT while the BAL did the LRT. Other factors include the natural history of the disease, concurrent medical condition and ongoing medical management. When interpreting within the clinical context, the BAL must not be excluded to result in false results.

Whatever of these scenarios was the case, the shown results warrant further studies aiming at improving the mask efficiency in sampling the LRT in disease.

4.4.5. Investigation of possible false-negative results

It was suggested that the face-masks collected from patients with suspected PJP showed a high degree of negativity resulted from technical or biological factors.

Generally, one should not expect to obtain neither a higher nor a lower frequency of positive or negative masks in order to avoid biased results as much as possible. However, substantial evidence exists on the widespread *P. jirovecii* colonization of healthy individuals and of patients without PJP (4.1.3).

P. jirovecii DNA was detectable by Le Gal and colleagues (2015) in 50% at 1m and 75% at 5m distance from the head of 4 PJP patients, and in 50% of air samples collected at 1–5m from 10 colonized hosts. While in our study, exhaled DNA was detected at 1.5–6cm in only 35% of 20 patients with likely PJP and in ~16% of 19 colonised ones. Comparing the results suggests that air sampling specificity increases with decreasing the distance from an exhaler source, taking into account that environmental air is different from exhaled air. A comprehensive series of experiments, however, was carried out investigating possible false-negativity.

The employed in-house TaqMan assay was originally developed by Meliani *et al.* (2003) to quantify *P. jirovecii* DNA in BAL and used here with modifications. These mainly included increasing the DNA template volume and optimising the cycling conditions on the available platform. The former has pros and cons as discussed in 2.4.5.

The diagnostic sensitivity of the original assay was 100% for BALs obtained from patients whose majority were HIV-infected (Table A.38). The high negativity could be due to the material analysed, where exhalable doses were probably below the limit of detection. This, however, highlights the necessity for standardizing analytical methods of respiratory specimens to allow more effective comparisons between different laboratories.

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MtLSU-rRNA gene was selected as a molecular target since it has a high degree of genetic conservation (Beard *et al.*, 2000) and exists in multicopies (15 copies) in *P. jirovecii* genome (Valero *et al.*, 2016; Damiani *et al.*, 2013; Jiancheng *et al.*, 2009; Robberts *et al.*, 2007). TaqMan rather SYBR Green chemistry was selected as the former is more specific due to its involvement of three hybridization oligonucleotide sequences. None of the used primer and probes, however, had degenerate bases. While this improves the overall specificity, it restricts the detectability when punctual mutations are expected. A probe labelled with a minor grove binder (MGB) was selected for three main reasons. First, it improves the assay specificity by preventing mismatches in the MGB region requiring a high melting temperature. Second, fluorescence quenching property has been shown to be more efficient with MGB probes resulting in better sensitivity (Kutyavin *et al.*, 2000). Third, the binding between the probe and the MGB can form hyper-stabilised conjugates allowing the use of short probes (Afonina *et al.*, 1997).

The specificity of the TaqMan assay was not in question since no cross-reactivity was detected upon testing against different microbial genomes and against human genome (Table A.45). Moreover, the lack of obtaining positive masks from the healthy volunteers suggests two possibilities: 1) the assay specificity for *P. jirovecii* was high 2) *P. jirovecii* was not exhalable or capturable in health. The latter is not fully consistent with published data (4.1.3) documenting *P. jirovecii* colonisation of healthy respiratory tract (but not exhalation) or with the findings of the Le Gal group detecting DNA in the air surrounding hospitalized colonized patients (not healthy subjects).

Intra-sample repeatability and reproducibility were achieved by including at least two technical replicates and matching the results to that of the external laboratory. To be more rigorous, however, at least six laboratories should have been involved to confirm the latter (Kralik and Ricchi, 2017).

Presence of PCR inhibitors in the extracts inducing full inhibition was excluded experimentally (Table A.40), in addition to our ability to amplify *HβG* in at least 80% of the 21 extracts (Table A.42).

To address the false negativity more accurately, one could investigate whether *P. jirovecii*-negative masks were saturated by other exhalable microbes. In fact, non-specific DNA template competition for reaction compounds has been discussed by Kalle *et al.* (2014). This may explain the absence of TaqMan amplification despite of the presence of the template revealed by DNA sequencing which further may be more sensitive (Figure 4.11).

Radomski *et al.* (2013) found that qPCR results for non-host targets were only reliable when the amount of host DNA was <3μg in PCR mixture (25μL) and the DNA purity was high. The latter was not prioritized as we found that DNA extraction yield from rigid cells is more vital for quantification than DNA purity (2.3.4).

The DNA extraction method optimised for mycobacteria was possibly over-extractive. The protocol showed higher extraction efficiency on mycobacteria compared to DNeasy plant mini Kit (Figure 2.12), a frequently used protocol for fungal DNA extraction; and was applied for *Pneumocystis* since both agents have rigid cell walls (Figure 2.16 and Figure 4.12). Despite it remains unknown whether what was found for mycobacteria holds for *Pneumocystis*, the protocol was proven successful in at least ten samples.
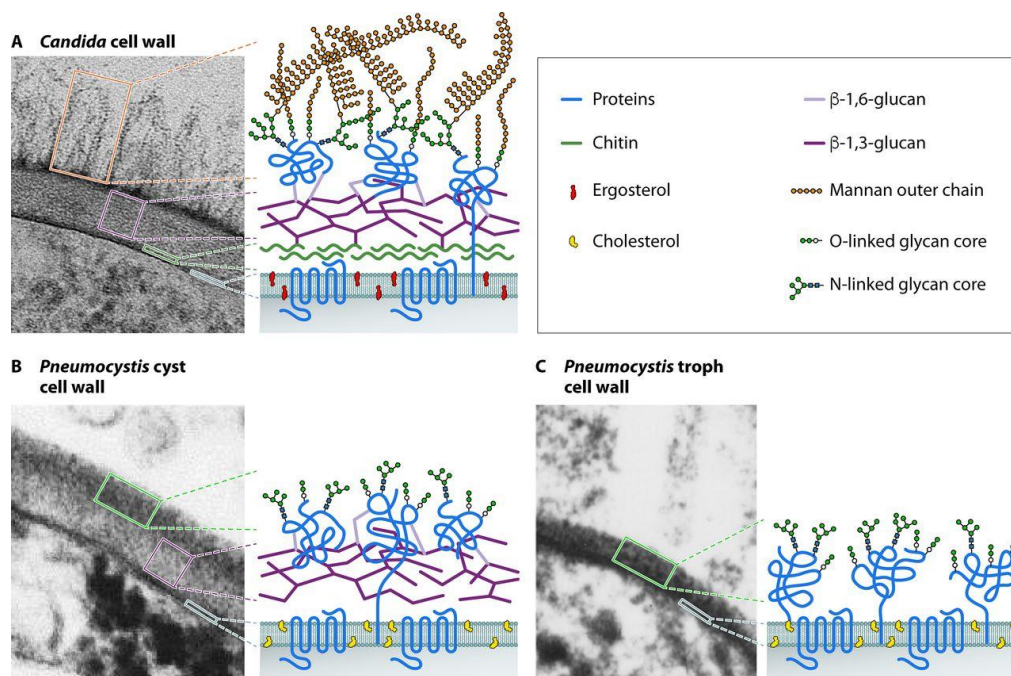
Figure 4.12 Schematic representation and **electron micrograph of the cell wall structure of pneumocystis trophic and cystic forms**
The cell wall is similar in both stages with the absence of β-glucans in the latter. This was reproduced from Ma *et al*. (2018) with permission from the American Society for Microbiology.

Going back to degenerate bases, a recent study applied the same assay was encountered by false negative results and revealed a previously not-reported mutation (C210T mutation of the *MtLSU* rRNA gene) (Le Gal *et al.*, 2017). If the false-negativity was entirely confined to polymorphism at this position, using the mixed base probe should have resolved this. However, only one false-negative was detected, suggesting other contributing factors. One could debate the assay design or thermodynamics of the used oligonucleotide primers and probes. However, this as a possibility was already excluded during optimising the assay.

In general, it is not desirable to use "N" in TaqMan probes as it would complicate detection of alternative alleles when the same dye is used for rare mutations. A multiple-template multiplex assay was designed using Beacon Designer 8.20 software (Premier Biosoft, USA), targeting *MtLSU*, *MSG and*

DHPS, in addition to *HβG* as a control. It was not possible, however, to be validated due to limited funding.

Using nested PCR has both advantages and disadvantages discussed in 4.1.5.2.

It was also investigated whether the clinical management of cases with suspected PJP was ever changed, particularly in terms of administrated medications, but no change was noted. Furthermore, *P. jirovecii* can be detected in clinical specimens for days or weeks after effective therapy is initiated (Roger *et al.*, 1998). Indeed, this is expected to widen the window for successful detection. Therefore, this as a possibility resulting in negative masks was excluded.

The breathing patterns performed during sampling were possibly not efficient to sample the infected zone which is typically the LRT, particularly the alveoli. Comparison of different patterns was detailed in health (Chapter Three). It might be worth highlighting that performing exertional efforts was not clinically feasible for this study cohort, supporting our earlier arguments. The quality of the collected samples was investigated as will be detailed next.

The sampling matrix was assumed to make no contribution, but it might have done so. If such impact, however, was significant, the two matrices used (gelatine and PVA) were then likely to show different performances but this was not the case.

Finally, it might be possible what have been encountered was a reflection of randomized selection and stochastic effects in a small ample size. This remains to be confirmed or excluded by this ongoing study.

4.4.6. Use of salivary amylase assays to assess sample quality

It was suggested that samples dominated by URT secretions may have contributed to obtaining false negative face-masks from cases with likely PJP. However, amylase results were positively correlated with the mask positivity.

It is known that *P. jirovecii* preferentially infects pneumocytes type-I of the LRT. *P. jirovecii* DNA has also been detected in the URT in health (Medrano *et al.*, 2005) and in disease (Vargas *et al.*, 2000; Miller *et al.*, 2001; Durand-Joly *et al.*, 2003). Since the known site of infection is the LRT while DNA was detected in these studies in the URT, one of the most likely scenarios is that micro-secretions lining the respiratory tract and carrying *P. jirovecii* DNA progressively contaminate the URT through aerosolisation and possibly other mechanisms. Indeed, such hypothesis might explain, if it was proven true, the trend of positive correlation found between exhaled *P. jirovecii* signals and that of amylase. This further supports our previous findings showing positive correlations between exhaled albumin and exhaled amylase in health (3.3.4). Therefore, the quality of sampling is not necessarily undermined merely because of detecting salivary amylase. Indeed, this re-opens the question what the desired target on a mask sample is (3.4.3 and 3.4.4).

The detectability of amylase in at least 56% of 39 masks collected from hospitalised patients and at least 70% of ten masks collected from healthy subjects (NB for 60 min) re-emphasises that α-amylase is a component of the material being captured by the face-mask.

Thus, while this undoubtedly indicates contamination with the URT secretions, detection of α-amylase may be devised as a quality control of recovery of the captured material and as an indicator of "productive" exhalation.

4.4.7. Correlations between mask results and selected blood analyses

BDG is a non-specific biomarker of invasive fungal infections, which negative predictive value has been appreciated.

This investigative test showed a strong correlation with the mask result which also added value to BDG's specificity and negative likelihood ratio. More interestingly, one case had positive BDG (377 pg/mL) during the period of PJP suspicion, however on the day of mask-sampling, the test was negative (49 pg/mL) and the mask was so (patient 14). Similarly, patient 23 had a positive test (110 pg/mL) downgraded to a borderline result (73 pg/mL) when the mask was collected negative. This might explain why the negative predicative value of the mask was slightly improved when the comparator BDG was the level obtained within seven days of mask sampling.

On the other hand, the same patient 14 was classified with unlikely PJP and had a history of *Pseudomonas* infection (Table 4.6). Whether his BDG was a true positive for fungal infection for which he was empirically treated with an incomplete anti-PJP course and showed improvement, or whether it was a false positive due to cross-reactivity (4.1.5.2) is open to debate. While the mask suggests this was false positive, one could argue a level as high as 377 to be false. It is possible, however, that the patient was concurrently infected and had been misdiagnosed.

This strong correlation could be devised for improving the mask diagnostic yeild. Synthesising this into a recommendation statement, the mask is recommended to be collected when serum or BAL BDG level is high.

This is not to claim, however, that the net sensitivity of the mask is based on BDG. A number of cases (16, 25, 26, 32–37 and 39) had a positive BDG but their masks were negative. As noted earlier, there are contributing technical and biological factors. Patient 25 and 26 had positive masks with nested PCR

while 32–34 had that with SYBR-Green chemistry. With regards to the shown monthly distribution of the mask results, patient 26 was sampled in May but his mask was positive only with nested PCR. Patients 23, 33, 35-37 and 39 were sampled during October and September but their masks were positive only with SYBR-Green. Interestingly, none of the remaining cases was sampled during these months. If these results were included, they would show a stronger correlation between the mask and BDG tests. However, a formal diagnostic study remains to be carried-out.

It was suggested that immune elements could have a role in controlling the burden of exhaled *P. jirovecii*. If this hypothesis holds true, one should expect a negative correlation between leukocytes counts and the quantities of exhaled *P. jirovecii*.

Since the counts in pulmonary tissue and BAL were not available, these were substituted with the serum counts. While using the serum counts could have undermined a prominence, this hypothesis was not statistically confirmed despite of the clear trends presented (Table A.46).

Circulation time of monocytes is between 24–72 hrs. After that, the monocytes migrate to different tissues within 8–12 hrs for differentiation into macrophages and dendritic cells then reside probably for weeks. Thus, peripheral monocytes counts can provide estimated data on the quantity of differentiated and activated monocytes. For neutrophils, the average half-life of non-activated neutrophils in peripheral blood is about 4–10 hrs before their spontaneous apoptosis.

Negative correlation trends were noted between the absolute serum counts of monocytes and neutrophils and the quantity of exhaled *P. jirovecii* for all the patients and were strengthened for cases with likely PJP (Table A.46 and Table A.47). This might reflect their phagocytic function againts *P. jirovecii* in the respiratory tract and consequently on the exhaled fungal signals.

Neutrophilia of BAL fluid in PJP has been correlated with pulmonary dysfunction and disease severity in HIV-infected (Smith *et al.*, 1988; Jensen *et al.*, 1991; Sadaghdar *et al.*, 1992) and HIV-uninfected patients (Lee *et al.*, 2015b) and in animal models (Swain *et al.*, 2004), however a cause and effect relationship was not observed between neutrophils and tissue damage or infection clearance.

In order to investigate the inflammatory impact, one could devise CRP level, a known inflammatory biomarker. Indeed, serum CRP has been correlated with the severity of PJP (Sage *et al.*, 2010). In our study cohort, serum CRP showed a trend of negative correlation with the exhaled fungal load, strengthened for cases with likely PJP (Table A.46 and Table A.47). This suggests that cases with an inflammatory status or more sever PJP exhale lower burden of *P. jirovecii*. Indeed, this can support further that these subjects suffer from the inflammation rather from the infection load and has a potential to invalidate postulations like in Cooley *et al.* (2014) that symptomatic patients are more "infectious".

Interestingly, the only serum leukocytes which did not show such negative trend were lymphocytes. This is relatively in agreement with Lee *et al.* (2015b)'s findings. They studied the cellular profile of BALs obtained from HIV-uninfected patients with PJP, showing correlations between the severity of PJP and neutrophil counts rather with lymphocyte counts.

If one would address these findings more rigorously may require correlating exhaled leukocytes with exhaled *P. jirovecii*. It would nonetheless be surprising if similar findings were not obtained. However, while different sample types were correlated here there are other factors might have contributed to undermining a statistical significance. These include concurrent medical condition, uninvestigated co-infections and the relatively small sample size, highlighting the need for further extensive studies.

4.5. Concluding remarks

It must be acknowledged first that the following conclusions are limited by the relatively small sample size of this study

- *P. jirovecii* was exhaled sufficiently to contaminate masks without exerting special efforts
- *P. jirovecii* was detectable by the mask from HIV-uninfected immunocompromised patients
- The exhalaled fungal burden fell within the range of $10^4 - 10^5$ copies per mask
- BDG level was correlated with the quantity of exhaled *P. jirovecii.* Thus, the mask collection was suggested with elevated BDG
- Neither clinical nor radiological features differentiated between *P. jirovecii* exhalers and non-exhalers
- The results presented here supported the need for infection control and protective isolation of patients with respect to PJP
- The face-mask in its current design was not shown able to replace bronchoscopies in providing a microbiological diagnosis on PJP; however, it showed great potential as a non-invasive intermediate step adding value to BDG specificity and negative likelihood ratio
- α-amylase was a component of the material captured on the mask in health and disease, and its detection implied productivity rather a merely contamination with URT secretions
- Formal diagnostic studies on using the face-mask in PJP are warranted

Chapter Five: Exhaled microbiome in health and disease

5.1. Introduction

After the explorations of mask samples collected with different breathing patterns and the diagnostic work with PJP, selected samples were analysed further by *16S* rDNA sequencing in order to determine their bacteriomes as a further step towards a more complete understanding of naturally exhaled microbiome.

5.1.1. Microbiome as a component of aerosolomics and breathomics

-omics studies have revolutionized different fields; however, little remains known, in health as well as in disease, about exhaled microbiome, a component of *aerosolomics* and *breathomics*.

Within aerosolomics, a number of studies have investigated bacteriome and mycobiome of environmental air. Qian *et al*. (2012) studied human-associated emission rates of bacteria and fungi in an occupied classroom. They reported significant increases in total particle mass and bacterial genome concentrations during the occupied period compared to the vacant one. These, though present, had lower concentrations for fungal genomes. Qian and colleagues found that around 20% of detected bacterial taxa were closely associated with the human skin microbiome.

On the other hand, Adams *et al*. (2015) studied the fungal and bacterial composition of air in an environmental chamber under different conditions including the chamber occupancy status and the activity of occupants. They found that while the number of occupants and their activity played a significant role in influencing that composition, the signature of human-associated taxa was relatively small compared to the stronger influencing signature of outdoor-derived particles.

Meadow *et al*. (2015) went further and investigated whether human "*microbial clouds*" emitted into environmental indoor air could sufficiently differ to allow

identification of individual occupants. Meadow's group confirmed that an occupied room was microbially distinct from a vacant one throughout different locations of the room space and the investigators were able to distinguish the individual occupants by their airborne bacterial signatures under different general activities such as sitting and walking.

Although these studies showed that microbiome signature of environmental air differed between occupied and unoccupied spaces, and when occupied, differed based on the number and activity of occupants, naturally exhaled air is different from environmental air which, further to including environmental elements, microbes can be suspended, and aerosols desiccated. In addition, the impact of the breathing activity itself on the surrounding environmental microbiome signature remains unknown.

One of the challenges in investigating the exhaled microbiome is the lack of a well-established and standardised measure in sampling. Therefore, different sampling tools can result in diverse and even discordant findings of the studied microbiome. Limitations of these tools and the advantages of utilizing the mask for this purpose were highlighted earlier (3.1.6).

5.1.2.   Exhaled mycobiome has been studied using EBC

A number of studies have investigated exhaled mycobiome rather than bacteriome, using EBC.

Carpagnano *et al*. (2014) aimed to analyse the EBC mycobiome of 43 lung cancer patients and 21 healthy controls from Puglia (Italy). Colonisations with *Aspergillus niger*, *A. ochraceus* and *Penicillium ssp.* were demonstrated in ~28% of these patients but not in their healthy controls.

In 2015 and 2016, Carpagnano's group extended the analysis to involve asthmatics with different disease severities and types (29 atopic asthma and 19 intrinsic asthma) and 20 healthy controls. Again, they showed, utilizing EBC, fungal colonizations in 70% of asthmatics while in none of the controls. The colonisation rates were significantly higher in patients with intrinsic, severe and uncontrolled asthma. In addition, Carpagnano's group observed complete overlapping of the EBC mycobiome with the sputum one.

Interestingly, in 2019, Carpagnano and colleagues focused their analysis on the EBC mycobiome of 27 healthy subjects from the same region. They found complex fungal communities in ten subjects in whom >10% of isolated species were *A. sydowii* and *Cladosporium spp.* Three subjects were later diagnosed with respiratory diseases.

Although these inconsistencies of EBC mycobiome in healthy subjects from studies conducted in the same region were accounted for different enrolment conditions (outpatient clinics vs community) and uninvestigated but supposed high fungal concentration in the ambient air, the dynamics of exhalation over time in terms of stability and mutability was not investigated.

5.1.3. Limited sets of bacterial and viral members have been studied in breathomics

On the other hand, certain exhaled bacteria and viruses have been investigated.

For example, Zakharkina *et al*. (2011) compared the detection of a number of targets in bacterial and viral nucleic acids in EBC and its paired spontaneous sputum of 29 COPD patients with acute exacerbations. Zakharkina and colleagues did not find significant correlations between the results of EBC and sputa, although *Legionella pneumophila* was only detected in the former and viral and *S. pneumoniae* nucleic acids were done so in the latter.

Similarly, Xu *et al*. (2012) collected EBC of seven patients with respiratory symptoms and analysed the samples using culturing, scanning electron microscope and qPCR targeting a number of bacterial and viral sequences. Xu's group reported high bacterial concentrations ($\sim$7000 CFU/m^3) of exhaled viable and dead cells.

May *et al*. (2015) paired EBC and BAL of 49 mechanically-ventilated patients with suspected pneumonia and analysed the paired samples by culturing and by qPCR for eight bacterial targets. They found strong correlations in DNA and culturing results between EBC and BAL.

However, beside exploring limited numbers of targets in the above studies, the main limitation of employing EBC is that a deliberate or condensate exhalation was being collected rather than natural aerosolization. Further limitations were previously highlighted (3.1.6).

5.1.4. Exhaled bacteriome has been studied in animals

On another hand, exhaled bacteriome has been performed in some animal studies.

For instance, Glendinning *et al*. (2017a) sought to compare the lung microbiota of sheep by analysing the microbiome in their EBC and protected specimen brushings. Glendinning and colleagues found the former had significantly less bacterial DNA than the latter and that there were differences in the bacterial community compositions of the two sample types, where 37 OTUs were differentially abundant in the EBC and the lung brushings.

Apprill et al. (2017) compared bacterial and archaeal microbiome in EBC samples collected from two geographically distinct populations of humpback whales. Interestingly, Apprill's group were able to differentiate the EBC microbiome from the seawater microbiome, detecting 25 phylogenetically diverse bacteria in all sampled whales and suggesting the presence of a large core microbiome assemblage shared in healthy marine mammals.

Similarly, Raverty et al. (2017) studied the fungal, bacterial and viral microbiome in EBC samples from a group of Southern Resident Killer Whales. Raverty and colleagues identified fungal and bacterial arrays including a group of antimicrobial agent-resistant microorganisms in EBC and seawater surface samples and suggested that these microbes can play a further role in endangering this population.

While these studies were performed in animals, they also did not collect a naturally exhaled microbiome.

5.1.5. Sputomics and dysbiosis of respiratory microbiome

Regarding materials excreted from the respiratory tract, most previous studies from this laboratory and broader have focused on bacteriome as a component of *sputomics*.

Wang *et al*. (2016) and Haldar *et al*. (2017) aimed, utilizing serial sputa of a group of COPD patients at stable, exacerbation, two weeks post-therapy and six weeks recovery points, to characterise their bacteriome dynamics. While they did not find significant within-subject changes in the bacteriome across these visits, significant increases of Gammaproteobacteria to Firmicutes ratios were observed from stable to exacerbation visits, suggesting a bacterial role, particularly accounted for *Haemophilus spp.*, in exacerbation pathogenesis. More interestingly, they found that the structure and diversity of bacterial community in sputa were correlated with sputum interleukin-8, a member of the CXC chemokine subfamily, suggesting potential use of the former as a biomarker for selective use of antibiotics in COPD.

Ghebre *et al*. (2018) sought to investigate the sputum cellular, mediator and bacteriome profiles of asthmatics and patients with exacerbating COPD. Ghebre and colleagues found that while these two groups had different clinical characteristics and inflammatory profiles, their bacterial ecologies were similar. Analysing sputum mediator and bacteriome profiles with clustering revealed three distinct subgroups, each with different percentages of asthmatics and COPD patients.

George *et al*. (2018) investigated whether *M. catarrhalis* strain was variable between COPD patients in order to explore whether acquisition of new strains was associated with COPD exacerbation. They found that sputum *M. catarrhalis* strains had significant diversity within and between subjects and that acquisition of new strains occurred in both stable and exacerbation events, excluding specific strain associations with COPD exacerbation.

However, as introduced earlier, exhaled aerosols are not fine particles of sputum since each holds a distinguishable biosignature (Nardell *et al*., 2016). Thus, while these studies explored the bacterial community in sputum, the findings must not be deemed applicable for aerosols.

The previous points have spotted the light on the need for a gap-filling study investigating naturally exhaled bacteriome from healthy and diseased human subjects over time and between different breathing patterns.

5.1.6. Bioinformatic approach

Next generation technology involves Roche 454 pyrosequencing, Illumina sequencing by synthesis including MiSeq and HiSeq types, and others. The latter is characterised by offering 10 to 100-fold higher sequencing depth and being more cost-effective, particularly the MiSeq platform, than the former (Caporaso *et al*., 2012). A major challenge with these large datasets, however, is the interpretation of the results using bioinformatic and statistical tools where exploratory methods can generate hypotheses and decisional methods test relationships between different datasets.

Building on molecular profiling techniques established in this laboratory, the overall aim here was to explore a potential complex and diverse array of naturally exhaled bacterial communities. Such different combinations might be an identifying key to distinguish individuals, or in other words, to hold a personalised signature.

In this study, the V4–V5 variable region of *16S* rDNA was targeted based on the recommendations of the Earth Microbiome Project (EMP; www.earthmicrobiome.org) made for several factors. More phylogenetic information can be yielded as this allows sequencing two 300bp reads per amplicon, or in other words, without significant compromise of the number of reads. Improved sequencing was sought by targeting this longer region (~412bp) compared, for example, to the frequently targeted V4 (~292bp). An improvement here was expected because significant loss of sensitivity and specificity in the taxonomic classification has been found to occur when short *16S* rRNA gene sequences were used. The EMP recommended primer set has been demonstrated to detect more environmentally or ecologically important taxonomic groups (including archaea) reducing therefore biases introduced by amplicon sequencing. In fact, selecting universal primers is a challenge as some taxonomic groups might not be well represented. Thus, and for more constancy, the EMP has designed a V4–V5 sequencing protocol with a primer

set thought to represent the emerging consensus sequence set. In addition, the emerged reads have been shown to completely overlap with the frequently generated V4 reads (Caporaso *et al*., 2012; Takahashi *et al*., 2014; Parada *et al*., 2015)

Terminologically, bacteriome rather than virome, mycobiome or archaeome was used to represent the microbiome, taking the advantage of available knowledge and expertise.

5.1.7. Hypotheses and Objectives

Accepting the shortcomings of this approach from sampling matrix background in characterising the microbiome, we hypothesised in this Chapter:

1. Exhaled microbiome signature differs over sampling time in healthy volunteers
2. Exhaled microbiome signature differs among various breathing patterns in healthy volunteers
3. Exhaled microbiome signature differs from BAL signature in patients with suspected PJP

Objectives
1. To explore using data mining tools the microbiome of masks obtained from healthy subjects during NB over 15, 30 and 60 min
2. To explore the microbiome of masks obtained from healthy subjects during NB, IB, IC and RL
3. To explore the microbiome in paired mask and BAL samples obtained from patients with suspected PJP

5.2. Materials and methods

5.2.1. BAL samples

Aliquots (1mL) of untreated BAL samples were fondly provided by Dr Perera; each was paired with one of the mask samples analysed in Chapter Four. The samples were centrifuged at 15,000xg for 10min and DNA extracted from the resultant pellets as per 2.2.7.4.

5.2.2. Illumina MiSeq samples

Two groups of samples were analysed: 1) 18 mask samples from four healthy volunteers who performed all four respiratory activities and 2) three mask samples and their paired BALs from the PJP study. Reagent controls were also included.

5.2.3. *16S*rDNA bacterial amplicon library generation

The *16S*rDNA bacterial amplicon library used for sequencing was produced as follows:

5.2.3.1. MiSeq primer design

Primers for Illumina MiSeq sequencing were designed based on the principles of Caporaso *et al*. (2011 and 2012), with kind assistance of Dr Haldar.

The primers were designed to target the V4 and V5 variable region of *16S*rDNA (amplicon size= 423bp) following the EMP guidelines.

The primers were composed of five parts for forward barcoded primers and of four for the reverse primer. These were:

1) Illumina adapter (for both forward (F) and reverse (R) primers)
2) Golay barcode (F)
3) Primer pad (F and R)
4) Primer linkers (F and R)
5) Template specific primers (515F and 939R)

In addition, sequencing primers were designed for the MiSeq platform as custom primers to read the target sequences. These were composed of

1) Primer pad (F and R)

2) Primer linker (F and R)

3) Primer sequence (F and R)

An index sequencing primer was designed having the MiSeq forward adaptor per the EMP guidelines.

The primer sequences are presented in Table 5.1.

Table 5.1 Primers used for MiSeq

Primer name	Illumina 5' Adapter	Golay Barcode	Primer Pad	Primer Linker	515F (forward) primer	Primer sequence for PCR	No. of bases
515F_G51	AATGATACGGCGACCACCGAGATCTACACGCT	CTCGCCCTCGCC	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTCTCGCCCTCGCCTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G52	AATGATACGGCGACCACCGAGATCTACACGCT	TCTCTTTCGACA	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTCTCTTTCGACATATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G53	AATGATACGGCGACCACCGAGATCTACACGCT	ACATACTGAGCA	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTACATACTGAGCATATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G54	AATGATACGGCGACCACCGAGATCTACACGCT	GTTGATACGATG	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTGTTGATACGATGTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G55	AATGATACGGCGACCACCGAGATCTACACGCT	GTCAACGCTGTC	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTGTCAACGCTGTCTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G56	AATGATACGGCGACCACCGAGATCTACACGCT	TGAGACCCTACA	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTGAGACCCTACATATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G57	AATGATACGGCGACCACCGAGATCTACACGCT	ACTTGGTGTAAG	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTACTTGGTGTAAGTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G58	AATGATACGGCGACCACCGAGATCTACACGCT	ATTACGTATCAT	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTATTACGTATCATTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G59	AATGATACGGCGACCACCGAGATCTACACGCT	CACGCAGTCTAC	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTCACGCAGTCTACTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G60	AATGATACGGCGACCACCGAGATCTACACGCT	TGTGCACGCCAT	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTGTGCACGCCATTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G61	AATGATACGGCGACCACCGAGATCTACACGCT	CCGGACAAGAAG	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTCCGGACAAGAAGTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G62	AATGATACGGCGACCACCGAGATCTACACGCT	TTGCTGGACGCT	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTTGCTGGACGCTTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G63	AATGATACGGCGACCACCGAGATCTACACGCT	TACTAACGCGGT	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTACTAACGCGGTTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G64	AATGATACGGCGACCACCGAGATCTACACGCT	GCGATCACACCT	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTGCGATCACACCTTATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G65	AATGATACGGCGACCACCGAGATCTACACGCT	CAAACGCACTAA	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTCAAACGCACTAATATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G66	AATGATACGGCGACCACCGAGATCTACACGCT	GAAGAGGGTTGA	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTGAAGAGGGTTGATATGGTAATTGTGCCAGCMGCCGCGGTAA	73
515F_G67	AATGATACGGCGACCACCGAGATCTACACGCT	TGAGTGGTCTGT	TATGGTAATT	GT	GCCAGCMGCCGCGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTGAGTGGTCTGTTATGGTAATTGTGCCAGCMGCCGCGGTAA	73

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	5' Illumina adapter	Barcode	Primer pad	Primer linker	515F primer	Primer sequence for PCR	
515F_G68	AATGATACGGCGACCACCGA GATCTACACGCT	TTACACAAA GGC	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTTACACAAAGGCTATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
515F_G69	AATGATACGGCGACCACCGA GATCTACACGCT	ACGACGCAT TTG	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTACGACGCATTTGTATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
515F_G70	AATGATACGGCGACCACCGA GATCTACACGCT	TATCCAAGC GCA	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTATCCAAGCGCATATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
515F_G71	AATGATACGGCGACCACCGA GATCTACACGCT	AGAGCCAA GAGC	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTAGAGCCAAGAGCTATGGTA ATTGTGCCAGCMGCCGCGGTAA	73
515F_G72	AATGATACGGCGACCACCGA GATCTACACGCT	GGTGAGCA AGCA	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTGGTGAGCAAGCATATGGTA ATTGTGCCAGCMGCCGCGGTAA	73
515F_G73	AATGATACGGCGACCACCGA GATCTACACGCT	TAAATATAC CCT	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTAAATATACCCTTATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
515F_G74	AATGATACGGCGACCACCGA GATCTACACGCT	TTGCGGACC CTA	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTTGCGGACCCTATATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
515F_G75	AATGATACGGCGACCACCGA GATCTACACGCT	GTCGTCCAA ATG	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTGTCGTCCAAATGTATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
515F_G76	AATGATACGGCGACCACCGA GATCTACACGCT	TGCACAGTC GCT	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTGCACAGTCGCTTATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
515F_G77	AATGATACGGCGACCACCGA GATCTACACGCT	TTACTGTGG CCG	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTTTACTGTGGCCGTATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
515F_G78	AATGATACGGCGACCACCGA GATCTACACGCT	GGTTCATGA ACA	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTGGTTCATGAACATATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
515F_G79	AATGATACGGCGACCACCGA GATCTACACGCT	TAACAATAA TTC	TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	AATGATACGGCGACCACCGAGATCTACACGCTTAACAATAATTCTATGGTAA TTGTGCCAGCMGCCGCGGTAA	73
Reverse complement of 3' Illumina adapter		Primer pad	Primer linker	939R (reverse) primer	Primer sequence for PCR		
939R	CAAGCAGAAGACGGCATACGAGAT	AGTCAGT CAG	CC	CTTGTGCGGGYC CCCGTCAAT	CAAGCAGAAGACGGCATACGAGATAGTCAGTCAGCCCTTGTGCGGGYCCCC GTCAAT	57

Sequencing primers

		Primer Pad	Primer Linker	F/R sequence	Read 1 and 2 sequencing primer	
Forward		TATGGTA ATT	GT	GCCAGCMGCCG CGGTAA	TATGGTAATTGTGCCAGCMGCCGCGGTAA	29
Reverse		AGTCAGT CAG	CC	CTTGTGCGGGYC CCCGTCAAT	AGTCAGTCAGCCCTTGTGCGGGYCCCCGTCAAT	33
					Index sequencing primer	
5' Illumina adapter extended					AATGATACGGCGACCACCGAGATCTACACGCT	32

5.2.3.2. *16S*rDNA amplicons generation

Conventional PCR was run to generate *16S*rDNA amplicons using the forward and the reverse MiSeq primers (4nmole Ultramer DNA Oligo, Integrated DNA Technologies, UK) and Phusion High-Fidelity PCR Kit (New England Biolabs, UK).

50μL PCR mixture of each reaction tube was prepared in 0.2mL PCR tubes containing 10μL of 5X Phusion High-Fidelity Buffer, 1μL of 10μM dNTP Solution Mix, 5μL of 100% v/v Dimethyl sulfoxide (DMSO), 0.5μL of Phusion High-Fidelity DNA Polymerase, 5μL of each 2.5μM forward (added individually) and 2.5μM reverse primers, 2μL of DNA template and the volume was completed with molecular grade water (21.5μL).

Cycling conditions were 98°C for 2min, 25 amplification cycles of 94°C for 30sec, 60°C for 30sec and 72°C for 30sec, and a final extension cycle at 72°C for 5min.

PCR amplicons (5μL) were subjected to 1.5% w/v agarose gel analysis alongside a broad range (10,000bp) DNA ladder (New England biolabs, UK) (4.2.4.5). Bands were shown at approximately 450bp. For samples which produced no or faint bands, PCR was performed in duplicate or triplicate, the amplicons were pooled, and the volume concentrated using a centrifugal evaporator (DNA mini, Heto Lab Equipment, Denmark).

5.2.3.3. Amplicon purification

AMPure XP for PCR purification (Beckman Coulter Life Sciences, USA) was used following the manufacturer's instructions. The purification was made in 1:1 of AMPure XP beads and PCR volumes. The final cleaned-up reaction was resuspended in 25μL molecular grade water.

5.2.3.4. Fluorometric DNA quantification

Purified samples (5.2.3.3) were quantified using QuantiFluor dsDNA System (E2670, Promega, UK) per the manufacturer's instructions. Fluorescence of samples at excitation/emission wavelength of 480/520nm was read in duplicate using FLUOstar Omega multi-mode microplate reader (BMG LABTECH Ltd., UK) with Omega Software (BMG LABTECH Ltd.), alongside 2-fold serially diluted standard DNA (1.56–100ng/μl). dsDNA concentration was interpolated from the standard curve using GraphPad Prism software (specified before).

5.2.3.5. Normalisation and pooling of amplicons

Quantified samples (5.2.3.4) were normalised to a standard DNA concentration (5ng/μL) with 1X TE buffer. Equal volumes (2μL) of the normalised samples were pooled and the amplicon library was dispatched to the Centre for Genomic Research (Liverpool, UK) for sequencing using the MiSeq Personal Sequencer platform (Illumina Inc., USA).

5.2.4. Sequence data analysis using Quantitative Insights Into Microbial Ecology (QIIME)

5.2.4.1. Sequence processing and curation

The sequencing reads were processed, and microbial community analysed using QIIME pipeline version 1.9 based on the principles established by Dr Haldar (2014). Rigorous criteria were applied to remove low-quality and chimeric reads based on trimming, primer matching and quality parameters, as follows.

MiSeq files were obtained from a paired-end run as a one Read 1 (R1) and one Read 2 (R2) FASTQ file created for each sample, with the extension *.fastq.gz. Trimmomatic tool of Bolger *et al*. (2014) was applied for removing adapters and quality filtering. Curated paired-end sequence files (R1 and R2)

were concatenated for downstream analysis. Chimera were detected and removed using UCHIME algorithm of Edgar *et al*. (2011) who developed this as a higher sensitive and more time-effective than the previously widely-used ChimeraSlayer. Human sequences were removed after BLAST analyses against the human genomic and transcriptomic database at the NCBI. These non-specific targets were amplifiable since the applied PCR conditions were flexible to maximise PCR yields and allow even representation of bacterial communities. The reads were then aligned, using the PyNAST tool of Caporaso *et al*. (2010), against the Greengenes reference taxonomies (http://greengenes.lbl.gov). Reads aligned against the well-characterised PhiX genome were removed using the PhiX Control v3 library (Illumina Inc.) utilised as a control for sequencing runs. Samples having <500 reads were removed, and the remaining reads were merged in a single cleaned file subjected to closed-source operational taxonomic unit (OTU) picking.

5.2.4.2. Taxonomic assignment and bacterial community ecological analysis

RDP Classifier of Wang *et al*. (2007), a naïve Bayesian classifier algorithm based on word size of 8 with a default confidence threshold of 80%, was used for taxonomic classification of sequence reads from phylum to genus level. 97% identity cut-off, assumed according to Drancourt *et al*. (2000) to represent sequence similarity at species level, was applied for grouping the reads and generating OTUs using the UCLUST clustering method of Edgar (2010). For each cluster, a representative sequence was picked and mapped to a taxonomic group. An assembled OTU table with taxonomic assignments was ultimately produced.

For determining community richness, Chao1 (S_{Chao1}) estimating species richness based on ratio of singletons to doubletons in samples was calculated (Chao, 1984; Gotelli and Colwell, 2011).

For determining within sample diversity (alpha diversity), indices of Shannon–Wiener (H) and Simpson (1-D) derived based on the number of OTUs and their distribution, and Shannon equitability (E_H) representing only the dispersion pattern of OTUs were calculated (Shannon, 1948; Simpson, 1949).

H is more sensitive to increases in species richness than 1-D and gives more weight to rarer groups by applying log transformation for relative abundance, while the latter squares the relative abundance giving more weight to abundant communities and thus can be yielding contradictory results (Harini, 2002).

For determining between sample diversity (beta diversity), Sørensen–Dice coefficient (S_S) and the traditional Jaccard coefficient (S_J) derived based on the observed richness, comparing the similarity of two samples in community membership (OTUs present) were calculated (Sørensen, 1948; Jaccard, 1912).

S_S gives more weight to common groups.

Differences in community structure (relative abundance of members of OTUs) between samples were explored using Bray-Curtis dissimilarity distance (Bray and Curtis, 1957).

Compositional dissimilarity between different communities (β-diversity) can be investigated using several types of distances for building a proximity matrix. These include Euclidian, Chi-square, Bray-Curtis, UniFrac distances and others. UniFrac is characterised by incorporating the relative relatedness or phylogenetic distances between community members and has two types: unweighted and weighted adding weight to dominant groups. Some studies recommend its use (Lozupone *et al*., 2011) and others disagree (Schloss, 2008). Whether or not to be implemented, however, depends on the research question. FastTree 2 tool of Price *et al*. (2010) can be, for example, used to

build on aligned sequences a phylogenetic tree required for obtaining the phylogenetic distance measures.

Exploratory multivariate data mining tools used to assist in reducing the dimensionality for data visualisation were Agglomerative Hierarchical Clustering (AHC), Principal Component Analysis (PCA), Principal Coordinate Analysis (PCoA) and Multidimensional Scaling (MDS). Each tool approaches a dataset differently and has its limitations, though they share the overall aim.

AHC works from the similarities or dissimilarities between samples when grouped together in a binary clustering tree (dendrogram). This was executed using the Euclidian distance measure.

PCA considers all the variance in a dataset when visualising the data on a low dimensional map, allowing to analyse the correlations between the variables to point out if the changes in a variable of a sample are different from the ones in other samples, and to identify trends of samples. PCA has several types based on different approaches like Pearson correlation coefficient (standardised) and covariance matrices that allocate more weight to variables with higher variances; both types were used in this study.

PCoA (metric MDS) considers the distance relationships between the samples described by a square matrix containing resemblance indices when visualising data on a low dimensional map. One could argue that PCoA is inferior to PCA since the former is based on approximation. However, such imprecision is negligible in practice, providing the approximation is close to the original dissimilarities. PCoA is recommended over PCA when there are missing data or fewer samples than variables (Rohlf, 1972).

MDS (non-metric) is better than PCoA in compressing the distance relationships between samples into two or three dimensions since it aims to minimize the stress, allowing a deformation in a 2D view of the 3D configuration in a way that the distances between samples are respected. On

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the other hand, PCoA (as well as PCA) allows rotations and projections in the 2D view of the 3D, that keeps as much variance as possible, representing only that Euclidean portion of the matrix even when non-Euclidean distances having negative eigenvalues are present.

5.2.5. Statistical analysis and graphical output

In addition to the methods applied in earlier Chapters, multivariate analyses were performed using R software 3.4.4 (R Development Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org) and XLSTAT 2018.7 (Addinsoft, 2018. XLSTAT statistical and data analysis solution, Boston, USA. https://www.xlstat.com.).

5.3. Results

5.3.1. Samples

Of 25 samples selected to produce the *16S*rDNA bacterial amplicon library, two samples obtained from one subject did not return any reads and were excluded.

Characteristics of the 23 included samples from three healthy volunteers and three patients with suspected PJP alongside the control background under different variables are presented in Table 5.2.

Table 5.2 Characteristics of included reads-generating samples

Group	Subject ID.*	Sample designation	Description
Healthy (1)	3	NB15_3	Normal breathing for 15 min
		NB30_3	Normal breathing for 30 min
		NB60_3	Normal breathing for 60 min
		IB60_3	Instructed breathing for 60 min
		IC60_3	Intermittent coughing for 60 min
		RL30_3	Reading-out loud for 30 min
	1	NB60_1	Normal breathing for 60 min
		IB60_1	Instructed breathing for 60 min
		IC60_1	Intermittent coughing for 60 min
		RL30_1	Reading-out loud for 30 min
	4	NB60_4	Normal breathing for 60 min
		IB60_4	Instructed breathing for 60 min
		IC60_4	Intermittent coughing for 60 min
		RL30_4	Reading-out loud for 30 min
PJP suspects (2)	1	MASS1	Mask sample +ive for *P. jirovecii*
		BAL1	Paired BAL +ive for *P. jirovecii*
	3	MASS3	Mask sample -ive for *P. jirovecii*
		BAL3	Paired BAL -ive for *P. jirovecii*
	7	MASS7a	Mask sample +ive for *P. jirovecii*
		BAL7	Paired BAL +ive for *P. jirovecii*
CTRL	G1	NC_I	Negative control extracted per Group 1's method
		NC_I_T	Negative control treated with PMA
	G2	NC_II	Negative control extracted per Group 2's method

(*) Correspondent with assigned study numbers in relevant Chapters.

The total number of sequences successfully assembled from paired-end reads across the set was 1,951,965 reads (Mean= 67,309.14; SD= 43,550.69).

Following quality filtering, truncation, chimera removal, human reads removal and PHiX reads removal, a total number of 1,531,784 sequences were advanced to OTUs picking and taxonomy assignment (Table A.50).

Unassigned OTUs had a considerable contribution. Since these were unavoidable within the employed databases and might be further not closely related, these were kept, and their relative abundances reported.

It should be noted that OTUs below 1.00E-03 level were assigned on the bases of very low reads (<10) in most cases and thus could be negligible (Janssen, 2006).

5.3.2. Background signals attributable to gelatine and reagents

5.3.2.1. Phylum level

Three reagent controls obtained by different processing methods on gelatine filters revealed in total 18 phyla (Figure 5.1A). These were 16, 5 and 9 phyla for the methods used for 1st) healthy volunteers (3.2.4.2), 2nd) PMA-treatment (3.2.4.2) and 3rd) PJP samples (4.2.4.2), respectively.

Eight phyla were unique to the first method and one each to the second and third, while sequences assigned to the Firmicutes, Proteobacteria and Actinobacteria were shared by all (Figure 5.1B).

Firmicutes and Proteobacteria, constituted over 90% of reads obtained. Contributions of these phyla were significantly different between these methods. A remarkable loss of Firmicutes signal is seen with PMA treatment, while the 3rd method seems to give less representation to this phylum. Spirochaetes and Bacteroidetes were also abundant in 3rd method, constituting around 6% of the community, but were not significant with 1st and 2nd methods (<1%).
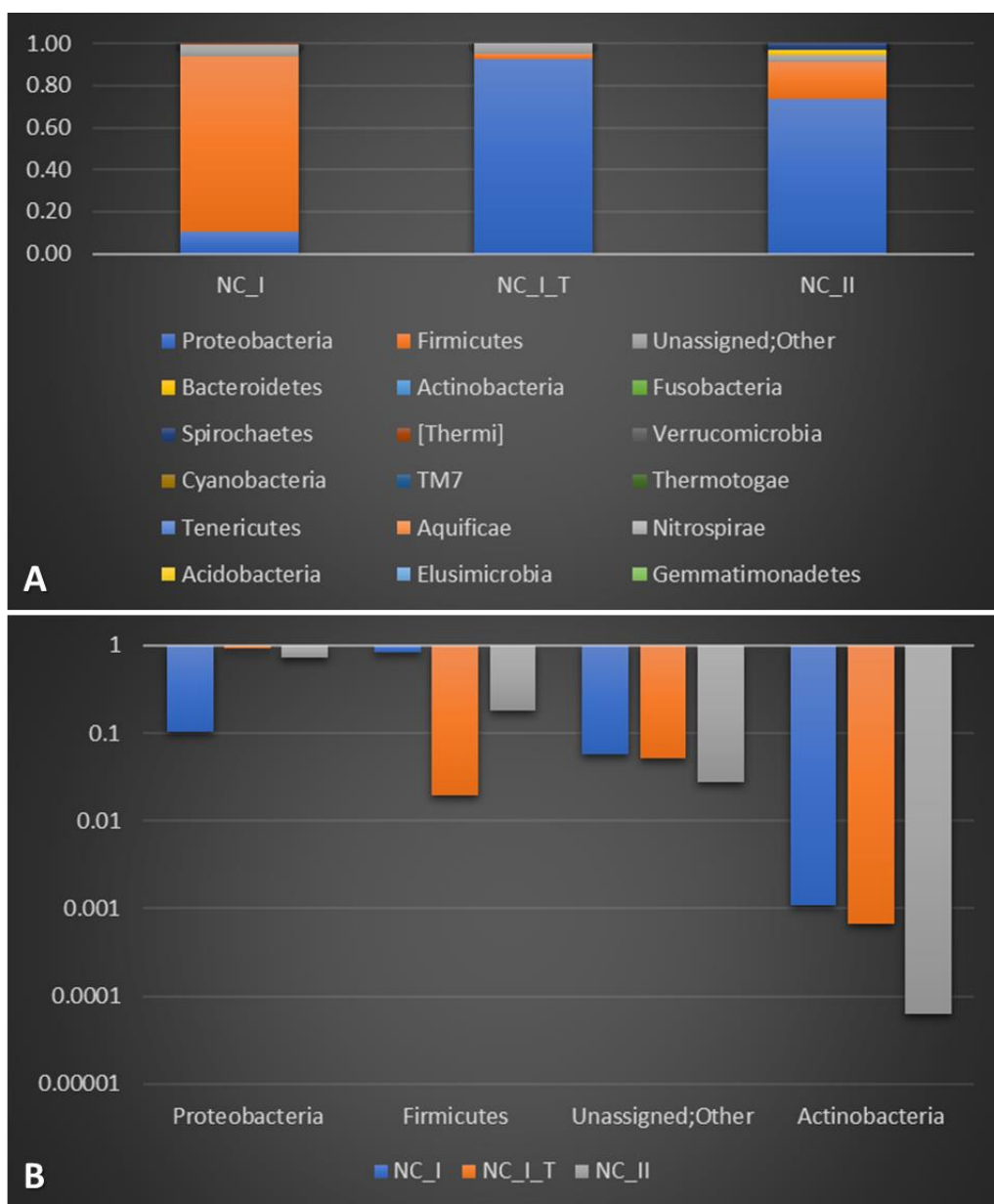
Figure 5.1 Relative abundance of background phyla detected from unexposed gelatine filters with three processing methods
A= Overall view; B= Shared phyla between the three processing methods

Calculated indices of bacterial communities' richness, α and β diversity are presented in Table 5.3.

Table 5.3 Richness, diversity and evenness indices at phylum level in different processing methods

	NC_I	NC_I_T	NC_II
α diversity indices			
S_{Chao1}	136.00	15.00	45.00
H	0.58	0.31	0.84
1-D	0.29	0.14	0.42
E_H	0.21	0.19	0.38
β diversity S_s I S_J			
NC_I		0.38 I 0.24	0.64 I 0.47

5.3.2.2. Genus level

Sequencing from the three reagent controls revealed 181 different genera (Table A.51). These were 179, 18 and 34 for processing by 1st, 2nd and 3rd methods, respectively, with 14 genera shared across all (Figure 5.2).

To be in accordance with the taxonomized phyla, the most abundant genera in 1st method were from Firmicutes: *Clostridium* and *Bacillus* and from Proteobacteria: *Halomonas* and *Shewanella*, constituting in total 89% of its bacterial community while 92% and 18% for 2nd and 3rd methods, respectively. In 2nd method, these were from Proteobateria: *Halomonas* and *Shewanella*, constituting 90% of its bacterial community while 4% and <1% for 1st and 3rd methods, respectively. In 3rd method, these were from Proteobacteria: *Rhizobium*, from Firmicutes: *Clostridium* and *Bacillus*, from Spirochaetes: *Leptospira* and from Bacteroidetes: *Sediminibacterium*, constituting 96% of its bacterial community while 84% and <2% for 1st and 2nd methods, respectively.
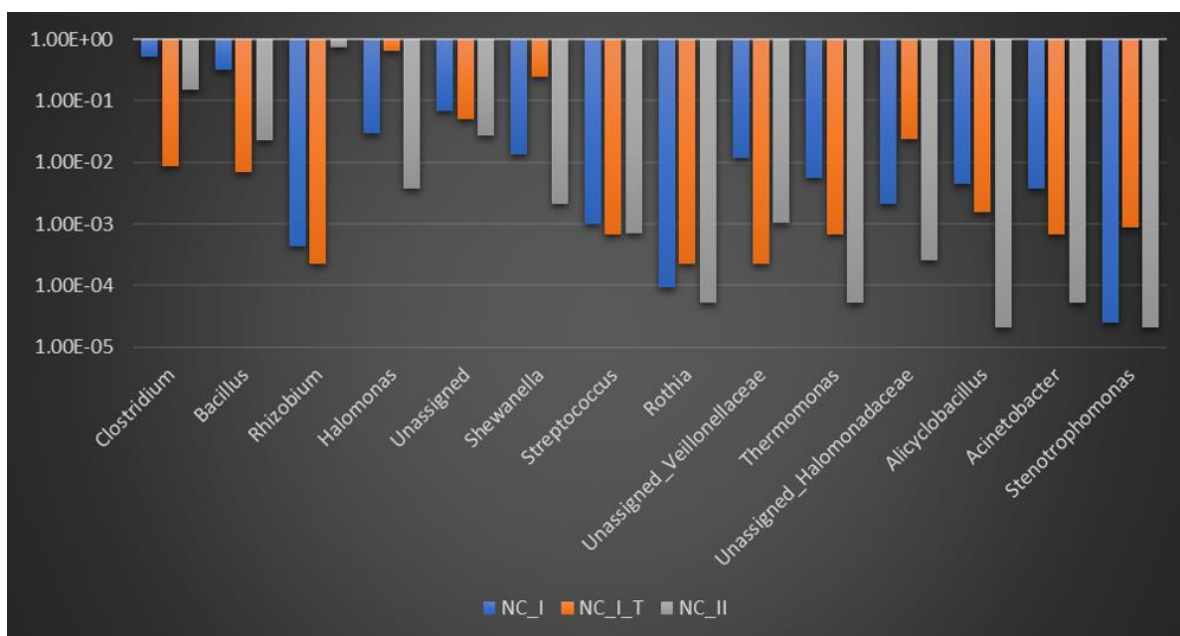
Figure 5.2 Relative abundance of background shared genera detected from unexposed gelatine filters with three processing methods

Calculated indices of bacterial communities' richness, α and β diversity are presented in Table 5.4.

Table 5.4 Richness, diversity and evenness indices at genus level in different processing methods

	NC_I	NC_I_T	NC_II
α diversity indices			
S_{Chao1}	16110.00	171.00	595.00
H	1.31	1.01	0.96
1-D	0.62	0.51	0.44
E_H	0.25	0.35	0.27
β diversity S_s I S_J			
NC_I		0.17 I 0.09	0.31 I 0.18

5.3.3. The exhaled bacteriome collected over different times with normal breathing

5.3.3.1. Phylum level

The phylum assignments of samples obtained during NB over 15, 30 and 60 min from one individual are shown in Figure 5.3A; the 60 min sample stands out as somewhat distinct. Only five phyla, Chlamydiae, Chloroflexi, Armatimonadetes, GN02 and OP11 were absent from the background profile. However, none of these were more frequently represented than at 0.01% of the total reads.

Nine phyla were detected at all three-time points, with the 60 min sample showing higher proportions of Bacteroidetes, Actinobacteria and Fusobacteria (Figure 5.3B). Several phyla were exclusive to particular time points, but these were at very low abundances represented by very few reads (Figure 5.3C).
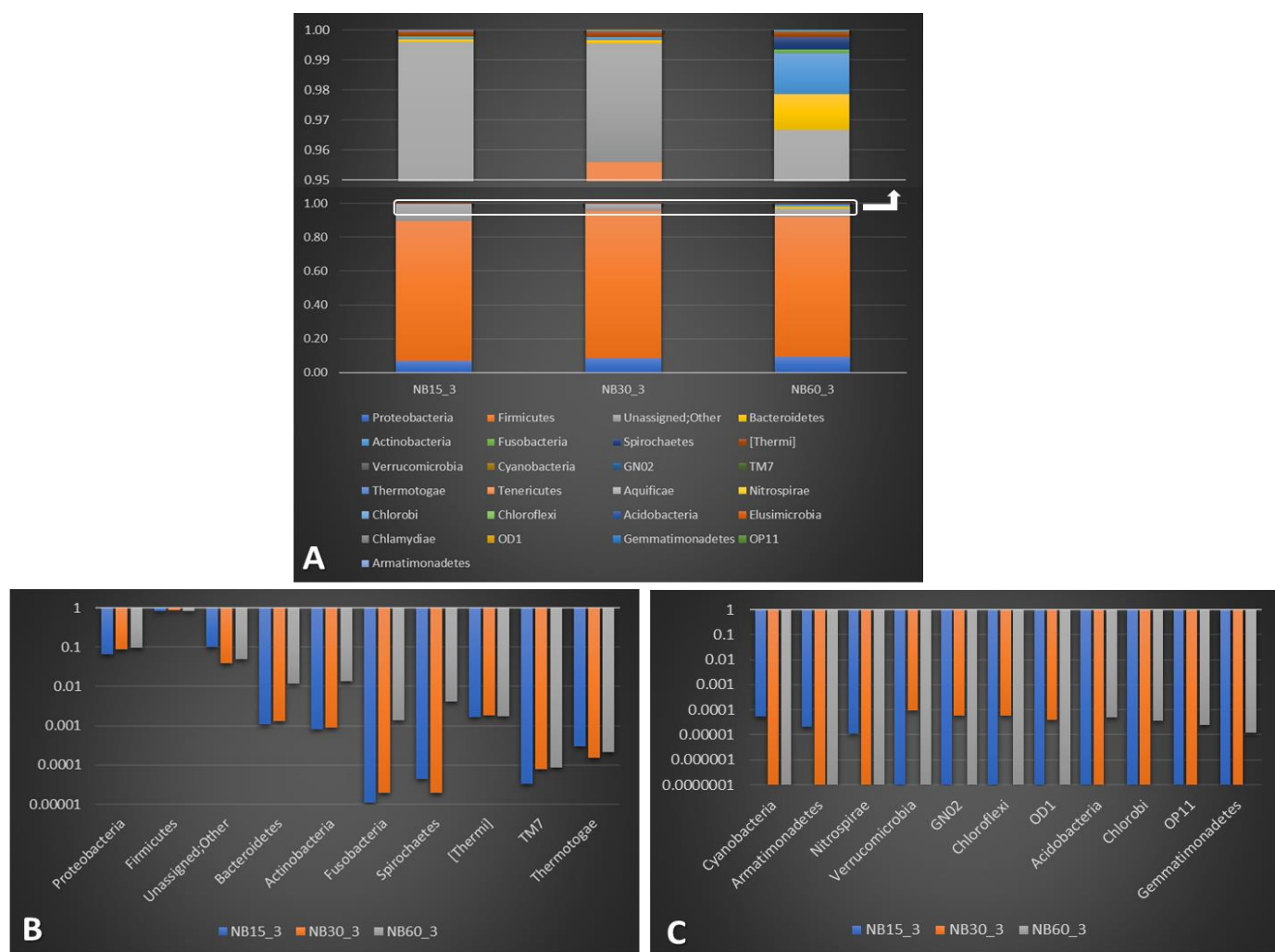
Figure 5.3 Relative abundance of phyla in different time periods
A= overall view; B= Shared phyla between the three time points; C= Phyla unique to a time point

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Calculated indices of bacterial communities' richness, α and β diversity are presented in Table 5.5.

Table 5.5 Richness, diversity and evenness indices at phylum level over the time of sampling

	NB15_3	NB30_3	NB60_3
α diversity indices			
S_{Chao1}	136.00	136.00	153.00
H	0.60	0.49	0.69
1-D	0.30	0.24	0.31
E_H	0.22	0.18	0.24
β diversity S_s I S_J			
NB15_3		0.69 | 0.52	0.73 | 0.57
NB30_3			0.67 | 0.50

Exploring the results with mining tools, AHC separated the samples into three clusters. PCA of Pearson correlation type projected the data on two axes (F1 and F2) corresponding to eigenvalues of 13.35 and 11.65, respectively, with cumulative variability of 100%. Similar findings were obtained with PCA using the covariance method (adding weight to phyla with higher variances). The two-dimensional maps plotted the samples far from each other, indicating different variabilities of phyla between the three sampling periods. PCoA and MDS separated the time periods far apart from each other. The Kruskal's stress for MDS was very close to zero (9.21E-06) indicating an optimal representation (data not shown).

5.3.3.2. Genus level

The three samples obtained from one individual during NB over three periods revealed in total 205 genera (Table A.52). These were 117, 92 and 152 genera over 15, 30 and 60 min, respectively. Overall, 75 detected genus signals were not among the background profile (Table A.52). However, none of these were more frequently represented than at 0.01% of the total reads.

The most abundant genera are presented in Figure 5.4. These were (median) from the Firmicutes: *Clostridium* (~55%) and *Bacillus* (~24%) constituting around 54% and 26%, 65% and 20%, and 52% and 23% of sequence reads of bacterial community over 15, 30 and 60 min, respectively; from Proteobacteria, these were *Halomonas* (~4%) and *Shewanella* (~2%) constituting around 4% and 1%, 5% and 2%, and 4% and 2% over 15, 30 and 60 min, respectively. *Streptococcus* and *Pseudomonas* were among the abundant genera (both ~5%) in NB over 60 min while they were not for 15 and 30 min (<0.5%). Further differences in genus representation across the three samples were all at very low abundance (Table A.52).
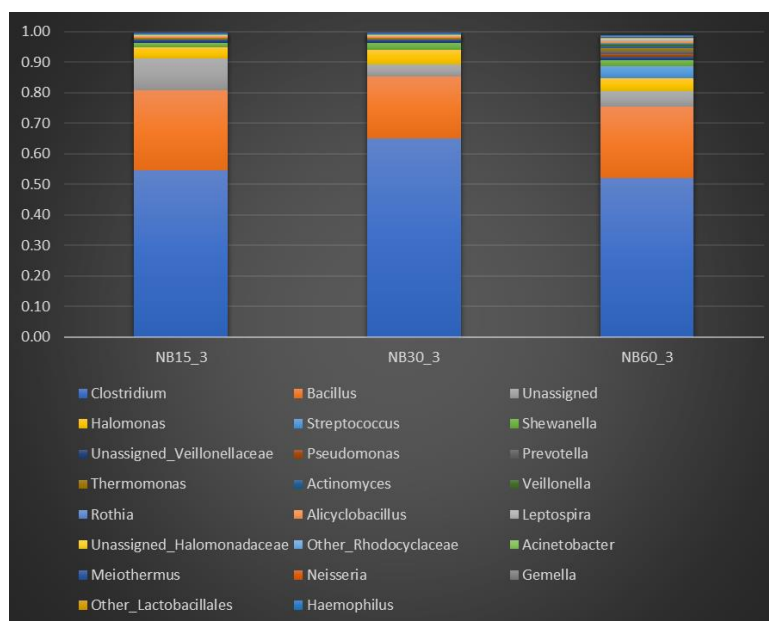
Figure 5.4 The most abundant genera detected over different times of NB

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Calculated indices of bacterial communities' richness, α- and β-diversity are presented in Table 5.6.

Table 5.6 Richness, diversity and evenness indices at genus level over the time of sampling

	NB15_3	NB30_3	NB60_3
α diversity indices			
S_{Chao1}	6903.00	4278.00	11628.00
H	1.33	1.19	1.69
1-D	0.62	0.53	0.67
E_H	0.28	0.26	0.34
β diversity S_s I S_J			
NB15_3		0.63 | 0.46	0.59 | 0.42
NB30_3			0.56 | 0.39

Using the mining tools for further exploration, AHC separated the samples into three clusters. PCA of Pearson correlation type projected the data on two axes (F1 and F2) corresponding to eigenvalues of 129.99 and 75.01, respectively, with cumulative variability of 100%. Similar findings were obtained with PCA using the covariance method (adding weight to genera with higher variances). The two-dimensional maps plotted the time periods far from each other, indicating different variabilities of genera between these periods. PCoA and MDS separated the time periods far from each other. The Kruskal's stress for MDS was very close to zero (8.08E-06) indicating an optimal representation (data not shown).

5.3.4. Differences in exhaled bacteriome between different respiratory activities

5.3.4.1. Phylum level

Twelve samples obtained from three healthy subjects over four respiratory activities lasting for longest tested periods revealed in total 27 phyla. These were 19, 21, 24 and 19 phyla in NB, IB, IC and RL, respectively. Overall, 4 detected phyla (Chlamydiae, Chloroflexi, GN02 and OP11) were not among the background profile but again, these were at low abundances.

The most abundant phyla among all breathing patterns were Firmicutes (~83%), Proteobacteria (~10%), Bacteroidetes (~0.4%) and Actinobacteria (~0.4%). Total reads of these constituted around 95%, 89%, 97% and 92% of sequence reads of bacterial communities of NB, IB, IC and RL, respectively (Figure 5.5).

13 phyla were shared between all four respiratory activities and inter-subject variation is shown in Figure 5.5. On the other hand, 14 phyla were unevenly distributed (Table 5.7) but, again, were at low abundances and showed inter-subject variation as well (Figure 5.5).

Table 5.7 Uneven distribution of phyla between the four respiratory activities

	NB	IB	IC	RL
Acidobacteria	+	–	+	–
Chlamydia	+	–	+	–
Chloroflexi	–	+	+	–
Elusimicrobia	+	–	+	–
Gemmatimonadetes	+	+	–	+
GN02	–	+	+	+
Nitrospirae	–	+	+	–
OD1	–	–	–	+
OP11	+	–	+	–
OP3	+	–	–	–
Synergistetes	–	+	+	+
Tenericutes	–	+	+	+
Verrucomicrobia	–	+	+	+
WPS-2	–	+	+	–
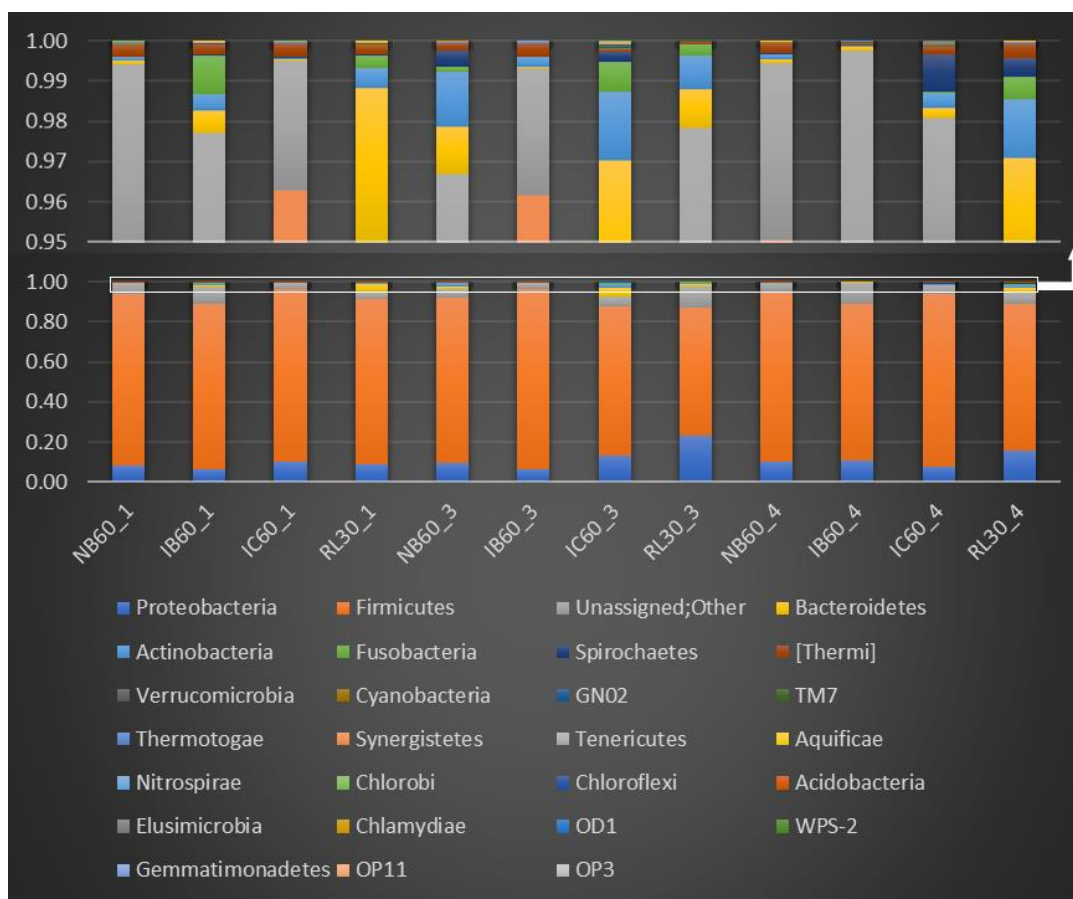
Figure 5.5 Relative abundance of phyla detected over four respiratory activities from three healthy subjects

Calculated indices of bacterial communities' richness, α and β diversity are presented in Table 5.8 and Table A.55. No statistically significant difference was found between α-diversity indices of different breathing patterns.

Table 5.8 Comparison of medians of richness, diversity and evenness indices at phylum level between different respiratory activities

α diversity	S_{Chao1}	H	1-D	E_H
NB	120.00	0.55	0.27	0.23
IB	78.00	0.67	0.31	0.23
IC	171.00	0.56	0.25	0.20
RL	120.00	0.92	0.44	0.34
P value*	0.91	0.15	0.19	0.20

β diversity	S_S		S_J	
NB vs IB	0.70		0.53	
NB vs IC	0.73		0.58	
NB vs RL	0.76		0.62	
IB vs IC	0.71		0.55	
IB vs RL	0.74		0.59	
IC vs RL	0.71		0.56	

(*) Friedman test (non-parametric ANOVA) was done

AHC did not separate the samples into groups, either based on the subject or on the respiratory activity (Figure 5.6A). PCA of Pearson correlation type projected the data on two axes (F1 and F2) corresponding to eigenvalues of 7.79 and 4.45, respectively, with cumulative variability of 45.31%. Squared cosines of IB and RL of subject 1 and of RL of subject 4 were low on F1 and F2 (Table A.57). Therefore, these were plotted on F6 and F8 (for subject 1) and on F2 and F7 (for subject 4). Each respiratory activity was plotted on the 2-dimensional maps far from other activities performed by the same subject (Figure 5.6A, B and C), indicating different variabilities of phyla between the activities. Similar findings were obtained with PCA using the covariance method (adding weight to phyla with higher variances) (Figure 5.6E). With PCoA, a 3-dimensional chart allowed a better visualisation clearly separating each respiratory activity for each subject (Figure 5.6F and G). For MDS, the Kruskal's stress for both the 2- and 3-dimensional configuration was close to zero indicating a good representation (Figure 5.6H and I). IB and RL were the activities most frequently separated. NB of different subjects were close to each other. IB and RL of subject 1 and subject 4 were far from their paired NB and IC. Activities of subject 3 were always far from each other.

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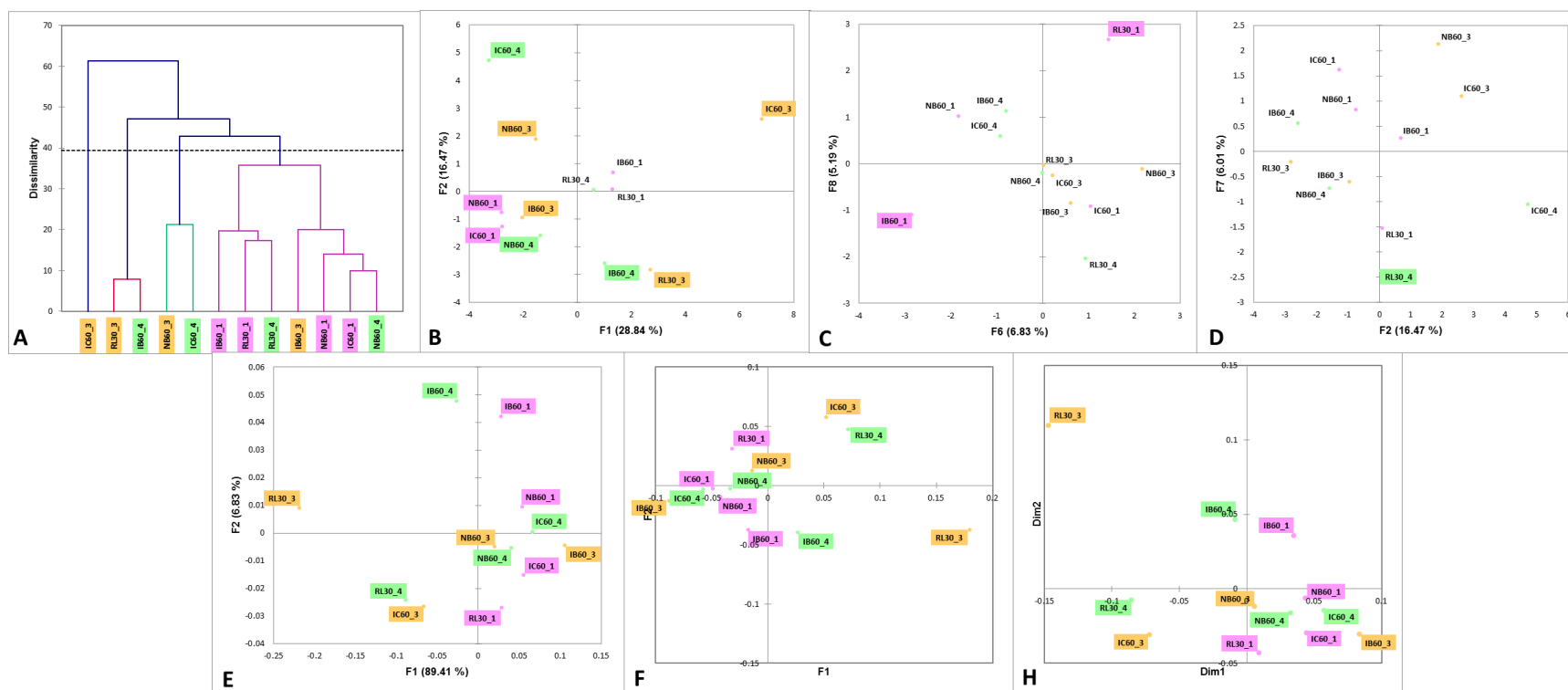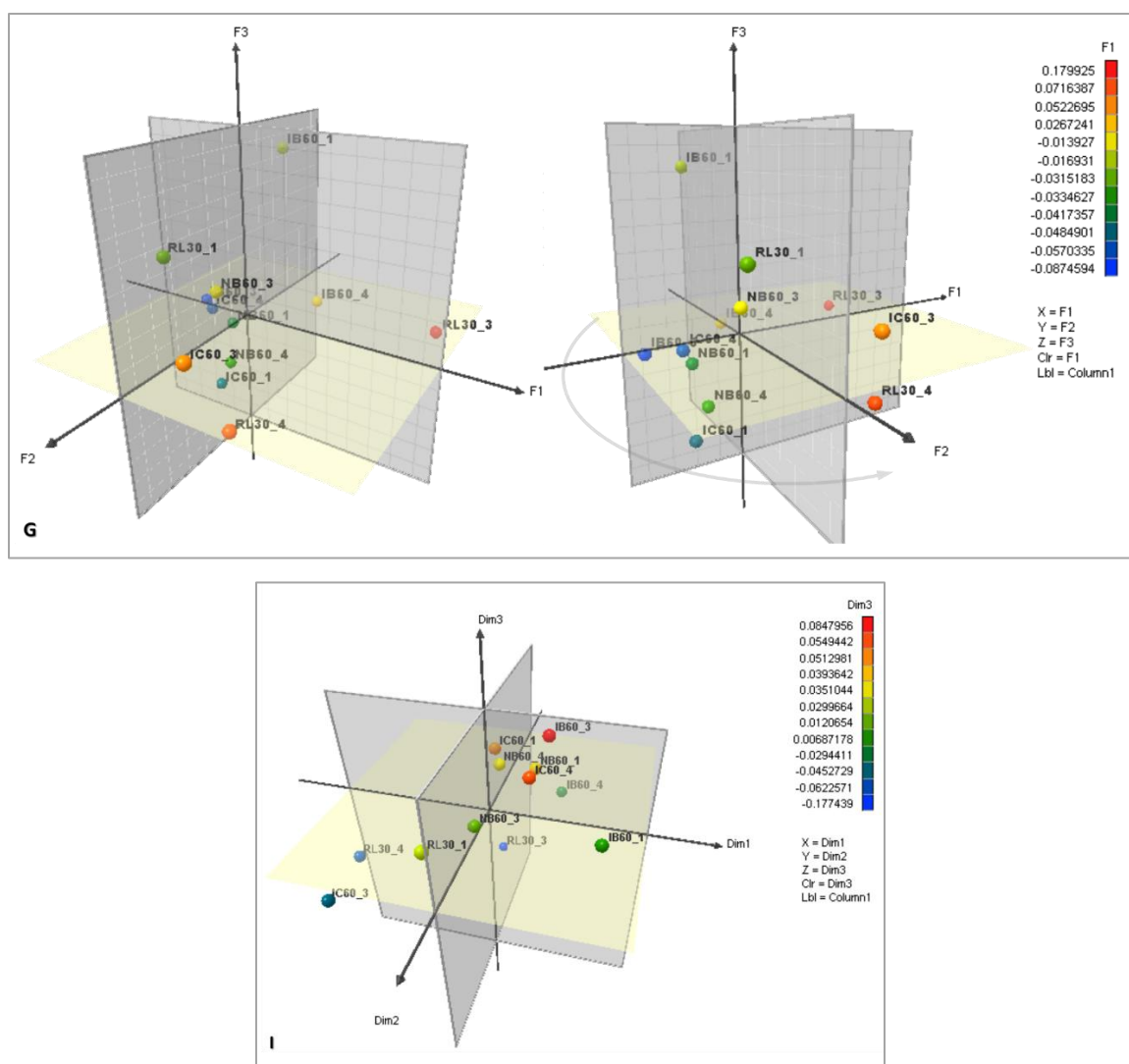
Figure 5.6 Exploratory tests done on phyla detected in masks over four respiratory activities from three healthy subjects
A= AHC using Euclidean distance with automatic truncation of clusters; B, C, D= PCA of Pearson correlation type plotted on F1 and F2, F6 and F8 and F2 and F7 axes, respectively; E= PCA using covariance method; F and G = PCoA using Bray and Curtis distance for creating dissimilatory matrices, plotted on 2 and 3 axes (principal coordinates), respectively; H and I= MDS using Bray and Curtis distance for dissimilatory matrices, configured on 2D (Kruskal's stress= 0.05) and 3D (Kruskal's stress= 0.03) space, respectively.

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5.3.4.2. Genus level

The twelve samples obtained from three healthy subjects over four respiratory activities revealed in total 316 genera. These were 215, 183, 193 and 217 genera in NB, IB, IC and RL, respectively. Overall, 155 detected genus signals were not in the background profile and only reached 0.01% representation in a few cases (Table A.53).

To be in accordance with the phyla, the most abundant genera among all breathing patterns are presented in Table 5.9. Total reads of these constituted around 91%, 85%, 80% and 94% of sequence reads of bacterial communities of NB, IB, IC and RL, respectively.

Table 5.9 Most abundant genera among the breathing patterns

Phylum	Genus	Median (%)
Firmicutes	*Clostridium*	46.20
	Bacillus	30.40
	Streptococcus	0.53
	Alicyclobacillus	0.50
	Veillonella	0.06
Proteobacteria	*Halomonas*	4.27
	Shewanella	1.72
	Thermomonas	0.76
	Pseudomonas	0.24
	Acinetobacter	0.17
	Neisseria	0.12
	Haemophilus	0.11
	Rhodanobacter	0.06
Bacteroidetes	*Prevotella*	0.07
	Porphyromonas	0.05
Actinobacteria	*Rothia*	0.12

109 genera were common across all four respiratory activities, while 38 were exclusive to NB, 19 to IB, 12 to IC and 26 to RL but, again, were at low abundances and showed inter-subject variation (Table A.53).

Calculated indices of bacterial communities' richness, α and β diversity are presented in Table 5.10 and Table A.56. No statistically significant difference was found between α-diversity indices of different breathing patterns.

Table 5.10 Comparison of medians of richness, diversity and evenness indices at genus level between different respiratory activities

α diversity	S_{Chao1}	H	1-D	E_H
NB	8385.00	1.41	0.65	0.30
IB	4095.00	1.29	0.62	0.29
IC	8646.00	1.44	0.62	0.31
RL	8778.00	1.84	0.70	0.38
P value*	0.52	0.21	0.34	0.30

β diversity	S_S		S_J	
NB vs IB	0.58		0.41	
NB vs IC	0.64		0.47	
NB vs RL	0.60		0.43	
IB vs IC	0.55		0.38	
IB vs RL	0.52		0.35	
IC vs RL	0.67		0.50	

(*) Friedman test (non-parametric ANOVA) was done

AHC did not separate the samples into groups based on the subject or on the respiratory activity (Figure 5.7A). PCA of Pearson correlation type projected the data on two axes (F1 and F2) corresponding to eigenvalues of 55.72 and 40.33, respectively, with cumulative variability of 30.39%. Squared cosines of NB and RL of subject 1 and of NB of subject 3 were low on F1 and F2 (Table A.57). Therefore, these were plotted on F4 and F5 (for both subjects). The two-dimensional maps plotted each respiratory activity far from other activities performed by the same subject (Figure 5.7B and C), indicating different variabilities of genera between the activities. PCA using the covariance method (adding weight to genera with higher variances) revealed that NB and IB of subject 1 was close to each other indicating no much variabilities of genera between these two activities, however IC and RL were far from each other and from NB and IB as well (Figure 5.7D), indicating different variabilities of genera between IC, RL and NB or IB. For other

subjects, each respiratory activity was plotted far from other activities performed by the same subject (Figure 5.7D), indicating different variabilities of genera between the activities. With PCoA, the 3-dimensional chart allowed a better visualisation separating each respiratory activity of each subject far apart from the others, except for NB and IB of subject 1 (Figure 5.7E and F). For MDS, the Kruskal's stress for both the 2- and 3-dimensional configuration was close to zero indicating a good representation (Figure 5.7G and H). RL was the most frequently-separated activity. NB and IB of subject 1 were far from their paired IC and RL, while IB and RL of subject 4 were far from their paired NB and IC. Activities of subject 3 were always far from each other.
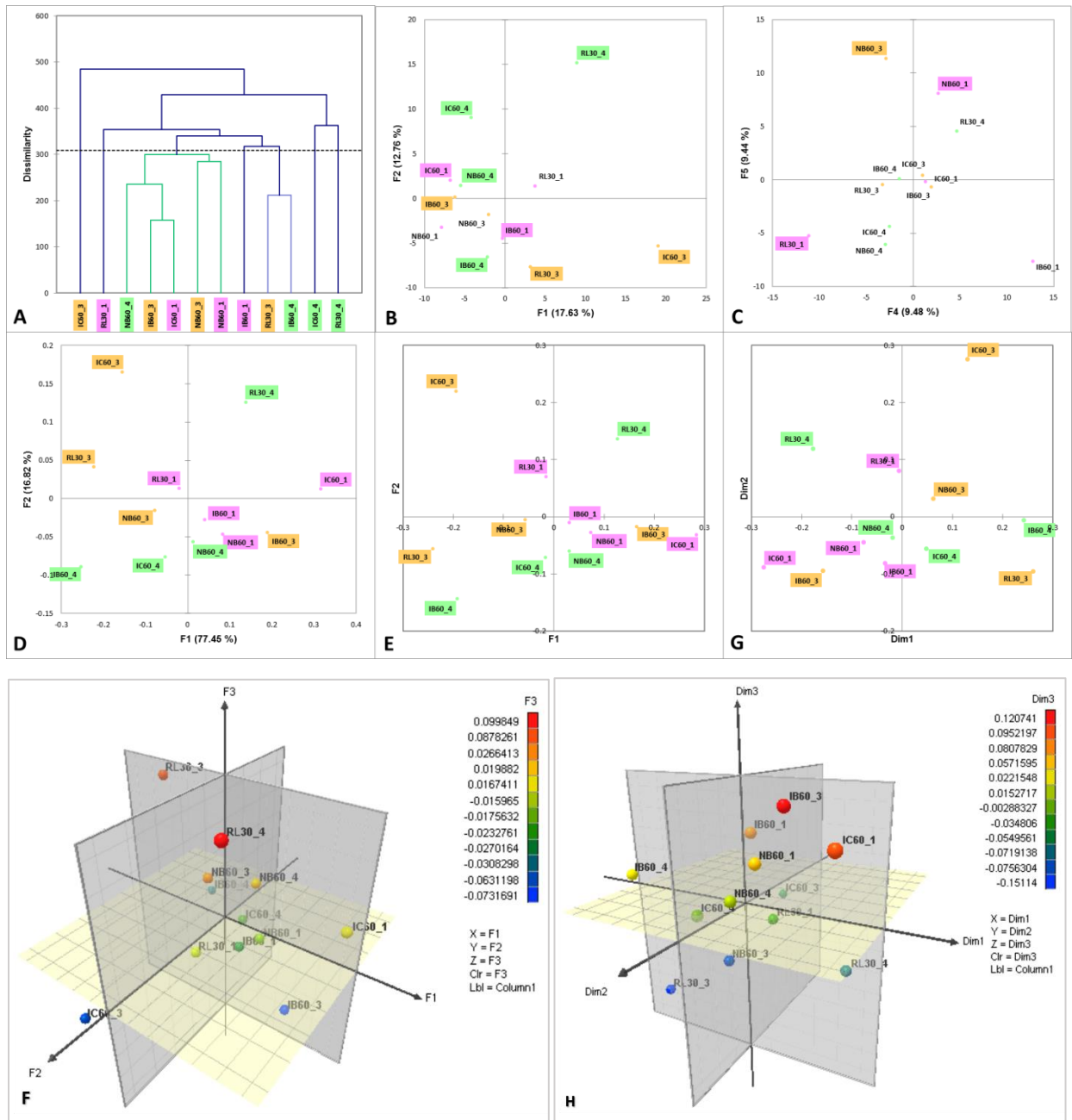
Figure 5.7 Exploratory tests done on genera detected in masks over four respiratory activities from three healthy subjects

A= AHC using Euclidean distance with automatic truncation of clusters; B and C= PCA of Pearson correlation type plotted on F1 and F2 and F4 and F5 axes, respectively; D= PCA using covariance method; E and F = PCoA using Bray and Curtis distance for creating dissimilatory matrices, plotted on 2 and 3 axes, respectively; G and H= MDS using Bray and Curtis distance for dissimilatory matrices, configured on 2D (Kruskal's stress= 0.11) and 3D (Kruskal's stress= 0.05) space, respectively.

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5.3.5. Comparison of the bacteriome signature between the mask and BAL in patients with suspected PJP

5.3.5.1. Phylum level

Three paired samples obtained by mask and BAL sampling from three patients with suspected PJP revealed in total 14 phyla. These were 10 and 13 phyla in masks and BALs, respectively and nine were shared (Figure 5.8A). All the detected phyla were among the background profile.

The most abundant phyla in the two sample types were Proteobacteria (~34%), Firmicutes (~31%), Bacteroidetes (~2%) and Spirochaetes (~1%). Total reads of these constituted around 94% in masks and 62% in BALs. Actinobacteria were more abundant in the BALs constituting 6% overall while <1% in the masks, although they were poorly represented in BAL 3 (Figure 5.8B).

There were dissimilarities in abundant phyla between masks and BALs, and between the patients within the same sample type. These are shown in Figure 5.8B and Figure 5.8C. At low abundance, one phylum (Thermi) was detected in the masks but not in the BALs, while three phyla (TM7, Thermotogae, Synergistetes) were detected in the latter but not in the former (Figure 5.8C).
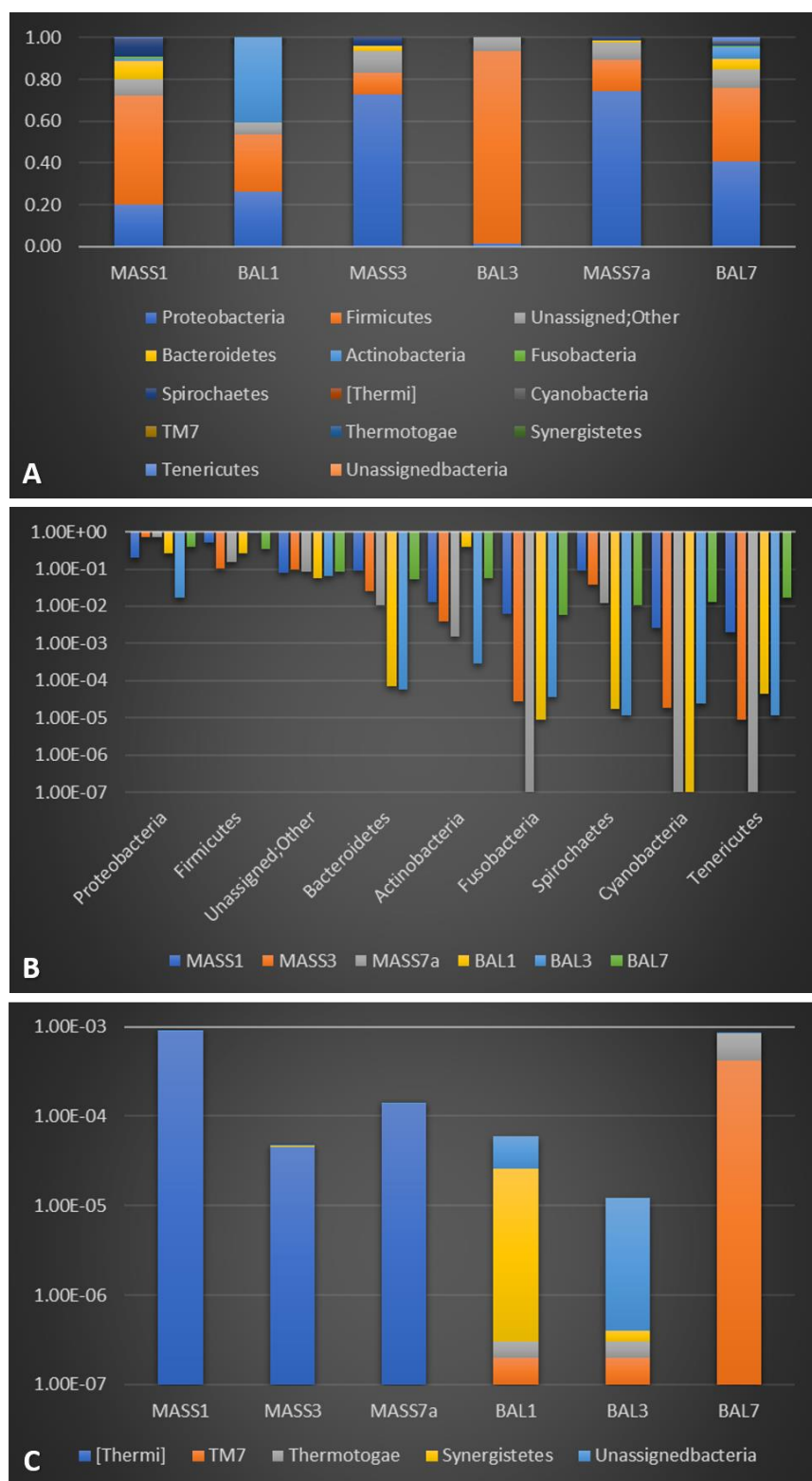
Figure 5.8 Relative abundance of phyla detected in masks and BALs
A= Overall view; B= Shared phyla detected in both masks and BALs; C= Phyla detected with
disparities between masks and BALs

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Calculated indices of bacterial communities' richness, α and β diversity are presented in Table 5.11. No statistically significant difference was found between α-diversity indices of the masks and the BALs (using Wilcoxon matched-pairs signed rank test).

Table 5.11 Richness, diversity and evenness indices at phylum level in paired mask and BAL samples

	MASS1	BAL1	MASS3	BAL3	MASS7a	BAL7
			α diversity			
S_{Chao1}	55.00	55.00	55.00	55.00	28.00	66.00
H	1.41	1.24	0.94	0.33	0.83	1.47
1-D	0.67	0.69	0.45	0.15	0.42	0.70
E_H	0.61	0.54	0.41	0.14	0.42	0.61
			β diversity S_S | S_J			
BAL1	0.80 | 0.67					
MASS3	1.00 | 1.00					
BAL3		0.90 | 0.82	0.90 | 0.82			
MASS7a	0.82 | 0.70		0.82 | 0.70			
BAL7		0.76 | 0.62		0.86 | 0.75	0.67 | 0.50	

Interestingly, AHC separated the samples into two major groups based on the sample type (Figure 5.9A). PCA of Pearson correlation type projected the data on two axes (F1 and F2) corresponding to eigenvalues of 5.75 and 3.66, respectively, with cumulative variability of 67.24%. The two-dimensional map plotted the masks far from their paired BALs (Figure 5.9B), indicating different variabilities of phyla between the masks and BALs. Similar findings were obtained with PCA using the covariance method (adding weight to phyla with higher variances) (Figure 5.9C). The 3-dimensional chart of PCoA allowed a better visualisation showing clear separation between the masks and their paired BALs (Figure 5.9D and E). For MDS, the Kruskal's stress for both the 2- and 3-dimensional configuration was close to zero indicating a good representation showing a further clear separation (Figure 5.9F and G).
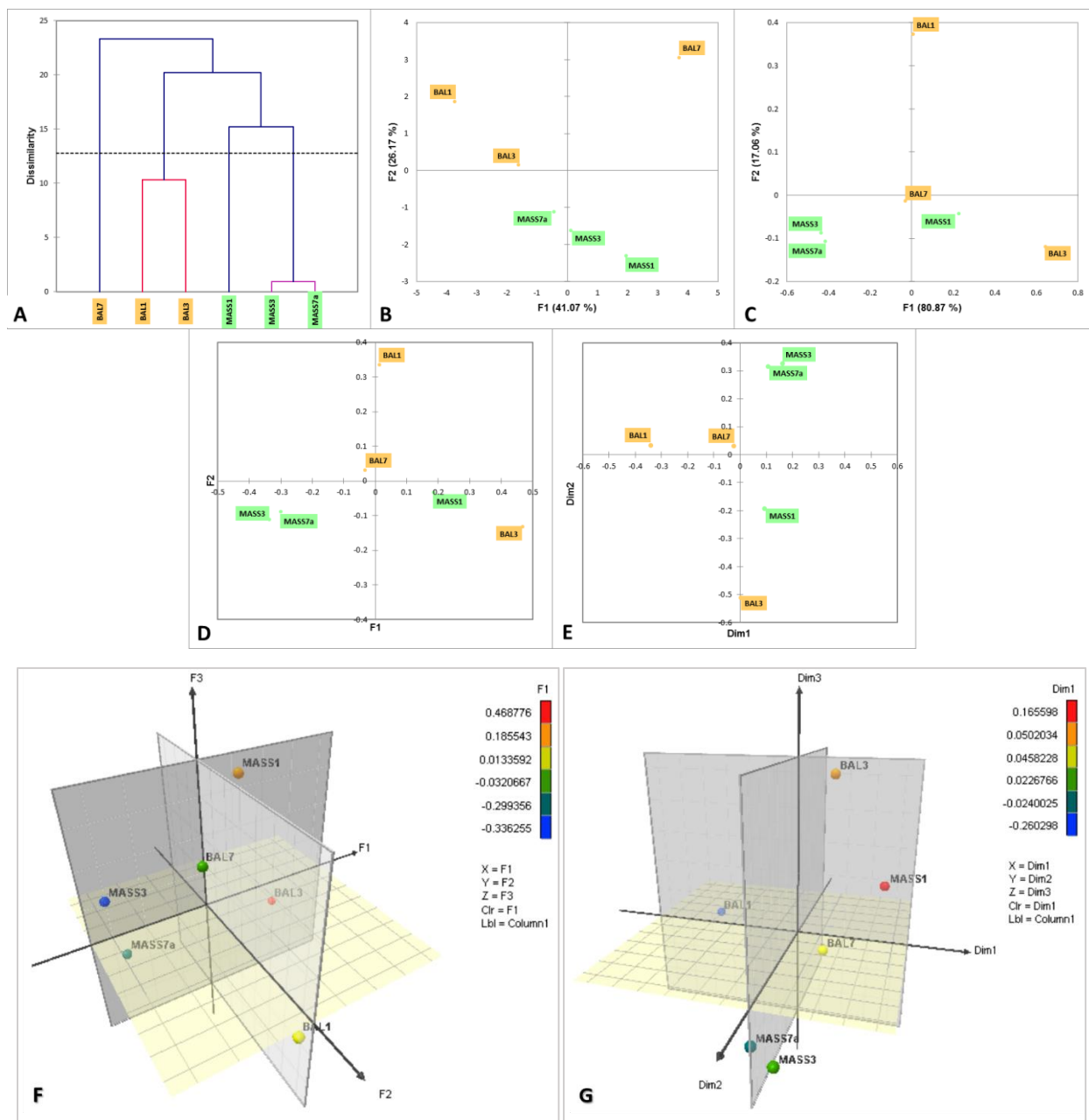
Figure 5.9 Exploratory tests done on phyla detected in masks and BALs
A= AHC using Euclidean distance with automatic truncation of clusters; B= PCA of Pearson correlation type; C= PCA using covariance method; D and E = PCoA using Bray and Curtis distance for creating dissimilatory matrices, plotted on 2 and 3 axes, respectively; F and G= MDS using Bray and Curtis distance for dissimilatory matrices, configured on 2D (Kruskal's stress = 0.06) and 3D (Kruskal's stress = 0.02) space, respectively.

5.3.5.2. Genus level

The three paired samples revealed in total 142 genera. These were 101 and 100 genera in the masks and BALs, respectively. Overall, 66 detected genus signals were not in the background profile but again, these were at low abundances. 60 genera were shared across both sample types (Table A.54).

The most overall abundant genera in both sample types were *Rhizobium*, *Halomonas*, *Shewanella*, *Clostridium*, *Bacillus*, *Streptococcus*, *Sediminibacterium*, *Leptospira*, and *Rothia*. Total reads of these constituted around 94% in masks but only 5% in BALs (Table A.54).

There were substantial dissimilarities in most abundant genera between the masks and BALs, and between the patients within the same sample type. At low abundance, 41 genera were detected in the masks but not in the BALs, while 40 genera were detected in the latter but not in the former (Table A.54).

Calculated indices of bacterial communities' richness, α and β-diversity are presented in Table 5.12. No statistically significant difference was found between α-diversity indices of the masks and the BALs (using Wilcoxon matched-pairs signed rank test).

Table 5.12 Richness, diversity and evenness indices at genus level in paired mask and BAL samples

	MASS1	BAL1	MASS3	BAL3	MASS7a	BAL7
α diversity						
S_{Chao1}	2415.00	1225.00	2628.00	861.00	253.00	2628.00
H	2.40	1.52	1.10	0.91	0.96	2.68
1-D	0.88	0.72	0.46	0.46	0.43	0.86
E_H	0.57	0.39	0.26	0.25	0.31	0.63
β diversity S_S | S_J						
BAL1	0.49 | 0.33					
MASS3	0.58 | 0.41					
BAL3		0.60 | 0.43	0.46 | 0.30			
MASS7a	0.42 | 0.26		0.45 | 0.29			
BAL7		0.51 | 0.34		0.41 | 0.26	0.28 | 0.16	

AHC did not clearly separate the samples based on their type (Figure 5.10A). PCA of Pearson correlation type projected the data on two axes (F1 and F2) corresponding to eigenvalues of 62.56 and 33.64, respectively, with cumulative variability of 67.75%. The masks of patient 1 and 7 were plotted far from their paired BALs on the two-dimensional map, while they were relatively close for patient 3 (Figure 5.10B), indicating different variabilities of genera between the masks and BALs in the former and less variabilities in the latter. The data were analysed with PCA using the covariance method (adding weight to genera with higher variances). Squared cosines of the mask of patient 1 were low on F1 and F2 (Table A.57), therefore this was plotted on F3 and F4 (Figure 5.10C and D). Similar findings were obtained indicating different variabilities of genera between the masks and BALs for patients 1 and 7, and with less variabilities for patient 3. The 3-dimensional chart of PCoA allowed a better visualisation separating each paired sample far apart from its pair (Figure 5.10E and F). For MDS, the Kruskal's stress for both the 2- and 3-dimensional configuration was close to zero indicating a good representation showing a further clear separation (Figure 5.10G and H).
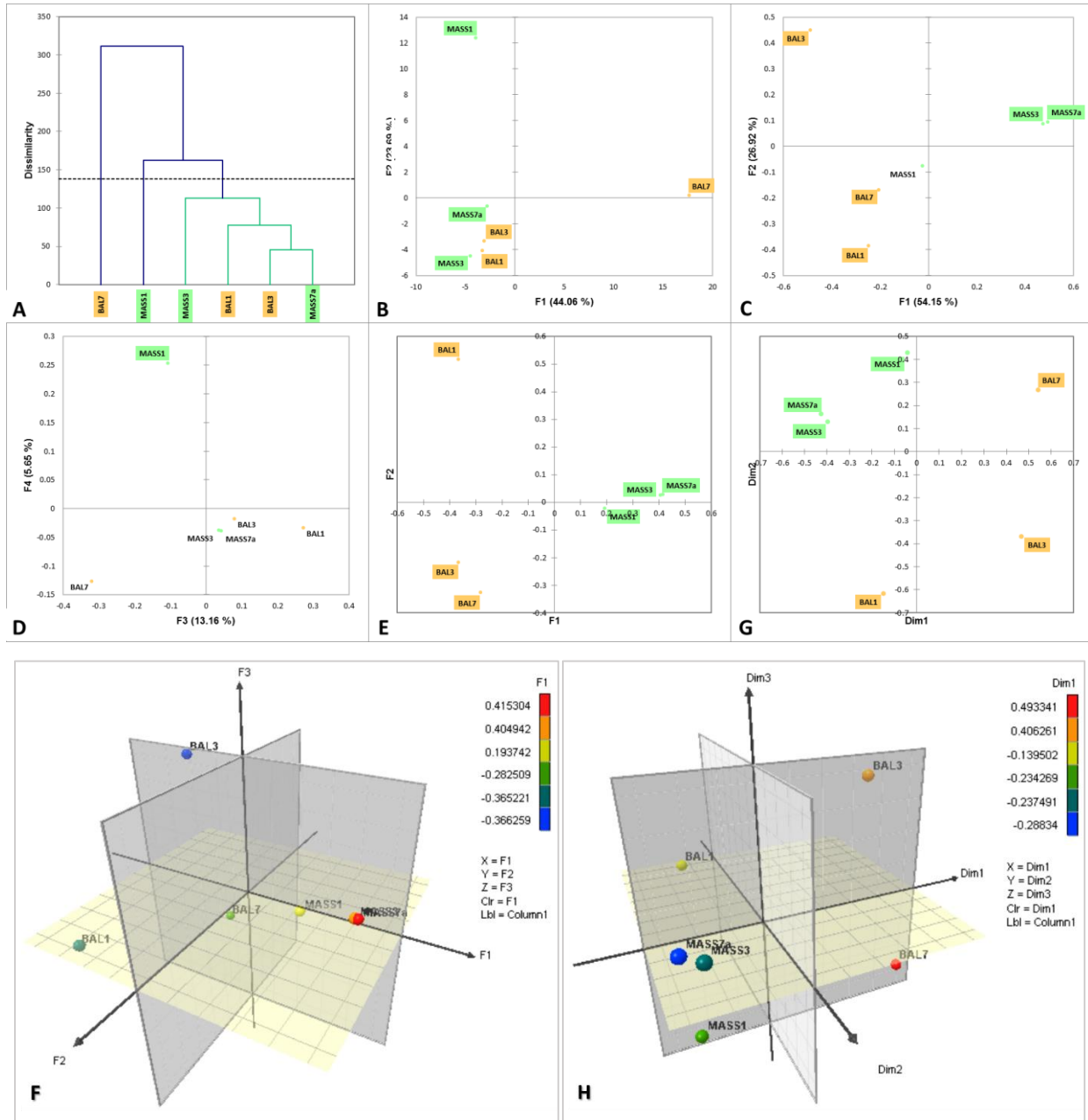
Figure 5.10 Exploratory tests done on genera detected in masks and BALs
A= AHC using Euclidean distance with automatic truncation of clusters; B= PCA of Pearson correlation type; C and D= PCA using covariance method plotted on F1 and F2 and F3 and F4, respectively; E and F = PCoA using Bray and Curtis distance for creating dissimilatory matrices, plotted on 2 and 3 axes, respectively; G and H= MDS using Bray and Curtis distance for dissimilatory matrices, configured on 2D (Kruskal's stress = 0.18) and 3D (Kruskal's stress = 0.09) space, respectively.

5.4. Discussion

5.4.1. Background signals attributable to gelatine and reagents

In order to be informative, interpreting sequencing data should start with defining a background profile to distinguish spurious from genuine signals. It was documented earlier that the gelatine filters harbour a heavy DNA background limiting their use in characterizing the exhaled microbiome. Nonetheless, it was considered potentially informative to study the impact of the different processing methods involving chemical lysis, PMA treatment followed by chemical lysis, and mechanical lysis (bead beating) on the studied bacteriomes.

The bacterial communities differed with the three processing methods, though this did not attain statistical significance. It was found using AHC, PCA, PCoA and MDS that the processing methods were well separated at both phylum and genus levels, indicating that each method posed a profound impact on the bacterial communities of the processed sample. However, in this occasion which demonstrated differences, no statistical values could be calculated due to absence of replicates (n=1).

At a general level, this finding is in agreement with Albertsen *et al*. (2015) and Guo and Zhang (2013) who studied the biasing effect of applying different DNA extraction methods, and reinforces the main points concluded in many studies concerned with assessing reagent contaminants (de Goffau *et al*., 2018; Jervis-Bardy *et al*., 2015; Salter *et al*., 2014; Weiss *et al*., 2014).

Detection of Firmicutes and Proteobacteria is in accordance with Salter *et al*. (2014) who investigated contaminating DNA in DNA extraction kits and other laboratory reagents. The most abundant genera detected in our study (*Clostridium*, *Halomonas* and *Shewanella*), however, were different and their source might be accounted for contamination of collagen-containing tissues during husbandry and in abattoirs, and, not surprisingly, for the use of

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collagenase derived from *C. histolyticum*. It would have been useful to test all the components used in the extractions separately to estimate the contribution of each.

The shared detectability of Firmicutes, Proteobacteria and Actinobacteria and 14 genera (Figure 5.2) could indicate these taxa are true components of gelatine and/or gelatine-processing reagents since their detections were repeatable among the three methods (de Goffau *et al*., 2018).

Regarding PMA treatment, it is unlikely that cells exposed to the manufacturer's sterilisation procedures would have maintained membrane integrity. The profiles obtained indicate that the effect of PMA was not even across the genera identified without PMA. Thus, Firmicutes (*Clostridium* and *Bacillus*) appeared "susceptible" while Proteobacteria (*Halomonas* and *Shewanella*) seemed "resistant". PMA is not selective and intercalates with any available dsDNA (or more broadly with any available binding sites) and, following UV exposure, is covalently bound. Thus, it must be presumed that genomes of Proteobacteria were less accessible for some reason. This might not require cell viability or PMA concentration as Seinige *et al*. (2014) showed that $>10^6$ nonviable cells remained detectable independently of PMA concentration. Internal factors may include lysability and GC content.

Rogers *et al*. (2013) showed that PMA treatment of sputa of CF patients decreased the abundance of total bacterial signals by around one log fold, altered the community structure and increased the diversity and evenness. Some of these features were observed here with a similar log-fold reduction and detection of Verrucomicrobia only when PMA was applied, while the indices showed that α-diversity was reduced. Despite these did not attain statistical significance and might be incomparable with the Rogers findings due to different study conditions, the disagreement might be due to a heterogeneity in gelatine. On the other hand, and going back to basics, while

the Rogers group applied a sophisticated meta-analysis approach for comparing PMA-treated and untreated communities, they did not explicitly characterise their reagents' microbiome.

Regarding bead-beating, we showed that this increased the total number of reads but changed the contribution of different community members and increased the community diversity and evenness, particularly at the phylum level. Signals from Proteobacteria, Bacteroidetes and Spirochaetes were more abundant with this method while Firmicutes signals were less. The detected signals should have emitted either from intracellular or extracellular DNA. In the case of the former, Proteobacteria, Bacteroidetes and Spirochaetes were better extracted by bead-beating while Firmicutes were degraded. At genus level, *Rhizobium* and *Leptospira* were better extracted while *Clostridium* and *Bacillus* did not. If the signals were of extracellular DNA, the shown pattern reflects the impact of exposing DNA to sharing forces. This is further supported by identifying an overall lower number of phyla and genera with the applied conditions and might explain why *Mycobacterium* was found in the first extraction method but not with bead-beating. If this was intracellular, contradictory results should have been expected. For instance, Macovei *et al*. (2015) extracted DNA using bead-beating for MiSeq to reveal the NTM component of URT microbiome in health. Another possibility is that the contaminating signals originated from both intra- and extra-cellular DNA. This might explain why Actinobacteria abundance was not found to increase with bead-beating as some studies on environmental samples suggest (Albertsen *et al*., 2015; Guo and Zhang, 2013), indicating their signals might have mainly originated from extracellular DNA, while Proteobacteria, Bacteroidetes, Nitrospirae, Chloroflexi did (Guo and Zhang, 2013), indicating their signals might have done so from intracellular components.

A heterogenetic background, or in other words, inconsistent raw biomaterials used for manufacturing the gelatine cannot be excluded. This experiment,

however, was not designed to evaluate the impact of extraction on different microbes rather to explore how different processing methods can impact on the bacterial communities and, more importantly, to define the background profile.

de Goffau *et al*. (2018) interestingly categorised the initial distinguishability between true and false microbial signals (reagent contaminants) on the basis of amounts of microbial biomass of samples of concern. Compared to other microbial communities in various niches, the exhaled component or a "true" mask sample is expected to harbour small microbial biomass. The challenges of the current design lie in discriminating genuine signals (expectedly low) diving in spurious signals (relatively high biomass). To complicate matters, the latter has been shown to dominate when the former attenuates (Salter *et al*., 2014). Therefore, special attention must be paid as genuine exhaled signals might be indistinguishable from contaminants. For example, Clostridia dynamics was reported in oronasal microbiome (Huang *et al*., 2014; Pragman *et al*., 2012; Dewhirst *et al*., 2010, Koskinen *et al*., 2018) and Sverrild *et al*. (2017) reported *Halomonas* and *Shewanella* in the airways' microbiome, while these were considered contaminants by others (Weyrich *et al*., 2019).

While it remains almost impossible to claim contaminant-free analyses (de Goffau *et al*., 2018; Salter *et al*., 2014) and is neither practical nor sufficient to combine adjunct tools like culturing for every taxon, it seems the most practical approach lies in making ecological sense of the studied profiles. However, this is of an acknowledgeable complexity, particularly for data lacking references, and cannot be taken for granted.

5.4.2. The exhaled bacteriome collected over different times with normal breathing

While it was previously shown that longer sampling did not increase *16S* yield, the structure of the bacterial community was found to differ among three samples obtained from one individual over 15, 30 and 60 min of NB. Increasing the sampling time to from 30 to 60 min resulted in increased richness, α-diversity and evenness. However, it must be recognised that, in contrast to Chapter Three, only one individual was sampled here, and thus the results reflect three separate sampling periods rather than one from which samples were withdrawn after different times.

Since the sample size is insufficient to statistically compare β-diversity indices, it is more reasonable to explore these communities with data mining tools. It was found using AHC, PCA, PCoA and MDS that different sampling periods were separated far from each other at both phylum and genus levels, indicating that the bacteriome constitutes were different between the three time points.

The within and between changes in bacterial communities could indicate that the sampling surface was not saturated after 15 min. The previously demonstrated bacterial-signal-degrading factors may have contributed and the detected signals may represent a balance between accumulation and degradation. In addition, degradation of the background signals from gelatine and other sources cannot be excluded. It is also recognised that variation in the bacterial community exhaled by this individual on separate occasions may have contributed. This possibility, individual output variation(s) with the same breathing pattern, was not tested here.

One could ask whether those showing uniqueness for time are differentially liable to degradation or whether they reflect temporal contributions from different anatomical zones during exhalation. However, the shown uniqueness

or differences are largely attributable to taxa assigned on the bases of very low reads which could be negligible (Janssen, 2006).

The impact of co-exhaled signal-degrading factors seems more apparent on settled rather on airborne particles. This might lead some to mis-relate the behaviour of aerosols even in environmental air. For example, Meadow *et al*. (2015) related the detectability of "*microbial cloud*" to increasing the chamber occupation period showing all the three individuals' clouds detectable after four hrs on air filters but only for two after two hrs on settling dishes during room air sampling of the same bacterial OTUs. However, this should not indicate that the impact is absent on airborne particles, possibly explaining why the Meadow's group detected through exhaust air from the chamber only two of the eight occupants, relating this to a sufficiency in "*human-associated taxa*".

While it was acknowledged that gelatine sampling matrix is heavily contaminated with DNA, if the obtained signals were all false (belonging to contaminants) they were likely to be stable between the three samples processed in one run using one batch of reagents, but this was not the case.

The presence of shared phyla and genera in the three time points should be interpreted against the balance of accumulation and degradation discussed above, together with the background reagent signals and natural variations in output. All the shared phyla and genera were also detected in the blank filters therefore a false component in their signals cannot be excluded. Relative abundances of Bacteroidetes, Actinobacteria, Fusobacteria and Spirochaetes were increased at 60 min while they were stable for Firmicutes, Proteobacteria, Thermi, TM7 and Thermotogae. The latter five could be classified as false signals while the dynamic pattern shown in the former four could reflect their exhaled contribution. Similarly, abundances of *Pseudomonas* and *Streptococcus* were relatively increased in 60 min, while

decreased for *Stenotrophomonas*, *Candidatus Rhodoluna* and *Burkholderia*, and steadied for the rest. If these were true signals, the shown dynamic pattern could have reflected an exhaled contribution of both signals and signal-degrading factors. However, again, these genera were also detected in the blanks and some are well-recognised contaminants (Tanner *et al*., 1998; Salter *et al*., 2014).

Excluding phyla detected in the blanks, the one phylum (Armatimonadetes), two phyla (GN02 and Chloroflexi) and the further one (OP11) uniquely detected in 15, 30 and 60 min, respectively, could reflect true signals. Having a wider view, however, these phyla pose a potential to be false. Armatimonadetes (or OP10) has been detected in lung microbiome of CF patients (de Dios Caballero *et al*., 2017) but its presence is also known in soil habitats (Lee *et al*., 2014). Similarly, OP11 have been described in the oral microbiome (Dewhirst *et al*., 2010). However, OP 1, 3, 5, 8, 9, 10 and 11 discovered in Obisidian Pool are potential components of gelatine microbial ecology. GN02 and Chloroflexi have been described in oral microbiome (Wade, 2013; Wade *et al*., 2016) but also in drinking water and soil bacterial communities (Janssen, 2006). Similarly, excluding genera detected in the blanks, the 16, 8, 39 genera uniquely detected in 15, 30 and 60 min, respectively, could nonetheless be false.

One manipulation for removing false signals can be through finding strong negative correlations between relative abundances of false OTUs and amplicon concentration (Jervis-Bardy *et al*., 2015). However, this was not helpful when tested at the phylum level yielding no statistically significant negative correlation. At the genus level, this approach revealed only *Halomonas* (Spearman r= -0.47, p= 0.02) and *Shewanella* (Spearman r= -0.43, p= 0.03).

Another manipulation for removing background signals can be through excluding these readings for the true samples. However, while such approach was bioinformatically feasible, it was not practically helpful, leaving OTUs at very low abundances (<10 reads) indicating extensive overlapping between true and false signals.

One could argue that it was worth including the control results with the relevant group when analysed. This was trialed but had masked the trends. Therefore, it was more plausible to present the trends separately alongside acknowledging the high background (Weiss *et al.*, 2014).

While the false signals were overwhelming, leading to inconclusive results, the shown patterns indicate that the exhaled microbiome signature has a potential to differ over time or sampling event and is worth being further explored.

5.4.3. Differences in exhaled bacteriome between different respiratory activities

Differences in the structure of the bacterial communities were found with the different breathing patterns. RL resulted in highest indices of richness, α-diversity and evenness at both phylum and genus levels. The difference, however, was not supported statistically, possibly due to the small sample size and the high background signals. Similarly, β-diversity indices were not found statistically different between the breathing patterns, despite exploring the data with the mining tools have revealed trends supporting distinguishability.

It was found using PCA and PCoA that the bacterial communities of different patterns were separated at phylum level. The separation was more complicated using the same tools at genus level and was furthermore individualised using MDS at both phylum and genus levels. On the other hand, AHC did not show a clear separation either at phylum or at genus level. While this might be due to the overwhelming background, it might have reflected a more complicated pattern of exhalation. Indeed, NB of different subjects were plotted close to each other at the phylum level, possibly reflecting the homogeneous nature of this activity. However, this was not clearly shown at the genus level, indicating personal variations. RL was the most frequently-separated activity far from others indicating its unique signature.

Detection of 13 phyla and 109 genera in the four respiratory activities may reflect all of the possibilities discussed in the previous section. A mixture of contributions from both false and true signals appears the most probable. Considering samples taken with three methods, Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Spirochaetes and Thermi are main components of mask samples, regardless whether their signals were true (exhalation) or false (reagents).

To make ecological sense, it was shown earlier that the first four phyla are components of aerosols (3.3.6). Spirochaetes is component of oral microbiome (Dewhirst *et al*., 2010; Wade, 2013), qualifying it to be a component of exhaled bacterial communities. Similarly, Thermi has been described in nasal and lung microbiome (Botero *et al*., 2014; Yu *et al*., 2016). However, both remain among the background profile. Fusobacteria, Cyanobacteria, TM7 and Thermotogae were not consistently detected suggesting that they are neither main false nor main true components. Aquificae has been claimed unique to sputa of TB patients (Cui *et al*., 2012) and Chlorobi has been described in the oral microbiome (Camanocha and Dewhirst, 2014). However, none of them was detected in NB over 15 and 30 min (one individual) while both are among the background profile, suggesting these are not main true components.

The four phyla (Chlamydiae, Chloroflexi, Gracilibacteria (GN02) and Microgenomates (OP11)) as well as the 155 genera (Table A.57) undetected in the background profile might be true signals. However, these were not dominant, prevalent or reproducible in a pattern. One should claim IC is equivalent to RL in order to claim GN02 reproducibility by this combined pattern. The other phyla were detected only in two of 12 samples for each. On the other hand, Chlamydiae, Chloroflexi and OP11 can be expected as contaminants on ecological grounds (Janssen, 2006). Nonetheless, all these phyla have been described in the oral microbiome (Dewhirst *et al*., 2010; Wade, 2013; Camanocha and Dewhirst, 2014) and their presence in lower anatomical zones should not be surprising. Therefore, these are might be true but infrequent components of exhaled bacteriome.

The detection of phyla and genera unique to breathing patterns supports our previous findings, suggesting aerosols generated by a specific respiratory pattern mirror the bacterial communities of the generating anatomical zone(s). The exact mechanism(s) involved in aerosolization remains unknown.

However, it is obvious that the contribution of respiratory muscles differs between differnt patterns, such as between NB and IC. Thus, based on the respiratory volumes and capacities involved in each breathing pattern one can expect a different exhalation output for different efforts. While it seems that the microbiome of the upper and lower respiratory tract is similar in terms of taxonomic identities, they are different in terms of taxon quantity (Charlson *et al*., 2011). Therefore, different breathing efforts were previously found to result in different quantities of tested phyla (3.3.6). For this Chapter, however, such conclusion is limited by two main factors. Firstly, the unique readings were occurred at low abundances due to the high background signals, while possibly a more manageable sampling surface would have shown more prominent uniqueness of some OTUs for certain breathing patterns. Secondly, the number of replicates were small and possibly a larger sample size would have shown a less inter-personal variation.

As noted in the previous section, some differences can be attributed to the length or occasion of sampling. For example, despite Tenericutes, Verrucomicrobia, Nitrospirae and Chloroflexi were not detected in NB over 60 min they were done so in 15 and 30 min (Figure 5.3A). Furthermore, OD1 (Parcubacteria) which was exclusively detected with 30 min of NB (Figure 5.3C) was also detected in RL over 30 min, suggesting its uniqueness for this period rather for the activity. Even though, these phyla remain among the background profile and lack prevalence and dominance criteria, despite of their membership in oral and respiratory microbiome. Nonetheless, it must be re-emphasised that OTUs recorded at the lower end of reads can be misleading, particularly for small libraries (Janssen, 2006).

The shown inter-subject variation is consistent with the results of Meadow *et al*. (2015) who demonstrated that occupants differ in their "*personal microbial cloud*" showing even a personal identifiability. This might be related to inter-subject variation in respiration, hygiene and other personal characteristics

including a personal microbiome. Therefore, a personalised rather a "procrustes" approach should be applied when investigating exhaled microbiome in health as well as in disease.

While RL was controlled at the rate and amplitude levels, it was not monitored at the sound articulation level. It is currently unknown whether the microbiological profile of particular articulations is distinct from the raw oral microbiome. Indeed, one could ask whether the exhaled microbiome varies between native speakers of different languages. On the other hand, contradictory views whether the URT and LRT microbiome are similar (Charlson *et al*., 2011; Bassis *et al*., 2015) or not (Man *et al*., 2017) have been discussed. Interestingly, Glendinning *et al*. (2017b) found that the LRT microbiota of lambs is dissimilar to that of the URT; and this suggests that, alongside the physiological and anatomical differences, human activities play a key role in shaping the LRT microbiome by means of microaspiration.

While it is understood the structural indices were not statistically different from that of other activities, the shown pattern in compositional structure of RL bacterial communities might be discussed further within two factors: the time which was longer for RL and the involved physiological and anatomical components characterising this activity. If the former was the case, one was likely to find comparable indices when other activities were conducted over the same period. This was not the case, however, when RL was compared to NB over 30 min performed by the same subject (Table 5.5, Table 5.6, Table 5.8 and Table 5.10), increasing the likelihood of the latter.

If, with a sufficient sample size and a sampling system with lesser background, the patterns shown here were confirmed and supported statistically, investigation of synergistic role(s) of co-exhaled bacteria in defining "infectiousness" with different respiratory activities might be warranted.

5.4.4. Comparison of the bacteriome signature between the mask and BAL in patients with suspected PJP

Differences in the compositional structure of the bacterial communities were found between paired mask and BAL samples. These, however, were not confirmed statistically, possibly because of the heterogeneous nature of patients' characteristics and the overwhelming background signals for the masks, in addition to the sample size factor. Similarly, β-diversity indices were not found statistically different between the paired samples, despite exploring the data with mining tools have revealed distinguishability.

It was found using AHC, PCA, PCoA and MDS that paired masks and BALs were clearly separated at phylum and to lesser extent at genus level. The revealed significant divergence indicates that the mask microbiome signature was distinguishable from the BAL one. Indeed, this can confirm that the material captured on the mask, which is assigned to be aerosols, is not solely from the LRT. On the other hand, PCA at the genus level separated the paired samples of patients 1 and 7 while it did not for patient 3. This indicates different variabilities of genera between the masks and BALs in the former two patients and lesser variabilities in the latter. Going back to PJP, the former two had yielded *P. jirovecii* positive masks and BALs, while the latter had a negative paired sample.

The contribution of Actinobacteria to the BAL bacteriome was higher than that of Kehrmann *et al*. (2017) who investigated the BAL bacteriome in PJP.

In agreement with the Kehrmann study, abundance of Proteobacteria was lower in BAL in the case with unlikely PJP while this was not replicated in the mask. However, the BAL α-diversity indices in this case was lower than those in the two likely cases, raising the possibility that a bacterial co-factor can mediates or facilitate a successful *P. jirovecii* infection of pulmonary tissue. These indices were higher in the mask of the PJP case who showed high

exhaled fungal burden than that of the case with a low burden, adding some weight to the notion of a permissive or co-operative bacteriome for *P. jirovecii* exhalation. Biologically, it is well documented that many fungal and bacterial members engage in complex interactions that lead to critical behavioural shifts of the microbes ranging from mutualism to antagonism. It is possible that attendance of such bacteria induces a local inflammation paving the way for an improved fungal growth and/or a higher exhalable fungal load.

Tenericutes are part of the oral microbiome in health (Dewhirst *et al*., 2010; Wade, 2013) and this phylum has been detected in BALs of patients with and without PJP (Kehrmann *et al*., 2017). In this study, the mask and the BAL Tenericutes were detected from one of two PJP-likely and one PJP-unlikely patients. This point is in agreement with the Kehrmann's group in that the lung bacteriome is not statistically different between patients with and without PJP while it is highly variable between PJP patients. Indeed, this adds to the Kehrmann study the exhaled component. Interestingly, it was shown earlier that NB for 60 min was not productive of Tenericutes whereas NB for 15 and 30 min, IB (60 min), IC (60 min) and RL (30 min) were. Despite the precise activity(ies) performed during one hr of sampling these patients was not monitored, it seems that Tenericutes signals can be devised to assess productivity in exhalation.

Since the 66 genus signals (Table A.54) were not detected in the blank filters while they were in both masks (gelatine-incorporated alongside processing reagents) and BALs (gelatine-free and reagents-rich), these might be classified true; however, they were in minor groups.

Generally, BALs are not a pure LRT sample due to both technical (BAL procedure) and physiological (microaspiration) factors, however they remain a gold standard. One limitation driven by the retrospective design is that the blank reagents used to obtain the BALs were not available although the blank

extraction reagents were studied. This might have led to over- rather to under-excluding signals for BALs, particularly those in the gelatine profile.

If the background signals were manageable, this study would have pioneered characterising the exhaled bacteriome in PJP and discriminating the exhaled signature from the BAL one. While this limitation was unavoidable, investigating the microbe-microbe interaction and the relationship(s) between exhaled bacteriome and mycobiome in PJP remain to be explored.

5.5. Concluding remarks

- Conclusions in this Chapter are limited by the small sample size and the high background signals of gelatine filters. Therefore, the proposed hypotheses could neither be proved or disproved, and the shown data may or may not be reproducible

- Different processing methods might affect the studied bacteriome and the reagent microbiome should be characterised in individual studies since the effects cannot be eliminated

- The mask bacteriome may change over time of sampling

- The mask bacteriome may change between different respiratory activities

- The mask bacteriome signature showed a potential difference from the BAL biosignature in disease

- Future work should investigate the exhaled microbiome on a background-manageable capturing surface

Chapter Six: General discussion, conclusions and future work

Several hypotheses were raised and empirically tested throughout this project. To sum up, we hypothesized that:

1. The established facemask processing methods were sub-optimal for the detection and quantification of captured Mtb
2. Different respiratory activities vary in their biological output, and a pattern exhaling largest amounts of SP-A and albumin might be productive of aerosols of an LRT origin, improving the efficiency of the face-mask as a non-invasive tool in sampling the LRT
3. The quantity of material collected is time-dependent
4. Different breathing activities yield microbiota reflecting that of the source zone to a greater or lesser extent
5. The developed protocol can detect *P. jirovecii* in HIV-uninfected patients with PJP and provide a further evidence that *P. jirovecii* is an airborne infection
6. Detection of *P. jirovecii* by the face-mask can provide a non-invasive means of approaching the microbiological diagnosis on PJP
7. Exhaled microbiome signature differs over sampling time in healthy volunteers
8. Exhaled microbiome signature differs among various breathing patterns in healthy volunteers
9. Exhaled microbiome signature differs from BAL signature in patients with suspected PJP

We found that:

1. In-vitro, the recovery rate of processing mycobacteria-contaminated gelatine filters was improved from less than 1% to more than 75% of captured mycobacteria, reaching a limit of detection of 163.81 genome copy/reaction, equivalent to 11 CFU per sample, with a limit of

quantification equivalent of 49 CFU per sample (600.67 genome copy/reaction). In-vivo, the developed method detected around 92% of 24 TB patients and 87% of 192 face-masks

2. Gelatine filter solubilization was achieved either with NaOH hydrolysis or collagenase digestion. Choosing a solubilization method, however, depends on the question being asked. For preservation of NaOH-susceptible particles, treatment with collagenase is suggested

3. Mycobacterial DNA extraction from gelatine filters was achieved with the in-house optimised extraction protocol combining bead-beating and chemical lysis. Choosing a proper DNA extraction method, however, depends on the question being asked. The optimised protocol was the most efficient among 15 protocols compared for qPCR detection and quantification of mycobacterial DNA from gelatine filters

4. Mycobacterial DNA detection and quantification from gelatine filters was achieved with TaqMan based assays i.e. Multiplex AmpliSens real-time qPCR assay and in-house 10ul *IS6110* qPCR TaqMan assay. However, the latter was more cost-effective

5. High temperatures posed a detrimental impact on the face-mask yield

6. SP-A was a consistently-detectable protein component of exhaled aerosols

7. Exhalation of particles containing largest amounts of SP-A and albumin was associated with that of salivary α-amylase

8. RL for 15 min was sufficient to sample the LRT should SP-A considered a specific biomarker of LRT. However, selecting a most efficient effort for sampling depends on the question being asked

9. The quantity of captured bacteria did not increase after 15 min of sampling and fell within the range of $10^5 - 10^8$ copies per mask

10. Bacteria belonging to different phyla and harboured in the respiratory tract were not exhaled in fixed proportions. The quantities differed, at least, according to the breathing pattern and the taxon identity

11. Exhaled particles were composed of, at least, two components: biochemical and microbiological, and there was a degree of interaction between them. Increasing the sampling time resulted in higher quantities of the former but not necessarily of the latter

12. The face-mask system can be used as a non-invasive tool to sample the LRT in health

13. The exhaled particles (aerosols and droplets) are a clinical specimen received a relatively-negligible attention and it is possible they are physiologically regulated like other human biological samples

14. Use of gelatine filters compromised the range of characterizable microbes because of significant background DNA contamination and thus conclusions on exhaled bacteriome were significantly limited

15. *P. jirovecii* was exhaled sufficiently to contaminate masks without exerting special efforts

16. *P. jirovecii* was detectable by the mask in HIV-uninfected immunocompromised patients with PJP and colonised or infected with *P. jirovecii*

17. The exhalaled fungal burden fell within the range of $10^4 - 10^5$ copies per mask

18. BDG level was correlated with the quantity of exhaled *P. jirovecii.* Thus, mask collection was suggested with elevated BDG level

19. Neither the clinical nor the radiological features were correlated with the mask results and thus none of them differentiated between *P. jirovecii* exhalers and non-exhalers

20. The shown results supported the need for infection control and protective isolation of patients with respect to PJP

21. The face-mask in its current design was not found capable to replace bronchoscopies in providing a microbiological diagnosis on PJP; however, it showed a great potential as a non-invasive intermediate step adding value to BDG specificity and negative likelihood ratio

22. α-amylase was a component of the material captured on the mask in health and disease and its detection implied productivity rather a merely contamination with URT secretions

23. Conclusions on the PJP diagnostic work were limited by the small sample size and a formal diagnostic study on using the face-mask in PJP remains warranted

24. Different processing methods affected the studied bacteriome and the reagent microbiome is suggested to be characterised in individual studies since the effects cannot be eliminated

25. The mask bacteriome may change over time of sampling

26. The mask bacteriome may change between different respiratory activities

27. The mask bacteriome signature showed a potential difference from the BAL biosignature in disease

A number of aspects of face-mask sampling have been developed to improve the overall performance and serviceability of this tool. We started from the basics suggesting there are technical gaps unaddressed in the previous studies. Such suggestion was judicious because using the mask, a well-known protective measure (Dharmadhikari *et al*., 2012; Nardell and Dharmadhikari, 2010; Fennelly and Nardell, 1998), is expected to sample, or at least to be contaminated with, what is being exhaled into the immediate microenvironment of wearer patients with TB, the archetypal example of airborne infections.

Indeed, the developed protocol has shown an ability to recover and detect 11 CFU and to quantify 49 CFU equivalents of Mtb, provided these are captured. For natural aerosolization of TB, the detectability has been rocketed to unmask infectivity in at least 92% of 24 infected subjects (Williams *et al*., 2018), confirming further that TB is an archetypal example. Technically, the detection rate of the optimised protocol has been comparable to that of RASC without the need to the latter's extensive infrastructure. More importantly, the developed tool has proved its ability to detect natural aerosolization of *P. jirovecii* from HIV-uninfected patients in whom the pulmonary fungal load is usually low.

As almost any other study, there will be, beside the essential limitations, some criticism. The most salient one could be directed toward the molecular-based detection of *P. jirovecii* DNA rather visualising the microorganism or confirming the source viability. HIV-uninfected patients with PJP are known to have low fungal load, rendering PCR more sensitive than microscopic staining in lung tissues and BALs. It is self-evident that the load in exhaled air is diluted further and as a result, visualising the airborne stage in exhaled air may necessitate prolonged sampling which is neither feasible nor ethical in this study cohort, assuming potentially co-exhaled degradative factors has minimal impact. Previous studies which detected *P. jirovecii* DNA in room air samples did so from either HIV-infected patients or AIDS patients (Bartlett *et al.,* 1997, Sing *et al.,* 1999b, Le Gal *et al.,* 2015 and Choukri *et al.,* 2010).

In addition, the applied processing method in this study increases the likelihood that detected DNA was extracted from cells, despite there remains a possibility that DNA was adhered to pelleted cells, suggesting potential further insight from PMA treatment of the extracts. However, beside the limitations of using PMA to discriminate between viable and non-viable cells (Taylor *et al.*, 2014; Seinige *et al.*, 2014; Li *et al.*, 2017), such approach would hinder the detection. We demonstrated how PMA can bias the results (3.3.5,

3.3.6 and 5.3.2). Detection of mRNA could solve the viability issue since is rapidly destroyed after cell death, however the time span between sampling, transporting and processing of samples could undermine its applicability for our case while DNA is stable for longer periods. Thus, detection of *P. jirovecii* DNA under these processing conditions, in HIV-uninfected patients, during one hr of sampling and without exerting special efforts must form a robust and defensible evidence that PJP is an airborne infection. In fact, detectability of *P. jirovecii* by the face-mask is a complicated process involved both technical and biological factors (4.3.5 and 4.3.7).

The developed tool has a great potential to answer critical questions within three dimensions: biological, transmission and diagnostic.

In biological terms and to understand the behaviour of aerosols, the exhaled particles were composed of two components, at least: biochemical and microbiological, and there was a degree of interaction between them. We demonstrated that increasing the sampling time can result in increasing the quantity of the former but not necessarily of the latter.

Although their diversity can increase, the quantity of captured bacteria did not increase after 15 min and fell within the range of $10^5 - 10^8$ copies per mask, while that for captured *P. jirovecii* fell within the range of $10^4 - 10^5$. These quantities are remarkable taking into account current insights that the number of bacteria in the body has the same order as the number of human cells. Furthermore, respiratory microbiota was not exhaled in fixed proportions. The quantities differed, at least, according to the breathing pattern and the identity factor where the mask bacteriome signature may change between different respiratory activities.

SP-A was a main component of respiratory aerosols. Exhaling particles containing largest amounts of SP-A and albumin was associated with that of salivary α-amylase which is a component of the material captured on the mask

in health and disease. While capturing this clearly indicates a contamination with URT secretions, α-amylase detection have implied productive exhalation. This supports Effros *et al*. (2005)'s conclusion that saliva is the source of around 10% of droplets generated from the RTLF.

To complicate matters in terms of infectious burden, the causative agents of both TB (Williams *et al*., 2018) and PJP (this study) are exhalable without exerting deliberate efforts and non-correlatable with the clinical and radiological pictures, rendering them deserving the "silent killers" term with respect to exhalation.

For infection control measures of PJP, providing isolation facilities for immunocompromised patients should be clinically recommended. Since BDG level was correlated with the exhaled fungal burden, this association might be considered in supporting provision of these facilities though this might be challenging in submerged circumstances.

Nevertheless, using the face-masks in those with suspected PJP should be recommendable for two main advantages 1) providing an infection-control measure of a continued public health importance aimed at patient and community level, particularly for susceptible groups, and 2) providing a non-invasive and time-effective utility with a significant potential in approaching the microbiological diagnosis, and as a result, in placing a more efficacious management on a case-by-case basis.

One of the paradoxes in clinical practice is that proactive measures are not considered when preliminary results that question dogma do not provide a conclusive picture. It remains difficult to challenge conventional dogma even when it is not evidence-based.

For example, the view that symptomatic patients are more infectious (Cooley *et al*., 2014) has been supported while some still reject applying isolation precautions merely because the evidence of airborne detection in symptomatic

and asymptomatic cases was molecular-based. Of course, one should question whether those with negative masks collected over one hr are genuinely non-exhalers in the remaining 23 hrs or on other days.

Three major messages must be clear, particularly for infection control authorities. First, the exhalation is taking place asymptomatically and without exerting any particular respiratory effort. Second, the exhalation is taking place independently from radiological features. Third, the clinical suspicion is not sufficient to recognise the fungal burden and the exhalers are being hosted in general wards instead of isolation rooms. If one would address isolation sensibly and realistically, two different aspects need attention: economic and psychological consequences. Utilising the mask might provide an economical tool to inform a discharge decision.

The non-invasive and cost-effective nature of this tool can enhance its applicability for a wider sector in the community and throughout different locations allowing identifying possible contacts with an infectious case.

Combining it with molecular fingerprinting and other established tools might address whether or not retrieving identical strains indicates recent transmission. Furthermore, it can identify regional and environmental conditions that favour transmission.

On the other hand, despite RL for 15 min was found sufficient and the developed system can be used as a non-invasive tool to sample the LRT, the current design cannot replace bronchoscopies for providing microbiological diagnoses in PJP. Moreover, the mask bacteriome signature can be different from the BAL one. Having said that, the mask shows a great potential as an intermediate step before proceeding to invasive investigations which ethics, at least, are debatable if the former was not taken into account.

Several limitations are warranted to be addressed in future studies. The developed aspects are aligned with recovery of mask-captured mycobacteria

rather than the mask capacity to capture, which might be improved in terms of the mask design and other variables mentioned earlier (2.1.1.2). Since reagent microbiome should be characterised as it cannot be eliminated, such design needs to consider molecular decontamination, or background-manageability, of the capturing surface, and the possibility of the mask to be integrated with nasal clips and saliva traps. The current design cannot differentiate in collection between aerosols and droplets since it does not select a size, otherwise size distribution of naturally generated infectious droplet nuclei could be estimated. The mask detectability of more specific biomarkers of the LRT, such as CC16 and CC10, for boosting the confidence in the capacity of IB as well as RL activities in exhaling aerosols from LRT should be then explored. The impact of shorting the sampling time further on understanding the behaviour of aerosols needs also to be investigated. After these are optimised, the diagnostic value of the face-mask in PJP and other airborne infections remains to be formally evaluated with a statistically-powered sample size.

Regarding exhaled microbiome in health and disease and beside the need for characterising the reagent microbiome, there are a number of variables related to different steps in sequencing workflow, each has a potential to introduce bias at the data analysis stage and thus should be highlighted. These include:

Firstly, sample collection which is dependent on the sample type. For collecting aerosols, the generating and the exact sampled site remains unknown thus the collection method can pose a major impact. The mask as a tool can collect natural aerosolization output while other sampling methods such as PEx and EBC collect a deliberate output (3.1.6).

Secondly, the processing method itself can bias the results. For example, different storage conditions can affect microbial community studies. McKain

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et al. (2013) found that samples frozen without glycerol (used as cryoprotectant) showed a significant loss of Bacteroidetes resulting in higher proportions of Firmicutes, compared to cryoprotected samples. This effect was less apparent on archaeal numbers and diversity. Rubin *et al*. (2013) found that both storage time and temperature had substantially affected bacterial community composition and structure. They demonstrated that frozen samples maintained a high α-diversity and showed low β-diversity differences. On the other hand, Rubin's group found three to seven days-stored samples showed both high α- and β-diversity differences. However, Lauber *et al*. (2010) stored soil, faecal and dermal samples at different temperatures and duration times and found that neither the storage temperature nor the storage duration posed significant impact on the phylogenetic structure and community diversity in individual samples or on relative abundances of the tested taxa. In fact, Fouhy *et al*. (2015) showed no significant changes in microbiota at the phylum or family level to take place following rapid freezing of faecal samples prior to DNA extraction while *Faecalibacterium* and *Leuconostoc* genera were significantly affected. Other studies showed no significant effects on microbial community structure or diversity when faecal samples were refrigerated for 24 hrs (Tedjo *et al*., 2015) or 72 hrs (Choo *et al*., 2015) before DNA extraction.

DNA extraction is an essential processing step that can influence both the quantity and quality of DNA. We have shown how different extraction methods had significantly affected the bacterial community structure and the abundances of many OTUs at both phylum and genus levels (5.3.2.1; 5.3.2.2). These finding are in agreement with multiple studies showing the abundances of different bacterial groups to vary based on different DNA extraction methods (Henderson *et al*., 2013; Guo and Zhang, 2013; Desneux and Pourcher, 2014; Kennedy *et al*., 2014; Hart *et al*., 2015; Wagner Mackenzie *et al*., 2015; Gerasimidis *et al*., 2016). Supposing the shown differences in

our study were originated from intracellular DNA, some microbial cells such as Gram-positive bacteria and cells with rigid walls are more resistant to lysis. Moreover, different inhibitors have been shown to negatively impact on DNA extraction efficiency (Schrader *et al*., 2012). Consequently, variations in DNA yield and quality from different microorganisms can lead to variable results in downstream analyses.

Thirdly, in addition to be affected by the choice of method for amplification, several factors can influence amplicon library preparation. These factors include choosing a hypervariable region for *16S* sequencing, PCR reagents, presence of PCR inhibitors and PCR conditions.

Studies of Walker *et al*. (2015) demonstrated that there was no universal *16S* rRNA gene primers in practical terms, where such allegedly-universal primers did not amplify a number of biologically relevant bacteria. Ghyselinck *et al*. (2013) and Yang *et al*. (2016) highlighted the crucial role of choosing a hypervariable region and PCR primers design in phylogenetic resolution. Similarly, Fouhy *et al*. (2016) showed differences in relative abundances and richness of bacterial community based on the primer choice alongside the DNA extraction method and the sequencing platform.

Biases introduced by PCR reagents, presence of PCR inhibitors and PCR conditions were demonstrated by multiple studies (Kanagawa, 2003; Wu *et al*., 2010; Ahn *et al*., 2012; Kennedy *et al*., 2014; Gohl *et al*., 2016). For example, formation of chimeras can occur in later PCR cycles but can be controlled with lowering the number of amplification cycles. Similarly, PCR artifacts can be reduced by using a high-fidelity polymerase. Other variations were reported between sequences generated from different libraries generated from the same material. The library preparation method and sequencing primers have been deemed responsible for this.

In fact, sequencing errors can rise before library preparation at different pre-amplification steps, during library preparation and amplification, and during sequencing. These errors are rarely investigated separately. For Illumina MiSeq, substitution errors occur more frequently than Indels (Insertion/deletion errors) and insertion errors are more frequent than deletions. Furthermore, substitution errors show biases where A and C substitutions occur more frequently than G and T substitutions. These errors increase toward the end or read and are higher in the second read.

Since the flow cell tiles in next generation platforms contain millions of sequence clusters, this creates a potential for flow cycle-specific errors. Substantial variations in error patterns were reported between different runs with the same library. Sequence–motif errors have been reported in GC-rich sequences and around inverted repeats. Another bias is overestimation of base-calling quality and this can be curated with quality trimming of errors at both ends of the read. Sequence-specific errors are strand-specific. A further bias is that the read coverage decreases in both extreme high and low GC content.

Fourthly, other biases can be related to the sequencing platform. Different sequencers were introduced (Roche, Illumina, Life Technologies, Beckman Coulter, Pacific Biosciences and Oxford Nanopore) where sequencing can be influenced by choice of chemistry. The user's choice usually depends on selecting a platform with most accurate longest reads, highest throughput, ability to assay several samples per run and with lowest cost. Illumina MiSeq has shown the lowest error rate between different sequencing platforms, although D'Amore *et al*. (2016) underlined that the choice of a sequencer depends on the research question. Caporaso *et al*. (2012) showed high reproducibility across Illumina HiSeq and MiSeq sequencing lanes.

Fifthly, choosing a pipeline can introduce further biases. Several pipelines are available, including QIIME, MG-RAST, UPARSE and mothur. The user's choice usually depends on the level of bioinformatics experience and on the resources made available by the host institution. Nilakanta *et al*. (2014) compared different packages (QIIME, WATERS, RDPipeline, VAMPS, Genboree, SnoWMan and mothur) and found that QIIME and mothur were superior due to the provided extensive documentations. Other studies found some differences between QIIME, mothur and MG-RAST in their taxonomic classification and diversity results, and the ease of use for each was deemed partially responsible. Furthermore, QIIME output sometimes shows organelle rRNA such as chloroblast (a descent from Cyanobacteria) and mitochondria (a descent from Rickettsiales) and these can be removed and filtered based on their taxonomic assignment during taxonomy classification, a feature planned to be supported by QIIME 2.

Additional aspects that show potentials to drive biased results are those related to quality control, alignment and taxonomic assignment. In fact, several tools have been developed for quality filtering by removing sequences with unexpected length, homopolymers or ambiguous bases, or by removing those non-alignable with a targeted gene region. This is a crucial step usually followed by removing chimeric sequences. After that, sequences are aligned to reference sequences using different databases such as RDP, Greengenes and SILVA. Using each of these databases can result in different quality results leading to different richness and diversity estimates. Some studies argued against using alignments that do not involve *16S* secondary structures since this can affect the diversity by overestimating the number of bacterial OTUs (Schloss, 2010).

Sequencing and/or PCR errors and mislabelling of studied sequences have been deemed responsible for introducing errors within these databases. Furthermore, a reliance on these databases for taxonomic assignment can

bias the results toward bacterial sequences which have been clinically and extensively investigated in humans. Other tools have been developed for improving classification depth by trimming the reference sequences to a certain primer region.

Sixthly, picking methods of OTUs can bias the results as well. Two main methods are available for defining OTUs: open-reference and closed-reference, or *de novo* and reference-based. As with almost any other aspect of sequencing, inconsistent evidences exist. Westcott and Schloss (2015) found that the former provided quality OTU assignments while He *et al*. (2015) did not find this stable. There is a room for others who found the results of both were comparable (Sul *et al*., 2011). Advantages of closed-reference include that this method is built-in with quality filtration and is easily parallelizable, where the OTUs are defined by high-quality trusted references (at least in theory) and the reference database is being improved over time. The main disadvantage, however, is that reads which did not align with the reference dataset are excluded, precluding observation of new OTUs.

Drancourt *et al*. (2000) showed that 97% identity cut-off can represent sequence similarity at species level, and this threshold is widely applied for clustering OTUs. However, some studies showed that as high as 99% similarity of *16S* rRNA gene sequences can still represent functionally distinguishable microorganisms (Patin *et al*., 2013). Therefore, sequence clustering is important for two main reasons, controlling for sequencing errors and controlling for recent evolutionary divergence.

Seventhly, other biases are related to correction for gene copy number. While different bacterial species have different copy numbers of *16S* rRNA gene, it is unusual for *16S* microbiome studies to accurately determine the *16S* copy numbers for the analysed OTUs. This can lead to inaccurate description of the bacterial community and inaccurate presentation of relative abundances of

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identified OTUs. In response to that, a number of tools like Copyrighter of Angly *et al*. (2014) and ribosomal RNA operon copy number database (rrnDB) of Stoddard *et al*. (2015) have been developed using sequence databases and phylogenetic information to correct variations for gene copy numbers. However, it could be argued that for comparing OTUs between samples rather within a sample the impact of such variation is acceptable even without correction when the same methodology is applied consistently. Nonetheless, even with applying correction tools which are based on databases, the taxonomic classification problem still exists.

While the above limitations are worth being highlighted and addressed whenever possible, face-mask sampling by its non-invasive and cost-effective nature opens the door for tremendous questions to which no answer is currently available.

Indeed, the exhaled particles are a measurable clinical sample received relatively negligible attention and it is possible that these particles are physiologically regulated like other human biological samples.

Several studies have provided evidence the respiratory tract in CF harbours arrays of complex microbial communities and that changes in the structure of these communities have an impact on the clinical status and CF progression in the lung (Sibley *et al*., 2011; Lynch and Bruce, 2013; Caverly *et al*., 2015; Aaron, 2016; Huang and LiPuma, 2016; Pittman *et al*., 2017; O'Toole, 2018; Hahn *et al*., 2018). It remains unknown, however, whether the exhaled microbiome in CF is consistently stable over time or whether a change(s) in its signature can indicate new infections or lung disease progression. Such uninvestigated insights may not only alter future clinical management of CF but also provide tremendous relief to CF patients for whom collecting a respiratory sample is nonetheless challenging.

Similarly, while many studies showed that dysbiosis of respiratory microbiome in COPD and asthma have potential implications on the clinical status of disease and its management (George *et al*., 2018; Ghebre *et al*., 2018; Haldar *et al*., 2017; Wang *et al*., 2016), none has investigated such dysbiosis in exhaled microbiome.

Since the upper and lower respiratory tract microbiome are similar in terms of taxonomic identities rather than quantities, future work can investigate quantitative differences of exhaled microbiota and their relation(s) to productive exhalation ability. For example, one may investigate whether a reduction in exhaled quantities of microbiota can indicate a deterioration of functions of respiratory muscles involved in exhalation, or even be implemented for monitoring disease progression or used as a prognostic factor.

Potential uninvestigated associations between exhaled microbiota and other physiological systems not only include those related to pulmonary functions but also those of different systems of the human body. One could ask whether different cognitive instructions e.g. retroback counting, or different neurological stimuli, can result in different exhalation signals or whether that can indicate sampling of exhalations from different stimulated zones of the respiratory tract. Does a reduction in exhaled quantities of microbiota indicate a deterioration in a cardiovascular function and can such change be used to monitor this function? Is there any association with the endocrine system? Could exhaled microbiome in terms of identity and quantity differ between different endocrine diseases? Tremendous questions not only remain unanswered but also even untouched.

In conclusion, an optimal sampling strategy for collecting and processing a mask sample depends on a number of factors:

1)   The purpose from collection and the question being asked. For example, the sample quality in terms of sampling the upper or lower airways does not outweigh a microbial detection for diagnostic purposes

2)   The breathing activity during sampling. RL can yield largest amounts of SP-A and thus is supposed to best sample the LRT. For microbial detection, the taxon identity can define an efficient breathing pattern for collection

3)   Time of sampling. The quantity of sampled exhaled bacteria using the current design does not seem to increase after 15 min in health

4)   Sample solubilization. Hydrolysis with NaOH is sufficient for exhaled Mtb detection and quantification. If protein studies are sought or NaOH impact on microbiome is to be avoided, digestion with collagenase is suggested

5)   DNA extraction method. For microbes with rigid-cell wall, the optimised bead beating is efficient while chemical/enzymatic lysis is suggested for extracting DNA from a microbial community

The work in this thesis has defined the technological feasibility to capture and analyse the exhaled microbiome and considered multiple biological variables for the potential implementation in clinical interventions, showing the exhaled particles are a measurable clinical sample. It also exemplifies a new area currently under-investigated within the context of the expanding and scientifically profound challenges to our understanding of the human microbiome in health and disease.

Appendix

A.1. Development of face-mask samples processing techniques

Table A.1 Common genomic DNA isolation protocols and commercial kits compared to the optimised protocol

No.	Protocol	Main principle
1	In-house DNA extraction by boiling	Thermal lysis
2	In-house DNA extraction by Chelex-100/ Nonidet P40 Substitute	Thermal lysis and chemical treatment
3	In-house DNA extraction by glass-beads	Mechanical lysis and chemical treatment
4	PowerLyzer PowerSoil DNA Isolation (MO-BIO Laboratories, Inc., USA)	Mechanical lysis and chemical treatment (thermal lysis optional)
5	BiOstic Bacteremia DNA Isolation (MO-BIO Laboratories, Inc., USA)	Mechanical and thermal lysis
6	PowerFecal DNA Isolation Kit (MO-BIO Laboratories, Inc., USA)	Mechanical lysis and chemical treatment (thermal lysis included)
7	QIAamp DNAmini kit (QIAGEN, UK)	Thermal and chemical lysis
8	DNeasy plant mini Kit (QIAGEN, UK)	Mechanical lysis, thermal lysis and chemical treatment
9	NucleoSpin Soil Kit (MACHEREY-NAGEL, GmbH & Co. KG, Germany)	Mechanical and chemical lysis Silica
10	NucleoSpin Tissue Kit (MACHEREY-NAGEL, GmbH & Co. KG, Germany)	Thermal lysis and chemical treatment
11	Magnetic Beads Genomic DNA Extraction Kit for Bacteria (Geneaid Biotech Ltd., Taiwan)	Thermal lysis and chemical treatment (magnetic bead separation for purification)
12	RIBO-prep nucleic acid extraction kit (InterLabService, Russia)	Chemical lysis and thermal treatment
13	DNA-sorb-C nucleic acid extraction kit (InterLabService, Russia)	Chemical lysis and thermal treatment
14	prepIT-MAX kit (DNA Genotek Inc., Canada)	Chemical lysis and thermal treatment
15	In-hose optimized DNA extraction protocol	Mechanical lysis and chemical treatment

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Table A.2 The average A260/A280 and A260/A230 ratios of the compared DNA extracts

Extraction protocol No.	A260/A280 (≥1.8)	A260/A230 (<2.0)
1	0.89	1.01
2	1.28	0.62
3	1.52	0.27
4	1.44	0.32
5	1.35	0.29
6	1.69	0.33
7	1.42	0.14
8	1.54	0.12
9	1.70	1.29
10	1.72	1.37
11	1.36	0.11
12	1.55	0.22
13	1.63	0.36
14	1.23	0.44
15	1.41	0.33

The values were estimated spectrophotometrically on Nanodrop 1000 (Thermo Scientific, UK) using 1μL per the manufacturer's instructions and DNA elution buffer as a blank.

Table A.3 Comparison of cost and time factors of the compared extraction protocols

Extraction protocol	Cost* (GBP)	Time (min)
1	0.05 (NR)	15
2	0.15 (NR)	30
3	0.15 (NR)	35
4	3.48	75
5	2.43	95
6	4.82	75
7	2.72	130
8	0.86	155
9	5.64	110
10	3.24	200

11	3.43	85
12	0.78	60
13	1.13	120
14	3.84 (D)	115
15	0.65 (NR)	35

The values were calculated per one preparation of relevant extraction method. Only the cost of reagents was included. The time of preparation of reagent stock was excluded. The time (and cost) of the downstream assay was not included.

Definition of abbreviations: GBP= Great British Pound; prep= Preparation; NR= No commercial kit required; D= Discontinued; *= Accurate at time of tabling the data.

Table A.4 Number of *IS6110* copies in some mycobacterial strains

Strain	Number of *IS6110* copies per genome
Mycobacterium abscessus ATCC 19977	0
Mycobacterium abscessus subsp. bolletii 50594	0
Mycobacterium africanum GM041182	7
Mycobacterium avium 104	0
Mycobacterium avium subsp. paratuberculosis MAP4	0
Mycobacterium avium subsp. paratuberculosis str. k10	0
Mycobacterium bovis BCG str. Korea 1168P	1
Mycobacterium bovis BCG str. Mexico	1
Mycobacterium bovis BCG str. Pasteur 1173P2	1
Mycobacterium bovis BCG str. Tokyo 172	2
Mycobacterium bovis subsp. bovis AF2122/97	1
Mycobacterium canettii CIPT 140010059	4
Mycobacterium canettii CIPT 140060008	2
Mycobacterium canettii CIPT 140070008	8
Mycobacterium canettii CIPT 140070010	0
Mycobacterium canettii CIPT 140070017	0
Mycobacterium chubuense NBB4	0
Mycobacterium gilvum PYR-GCK	0

Mycobacterium indicus pranii MTCC 9506	0
Mycobacterium intracellulare ATCC 13950	0
Mycobacterium intracellulare MOTT-02	0
Mycobacterium intracellulare MOTT-64	0
Mycobacterium kansasii ATCC 12478	0
Mycobacterium leprae Br4923	0
Mycobacterium leprae strain TN	0
Mycobacterium liflandii 128FXT	0
Mycobacterium marinum M	0
Mycobacterium massiliense str. GO 06	0
Mycobacterium neoaurum VKM Ac-1815D	0
Mycobacterium rhodesiae NBB3	0
Mycobacterium smegmatis JS623	0
Mycobacterium smegmatis MKD8	1
Mycobacterium smegmatis str. MC2 155	0
Mycobacterium smegmatis str. MC2 155	0
Mycobacterium sp. JDM601	0
Mycobacterium sp. JLS	0
Mycobacterium sp. KMS	0
Mycobacterium sp. MCS	0
Mycobacterium sp. MOTT36Y	0
Mycobacterium sp. Spyr1	0
Mycobacterium tuberculosis 7199-99	9
Mycobacterium tuberculosis CAS NITR204	12
Mycobacterium tuberculosis CCDC5079	14/21
Mycobacterium tuberculosis CCDC5180	18
Mycobacterium tuberculosis CDC1551	4
Mycobacterium tuberculosis CTRI-2	15
Mycobacterium tuberculosis EAI5	1
Mycobacterium tuberculosis EAI5 NITR206	1
Mycobacterium tuberculosis F11	17
Mycobacterium tuberculosis H37Ra	17
Mycobacterium tuberculosis H37Rv	16
Mycobacterium tuberculosis KZN 1435	14

Mycobacterium tuberculosis KZN 4207	12
Mycobacterium tuberculosis KZN 605	15
Mycobacterium tuberculosis RGTB327	16
Mycobacterium tuberculosis RGTB423	16
Mycobacterium tuberculosis UT205	0
Mycobacterium tuberculosis str. Beijing NITR203	16
Mycobacterium tuberculosis str. Erdman = ATCC 35801 DNA	11
Mycobacterium tuberculosis str. Haarlem	9
Mycobacterium tuberculosis str. Haarlem NITR202	4
Mycobacterium ulcerans Agy99	0
Mycobacterium vanbaalenii PYR-1	0
Mycobacterium yongonense 05-1390	0

In silico PCR amplification was done with *IS6110*-specific primers and probe. Mismatches were not allowed.

A.2. A healthy volunteer study: novel insights into face-mask sampling

Definition of abbreviations

16S	Universal *16S*rDNA
Actin	Actinobacteria
Alb	Albumin
Bact	Bacteroidetes
Beta	Beta-proteobacteria
Firm	Firmicutes
Gamma	Gamma-proteobacteria
Green-highlighted P	p-value >0.05 – <0.10
IB	Instructed breathing
IC	Intermittent coughing
IQR	Interquartile range
Lys	Active lysozyme
NA	No data available
NB	Normal breathing
P	P value (Two-tailed for correlations)
Prot	Protease
r	Spearman's rank correlation coefficient
Red-highlighted P	p-value <0.01
RL	Reading-out loud
SP-A	Surfactant Protein-A
Subj	Subject
Yellow-highlighted P	p-value >0.01 – <0.05
α-A	α-Amylase
15	15 minutes session
30	30 minutes session
60	60 minutes session

Heatmap colour Key

Highest — Lowest

A.2.1. Culture preparation method

1. Brain heart infusion (BHI) broth

BHI broth was prepared by dissolving 37g BHI broth powder in 1L of distilled water. The solution was sterilized by autoclaving.

2. Blood Agar

40g of blood agar base was dissolved in 950mL distilled water. The solution was sterilized by autoclaving then 5% v/v blood was added after the solution cooled down to 40°C. The media was thoroughly mixed and poured into Petri dishes inside the laminar flow cabinet. The plates were stored at 4°C for 4 weeks.

3. Cultivation of *S. pneumoniae*

A glycerol laboratory stock was streaked on to blood agar and incubated in a candle-jar at 37°C until single colonies were visible. The genus was confirmed by haemolytic activity and Gram staining. Single colonies were used to inoculate 10mL BHI broth and the latter was incubated statistically at 37°C overnight.

4. Cultivation of *M. catarrhalis*

The same method used for *S. pneumoniae.*

5. Cultivation of *B. fragilis*

In an anaerobic chamber, a glycerol laboratory stock was streaked on to blood agar where the agar was incubated at 37°C until single colonies were visible. Single colonies were used to inoculate 10mL BHI broth and the latter was statistically incubated in the chamber at 37°C overnight.

A.2.2. Data tables

Table A.5 Amount of exhaled SP-A of different respiratory activities and time periods in pg/mL

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	133.28	145.99	87.39	155.21	121.03	151.02	75.86	137.12	141.95	135.81	250.02
2	138.37	212.87	159.93	140.56	165.00	207.17	121.03	148.93	192.75	165.41	242.92
3	105.46	129.91	287.73	123.12	88.67	149.64	131.61	167.84	177.63	123.99	132.24
4	176.35	147.27	126.94	94.33	144.73	129.49	167.52	158.61	242.92	159.79	160.27
5	109.11	139.66	140.92	112.11	126.95	154.87	164.65	149.80	127.37	158.61	141.97
6	128.21	132.03	161.19	105.46	123.13	92.24	NA	NA	NA	162.47	148.88
7	108.90	149.80	139.66	NA	NA	NA	184.69	171.73	162.17	247.28	217.88
8	NA	NA	NA	126.10	174.44	232.91	156.34	170.27	183.26	175.10	167.04
9	NA	NA	NA	93.79	123.74	175.10	180.17	158.52	156.72	170.05	116.76
10	NA	NA	NA	122.90	135.81	144.73	106.56	152.34	124.40	NA	NA
11	NA	NA	NA	205.33	172.58	202.82	NA	NA	NA	170.88	240.42
12	197.79	159.94	180.14	NA	NA	NA	NA	NA	NA	NA	NA
13	170.05	167.51	116.74	NA	NA	NA	NA	NA	NA	NA	NA
14	127.80	104.01	93.79	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	139.66	162.47	174.74	NA	NA
Median (IQR)	130.75 (171.63-113.78)	146.63 (161.83-133.94)	140.29 (165.93-119.29)	123.01 (144.22-107.12)	131.38 (166.89-123.28)	152.95 (203.90-145.96)	148.00 (167.52-121.03)	158.56 (168.45-150.44)	168.45 (185.64-145.64)	163.94 (171.93-158.90)	163.65 (241.04-143.69)

Definition of abbreviations is listed in page 343

Table A.6 Amount of exhaled albumin of different respiratory activities and time periods in ng/mL

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	<LLD	<LLD	0.73	0.99	2.48	25.38	<LLD	<LLD	0.42	13.39	21.78
2	1.13	7.52	1.98	12.12	9.67	24.82	16.09	7.25	12.50	34.87	132.05
3	0.48	0.30	5.93	0.22	0.32	3.64	40.73	128.95	277.87	25.76	77.37
4	<LLD	0.12	2.84	0.24	<LLD	<LLD	1.51	2.91	6.70	7.88	21.78
5	1.50	<LLD	0.55	2.30	7.84	1.13	34.71	53.49	57.67	4.94	10.25
6	0.91	0.30	1.40	0.83	<LLD	11.08	NA	NA	NA	131.84	115.64
7	2.46	0.04	0.20	NA	NA	NA	4.17	5.25	5.60	58.34	133.50
8	NA	NA	NA	0.26	<LLD	37.80	25.27	19.77	33.34	30.67	46.53
9	NA	NA	NA	6.73	0.96	4.40	12.28	15.42	172.87	6.36	10.47
10	NA	NA	NA	0.34	18.47	0.46	0.99	5.12	23.20	NA	NA
11	NA	NA	NA	<LLD	0.28	0.74	NA	NA	NA	24.77	87.30
12	<LLD	0.02	0.87	NA	NA	NA	NA	NA	NA	NA	NA
13	<LLD	1.94	0.79	NA	NA	NA	NA	NA	NA	NA	NA
14	7.60	88.33	254.72	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	8.02	26.52	56.27	NA	NA
Median (IQR)	0.69 (1.74-0.01)	0.21 (3.33-0.03)	1.14 (3.61-0.75)	0.58 (3.41-0.25)	0.64 (8.30-0.07)	4.02 (24.96-0.84)	10.15 (25.27-1.51)	11.33 (33.26-5.16)	28.27 (86.47-8.15)	25.26 (40.73-9.26)	61.95 (119.74-21.78)

Definition of abbreviations is listed in page 343

Table A.7 Amount of exhaled protease of different respiratory activities and time periods in nU/mL

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	22.99	22.83	46.75	26.46	34.15	37.13	30.44	23.83	<LLD	319.86	29.19
2	43.04	54.45	67.47	32.19	451.34	42.53	26.81	182.93	28.04	<LLD	<LLD
3	39.89	4.93	37.95	25.20	24.10	218.11	205.71	103.55	27.35	48.59	<LLD
4	190.72	<LLD	33.52	44.46	47.20	44.69	298.76	18.27	42.90	13.49	35.81
5	0.12	42.98	33.52	45.02	30.07	29.03	36.11	<LLD	34.11	<LLD	62.60
6	27.92	40.50	33.58	26.79	<LLD	39.11	NA	NA	NA	38.66	31.08
7	156.75	30.62	8.87	NA	NA	NA	<LLD	18.42	39.28	348.94	145.50
8	NA	NA	NA	51.40	47.03	79.81	38.45	<LLD	36.96	22.44	<LLD
9	NA	NA	NA	269.60	48.83	35.53	27.05	282.80	29.77	50.36	3.13
10	NA	NA	NA	22.42	29.73	37.28	14.03	29.34	<LLD	NA	NA
11	NA	NA	NA	32.84	175.97	23.92	NA	NA	NA	<LLD	32.66
12	54.31	41.25	35.56	NA	NA	NA	NA	NA	NA	NA	NA
13	<LLD	27.58	16.91	NA	NA	NA	NA	NA	NA	NA	NA
14	30.87	257.32	35.02	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	<LLD	9.03	40.34	NA	NA
Median (IQR)	35.38 (79.92-24.22)	35.56 (45.85-24.02)	34.30 (40.15-33.52)	32.51 (46.62-26.55)	40.59 (80.61-29.82)	38.20 (53.47-35.93)	28.74 (38.45-14.03)	21.12 (123.40-11.34)	31.94 (39.55-27.52)	30.55 (117.73-3.37)	30.13 (42.51-0.78)

Definition of abbreviations is listed in page 343

Table A.8 Amount of exhaled active α-amylase of different respiratory activities and time periods in mU/mL

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	21.71	12.61	114.77	79.47	390.15	868.39	25.56	13.56	<LLD	754.21	861.16
2	35.90	87.22	23.24	93.25	152.66	159.03	531.54	133.20	146.98	748.36	958.46
3	39.69	31.25	<LLD	13.99	15.49	617.82	859.78	1031.13	1162.88	791.24	1038.88
4	27.46	22.29	18.71	24.36	27.29	49.16	549.96	511.73	670.86	760.07	932.97
5	20.57	32.63	49.50	<LLD	41.41	35.38	827.23	1241.75	1039.92	325.57	625.05
6	31.83	89.63	282.17	24.67	32.63	295.26	NA	NA	NA	1004.96	1067.30
7	138.02	33.95	38.65	NA	NA	NA	289.74	104.78	345.71	744.05	852.20
8	NA	NA	NA	23.45	58.11	57.60	620.92	587.51	718.73	854.44	781.25
9	NA	NA	NA	527.75	238.60	703.23	702.20	734.92	1077.46	744.57	838.25
10	NA	NA	NA	80.85	145.08	16.44	85.67	272.69	572.70	NA	NA
11	NA	NA	NA	23.86	67.59	<LLD	NA	NA	NA	408.40	847.72
12	54.67	48.47	414.60	NA	NA	NA	NA	NA	NA	NA	NA
13	<LLD	47.78	13.92	NA	NA	NA	NA	NA	NA	NA	NA
14	44.59	364.14	664.83	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	611.62	887.51	811.90	NA	NA
Median (IQR)	33.87 (44.59-21.71)	40.87 (87.22-31.25)	44.08 (282.10-18.71)	24.51 (80.85-23.45)	62.85 (152.60-32.63)	108.31 (617.80-35.38)	580.79 (702.20-289.74)	549.62 (887.51-133.20)	694.80 (1039.90-345.71)	751.28 (791.24-744.05)	856.68 (958.46-838.25)

Definition of abbreviations is listed in page 343

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Table A.9 The quantity of exhaled bacterial *16S* of different respiratory activities and time periods in copy/sample

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	2.88E+06	4.00E+05	1.29E+06	<LLD	<LLD	<LLD	<LLD	3.33E+05	1.12E+06	3.60E+05	<LLD
2	8.40E+05	4.75E+05	7.14E+05	6.45E+05	3.52E+05	4.43E+05	<LLD	5.42E+05	<LLD	<LLD	8.68E+05
3	3.32E+05	3.32E+05	3.20E+05	5.61E+05	<LLD	6.35E+05	<LLD	<LLD	3.96E+05	3.92E+05	<LLD
4	<LLD	3.42E+05	6.89E+05	3.02E+05	3.59E+05	<LLD	<LLD	<LLD	4.52E+05	9.96E+05	3.38E+05
5	3.11E+05	<LLD	3.65E+06	5.41E+05	5.02E+05	3.56E+05	<LLD	<LLD	3.41E+05	5.25E+05	<LLD
6	1.09E+06	7.66E+05	6.37E+05	3.55E+05	3.28E+05	<LLD	NA	NA	NA	<LLD	<LLD
7	<LLD	<LLD	3.11E+05	NA	NA	NA	7.72E+05	<LLD	<LLD	<LLD	<LLD
8	NA	NA	NA	<LLD	3.41E+05	3.97E+05	<LLD	<LLD	<LLD	3.74E+05	4.99E+05
9	NA	NA	NA	<LLD	<LLD	3.19E+05	<LLD	<LLD	<LLD	3.50E+05	9.71E+05
10	NA	NA	NA	<LLD	<LLD	3.13E+05	<LLD	<LLD	3.09E+05	NA	NA
11	NA	NA	NA	3.29E+05	<LLD	1.40E+07	NA	NA	NA	<LLD	<LLD
12	5.59E+05	2.32E+06	4.99E+05	NA	NA	NA	NA	NA	NA	NA	NA
13	<LLD	5.01E+05	8.82E+05	NA	NA	NA	NA	NA	NA	NA	NA
14	<LLD	3.05E+05	4.28E+05	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	<LLD	<LLD	3.79E+05	NA	NA
Media n (IQR)	3.22E+05 (9.02E+05 - 0.00E+00)	3.71E+05 (5.67E+05 - 3.12E+05)	6.63E+05 (9.83E+05 - 4.46E+05)	3.16E+05 (5.46E+05 - 0.00E+00)	1.64E+05 (3.54E+05 - 0.00E+00)	3.38E+05 (4.91E+05 - 7.82E+04)	0.00E+00 (0.00E+00 - 0.00E+00)	0.00E+00 (8.34E+04 - 0.00E+00)	3.25E+05 (4.10E+05 - 0.00E+00)	3.55E+05 (4.25E+05 - 0.00E+00)	0.00E+00 (5.91E+05 - 0.00E+00)

Definition of abbreviations is listed in page 343

Table A.10 The quantity of exhaled Firmicutes of different respiratory activities and time periods in copy/sample

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	1.02E+07	3.98E+05	2.46E+06	<LLD	<LLD	<LLD	<LLD	<LLD	3.20E+06	1.35E+07	<LLD
2	1.74E+06	4.65E+05	1.36E+06	7.05E+05	<LLD	7.26E+05	<LLD	1.66E+06	<LLD	<LLD	2.80E+06
3	<LLD	<LLD	<LLD	1.10E+06	<LLD	4.69E+05	3.05E+05	<LLD	<LLD	<LLD	<LLD
4	<LLD	<LLD	6.24E+05	<LLD	3.87E+05	<LLD	3.89E+05	<LLD	1.14E+06	2.61E+06	3.71E+05
5	<LLD	<LLD	7.71E+06	<LLD	3.87E+05	<LLD	<LLD	<LLD	<LLD	9.09E+05	<LLD
6	2.08E+06	8.15E+05	1.30E+06	<LLD	<LLD	<LLD	NA	NA	NA	<LLD	<LLD
7	<LLD	<LLD	<LLD	NA	NA	NA	1.65E+06	<LLD	<LLD	<LLD	<LLD
8	NA	NA	NA	<LLD	3.48E+05	<LLD	<LLD	<LLD	<LLD	4.17E+05	<LLD
9	NA	NA	NA	<LLD	<LLD	4.69E+05	<LLD	<LLD	<LLD	3.39E+05	2.58E+06
10	NA	NA	NA	<LLD	<LLD	<LLD	<LLD	<LLD	3.31E+05	NA	NA
11	NA	NA	NA	<LLD	<LLD	2.74E+07	NA	NA	NA	4.16E+05	3.07E+05
12	3.80E+05	3.79E+06	6.70E+05	NA	NA	NA	NA	NA	NA	NA	NA
13	<LLD	4.14E+05	1.12E+06	NA	NA	NA	NA	NA	NA	NA	NA
14	<LLD	<LLD	3.55E+05	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	<LLD	<LLD	<LLD	NA	NA
Median (IQR)	0.00E+00 (1.83E+06 - 0.00E+00)	1.99E+05 (5.53E+05 - 0.00E+00)	8.93E+05 (1.63E+06 - 4.22E+05)	0.00E+00 (1.76E+05 - 0.00E+00)	0.00E+00 (3.57E+05 - 0.00E+00)	0.00E+00 (5.34E+05 - 0.00E+00)	0.00E+00 (3.05E+05 - 0.00E+00)	0.00E+00 (0.00E+00 - 0.00E+00)	0.00E+00 (5.34E+05 - 0.00E+00)	3.77E+05 (1.33E+06 - 0.00E+00)	0.00E+00 (9.22E+05 - 0.00E+00)

Definition of abbreviations is listed in page 343

Table A.11 The quantity of exhaled Actinobacteria of different respiratory activities and time periods in copy/sample

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	<LLD	<LLD	<LLD	5.20E+05	7.82E+05	5.02E+05	4.69E+05	3.13E+05	<LLD	<LLD	<LLD
2	<LLD	<LLD	<LLD	5.43E+05	5.32E+05	4.90E+05	9.48E+05	2.70E+05	4.62E+05	<LLD	8.71E+05
3	4.77E+05	3.29E+05	<LLD	<LLD	<LLD	4.03E+05	3.13E+05	3.90E+05	8.74E+05	<LLD	<LLD
4	2.34E+05	<LLD	<LLD	4.00E+05	<LLD	<LLD	<LLD	<LLD	3.32E+05	2.56E+05	6.96E+05
5	<LLD	<LLD	<LLD	8.49E+05	5.17E+05	<LLD	<LLD	<LLD	<LLD	3.83E+05	<LLD
6	<LLD	<LLD	<LLD	3.03E+05	<LLD	<LLD	NA	NA	NA	3.50E+05	3.54E+05
7	<LLD	<LLD	<LLD	NA	NA	NA	<LLD	<LLD	2.12E+05	4.96E+05	3.91E+05
8	NA	NA	NA	4.01E+05	<LLD	5.21E+05	<LLD	<LLD	<LLD	5.63E+05	<LLD
9	NA	NA	NA	<LLD	<LLD	<LLD	2.03E+05	<LLD	3.04E+05	<LLD	<LLD
10	NA	NA	NA	<LLD	<LLD	2.90E+05	4.90E+05	4.48E+05	5.44E+05	NA	NA
11	NA	NA	NA	2.08E+05	7.67E+05	4.77E+05	NA	NA	NA	<LLD	<LLD
12	<LLD	<LLD	2.00E+05	NA	NA	NA	NA	NA	NA	NA	NA
13	<LLD	2.77E+05	3.44E+05	NA	NA	NA	NA	NA	NA	NA	NA
14	<LLD	5.92E+05	1.13E+06	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	<LLD	<LLD	<LLD	NA	NA
Median (IQR)	0.00E+00 (5.84E+04 - 0.00E+00)	0.00E+00 (2.90E+05 - 0.00E+00)	0.00E+00 (2.36E+05 - 0.00E+00)	3.52E+05 (5.25E+05 - 5.20E+04)	0.00E+00 (5.91E+05 - 0.00E+00)	3.46E+05 (4.93E+05 - 0.00E+00)	1.02E+05 (4.69E+05 - 0.00E+00)	0.00E+00 (3.32E+05 - 0.00E+00)	2.58E+05 (4.83E+05 - 0.00E+00)	1.28E+05 (4.12E+05 - 0.00E+00)	0.00E+00 (4.67E+05 - 0.00E+00)

Definition of abbreviations is listed in page 343

Table A.12 The quantity of exhaled Bacteroidetes of different respiratory activities and time periods in copy/sample

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	4.25E+05	4.79E+05	5.76E+05	3.18E+05	1.27E+06	9.41E+05	4.03E+05	5.59E+05	3.67E+05	2.06E+06	3.29E+06
2	5.58E+05	7.88E+05	<LLD	4.05E+06	4.26E+06	3.17E+06	5.59E+05	6.15E+05	6.52E+05	1.17E+06	7.43E+06
3	2.79E+05	<LLD	6.37E+05	6.23E+05	5.19E+05	2.40E+05	1.66E+06	4.36E+06	1.48E+07	5.10E+05	5.98E+05
4	2.94E+05	5.99E+05	<LLD	2.83E+05	2.00E+05	2.04E+05	3.78E+05	3.03E+05	1.05E+06	2.90E+06	5.16E+06
5	6.84E+05	5.02E+05	5.63E+05	4.19E+05	2.71E+05	<LLD	6.20E+05	5.08E+05	4.85E+05	1.06E+06	3.49E+05
6	5.51E+05	5.46E+05	4.17E+05	2.38E+05	2.25E+05	<LLD	NA	NA	NA	8.28E+06	5.63E+06
7	7.32E+06	5.41E+06	7.31E+06	NA	NA	NA	9.02E+06	5.56E+06	1.54E+07	1.93E+07	1.70E+07
8	NA	NA	NA	3.10E+05	2.69E+05	2.51E+05	2.13E+06	1.99E+06	2.82E+06	1.40E+07	3.41E+06
9	NA	NA	NA	6.04E+06	6.86E+06	6.06E+06	1.04E+07	6.41E+06	8.36E+06	5.63E+06	2.74E+06
10	NA	NA	NA	2.51E+05	3.11E+05	2.36E+05	3.35E+05	3.24E+05	4.19E+05	NA	NA
11	NA	NA	NA	1.74E+06	2.42E+06	2.89E+06	NA	NA	NA	1.75E+06	2.94E+06
12	5.31E+05	6.48E+05	3.31E+05	NA	NA	NA	NA	NA	NA	NA	NA
13	3.60E+06	5.41E+06	4.17E+06	NA	NA	NA	NA	NA	NA	NA	NA
14	4.69E+06	1.03E+07	1.15E+07	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	2.92E+06	2.54E+06	3.10E+06	NA	NA
Median (IQR)	5.55E+05 (3.87E+06 - 4.52E+05)	6.23E+05 (5.41E+06 - 5.13E+05)	5.69E+05 (4.96E+06 - 3.52E+05)	3.69E+05 (2.31E+06 - 2.90E+05)	4.15E+05 (2.88E+06 - 2.70E+05)	2.46E+05 (2.96E+06 - 2.12E+05)	1.14E+06 (2.92E+06 - 4.03E+05)	1.30E+06 (4.66E+06 - 5.21E+05)	1.93E+06 (9.97E+06 - 5.27E+05)	2.48E+06 (9.72E+06 - 1.32E+06)	3.35E+06 (6.08E+06 - 2.79E+06)

Definition of abbreviations is listed in page 343

Table A.13 The quantity of exhaled β-proteobacteria of different respiratory activities and time periods in copy/sample

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	1.17E+06	1.61E+06	1.72E+06	4.05E+06	2.19E+06	3.90E+06	1.86E+07	1.61E+06	1.70E+06	1.85E+06	1.17E+06
2	6.66E+05	6.35E+05	5.26E+05	3.99E+06	3.86E+06	3.59E+06	9.90E+07	1.43E+06	6.73E+07	6.87E+05	7.53E+06
3	3.38E+06	3.04E+06	4.03E+06	1.17E+06	5.84E+05	3.00E+06	3.66E+06	4.19E+06	1.47E+07	1.15E+06	6.33E+05
4	1.86E+06	1.62E+06	1.09E+06	1.19E+07	1.31E+06	1.32E+06	6.36E+05	7.96E+04	2.16E+06	1.49E+06	1.90E+06
5	1.52E+06	2.00E+05	2.38E+05	8.29E+06	1.90E+06	2.16E+05	4.23E+05	1.66E+05	1.49E+05	1.85E+06	1.21E+05
6	1.55E+06	1.17E+06	1.04E+06	2.49E+06	3.70E+05	1.63E+05	NA	NA	NA	1.26E+06	1.01E+06
7	1.51E+06	1.19E+06	1.82E+06	NA	NA	NA	7.50E+05	2.69E+05	1.70E+06	1.14E+07	1.36E+07
8	NA	NA	NA	3.23E+06	1.13E+06	3.49E+06	1.34E+05	9.22E+04	1.51E+05	2.40E+06	6.29E+05
9	NA	NA	NA	8.32E+05	8.77E+05	5.11E+05	1.32E+06	4.26E+05	1.84E+06	7.19E+05	2.07E+05
10	NA	NA	NA	1.53E+06	3.35E+06	2.07E+06	4.09E+06	3.35E+06	3.74E+06	NA	NA
11	NA	NA	NA	1.98E+06	6.36E+06	3.68E+06	NA	NA	NA	5.89E+05	9.46E+05
12	1.03E+06	5.42E+05	2.12E+06	NA	NA	NA	NA	NA	NA	NA	NA
13	8.76E+05	2.54E+06	1.23E+06	NA	NA	NA	NA	NA	NA	NA	NA
14	2.73E+06	2.12E+06	9.96E+06	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	7.16E+05	4.71E+05	6.01E+05	NA	NA
Median (IQR)	1.52E+06 (2.07E+06 - 1.06E+06)	1.40E+06 (2.23E+06 - 7.68E+05)	1.48E+06 (2.60E+06 - 1.05E+06)	2.86E+06 (5.11E+06 - 1.64E+06)	1.61E+06 (3.48E+06 - 9.41E+05)	2.53E+06 (3.61E+06 - 7.14E+05)	1.03E+06 (4.09E+06 - 6.36E+05)	4.49E+05 (2.05E+06 - 1.92E+05)	1.77E+06 (6.49E+06 - 8.75E+05)	1.38E+06 (1.99E+06 - 8.27E+05)	9.80E+05 (3.31E+06 - 6.30E+05)

Definition of abbreviations is listed in page 343

Table A.14 The quantity of exhaled γ-proteobacteria of different respiratory activities and time periods in copy/sample

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	2.45E+06	3.10E+06	3.15E+06	5.26E+06	1.55E+07	3.84E+06	1.51E+07	5.44E+06	4.16E+06	3.88E+06	1.91E+06
2	1.57E+06	1.17E+06	1.25E+06	4.53E+06	4.87E+06	4.66E+06	1.00E+08	4.17E+06	1.64E+07	3.02E+06	1.37E+07
3	4.04E+06	3.53E+06	4.57E+06	2.40E+06	1.54E+06	3.32E+06	9.51E+06	9.28E+06	2.18E+07	3.09E+06	9.93E+05
4	3.82E+06	5.09E+06	2.32E+06	6.81E+06	8.50E+06	1.08E+06	2.12E+06	3.51E+05	6.05E+06	7.39E+06	4.70E+06
5	2.57E+06	4.77E+05	4.90E+05	4.58E+06	2.61E+06	2.66E+05	1.71E+06	9.77E+05	6.97E+05	2.66E+06	4.57E+05
6	2.81E+06	2.56E+06	2.42E+06	2.64E+06	1.31E+06	1.48E+05	NA	NA	NA	5.33E+06	4.32E+06
7	3.05E+06	2.80E+06	3.88E+06	NA	NA	NA	2.17E+06	8.94E+05	4.12E+06	1.47E+07	1.68E+07
8	NA	NA	NA	4.04E+06	1.48E+06	4.26E+06	5.22E+05	4.92E+05	7.55E+05	5.28E+06	2.13E+06
9	NA	NA	NA	9.55E+05	8.93E+05	6.85E+05	4.51E+06	1.44E+06	6.29E+06	3.85E+06	1.49E+06
10	NA	NA	NA	2.94E+06	4.47E+06	2.44E+06	5.02E+06	4.26E+06	4.56E+06	NA	NA
11	NA	NA	NA	2.18E+06	6.88E+06	4.34E+06	NA	NA	NA	2.54E+06	4.09E+06
12	2.22E+06	1.18E+06	4.41E+06	NA	NA	NA	NA	NA	NA	NA	NA
13	1.67E+06	5.27E+06	2.77E+06	NA	NA	NA	NA	NA	NA	NA	NA
14	1.13E+07	5.48E+06	9.62E+06	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	2.08E+06	1.16E+06	1.71E+06	NA	NA
Median (IQR)	2.69E+06 (3.88E+06 - 2.28E+06)	2.95E+06 (5.14E+06 - 1.53E+06)	2.96E+06 (4.45E+06 - 2.34E+06)	3.49E+06 (4.75E+06 - 2.46E+06)	3.54E+06 (7.29E+06 - 1.50E+06)	2.88E+06 (4.28E+06 - 7.82E+05)	3.34E+06 (9.51E+06 - 2.08E+06)	1.30E+06 (4.55E+06 - 9.15E+05)	4.36E+06 (8.81E+06 - 2.31E+06)	3.86E+06 (5.84E+06 - 3.03E+06)	3.11E+06 (6.95E+06 - 1.60E+06)

Definition of abbreviations is listed in page 343

Table A.15 Amount of exhaled active lysozyme of different respiratory activities and time periods in U/mL

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	<LLD	<LLD	70.35	75.40	<LLD	62.72	<LLD	<LLD	<LLD	<LLD	64.07
2	63.55	<LLD	<LLD	<LLD	<LLD	62.28	67.74	96.94	63.12	<LLD	101.70
3	<LLD	<LLD	<LLD	79.39	62.59	<LLD	85.52	152.63	262.08	66.06	95.14
4	<LLD	84.82	<LLD	<LLD	96.86	<LLD	<LLD	63.64	84.36	77.22	99.77
5	<LLD	<LLD	71.15	<LLD	<LLD	<LLD	93.36	88.99	118.99	<LLD	<LLD
6	<LLD	69.49	<LLD	<LLD	<LLD	<LLD	NA	NA	NA	124.27	126.94
7	64.29	68.03	<LLD	NA	NA	NA	<LLD	<LLD	<LLD	64.37	79.55
8	NA	NA	NA	<LLD	<LLD	94.87	89.83	81.20	110.32	95.06	98.61
9	NA	NA	NA	<LLD	<LLD	65.61	<LLD	<LLD	118.51	<LLD	69.92
10	NA	NA	NA	<LLD	<LLD	78.71	<LLD	<LLD	70.14	NA	NA
11	NA	NA	NA	<LLD	<LLD	<LLD	NA	NA	NA	<LLD	90.05
12	67.75	<LLD	<LLD	NA	NA	NA	NA	NA	NA	NA	NA
13	74.79	63.81	<LLD	NA	NA	NA	NA	NA	NA	NA	NA
14	<LLD	97.24	144.78	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	62.39	62.90	71.17	NA	NA
Median	0.01	31.90	0.01	0.01	0.01	31.14	31.20	63.27	77.77	32.18	92.59
(IQR)	(65.16-0.01)	(73.32-0.01)	(70.55-0.01)	(18.85-0.01)	(15.65-0.01)	(68.89-0.01)	(85.52-0.01)	(90.98-0.01)	(118.63-64.87)	(81.68-0.01)	(100.25-72.33)

Definition of abbreviations is listed in page 343

Table A.16 The relative abundance of Actinobacteria exhaled in different respiratory activities and time periods (percentage)

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	0.00	0.00	0.00	5.12	3.97	5.47	1.36	3.95	0.00	0.00	0.00
2	0.00	0.00	0.00	4.14	3.94	4.11	0.47	4.16	0.54	0.00	2.95
3	5.83	4.77	0.00	0.00	0.00	5.79	2.07	2.14	1.67	0.00	0.00
4	3.77	0.00	0.00	2.07	0.00	0.00	0.00	0.00	3.46	2.13	5.59
5	0.00	0.00	0.00	6.00	9.76	0.00	0.00	0.00	0.00	6.44	0.00
6	0.00	0.00	0.00	5.35	0.00	0.00	NA	NA	NA	2.30	3.13
7	0.00	0.00	0.00	NA	NA	NA	0.00	0.00	0.99	1.08	0.82
8	NA	NA	NA	5.02	0.00	6.11	0.00	0.00	0.00	2.53	0.00
9	NA	NA	NA	0.00	0.00	0.00	1.23	0.00	1.81	0.00	0.00
10	NA	NA	NA	0.00	0.00	5.75	4.93	5.35	5.88	NA	NA
11	NA	NA	NA	3.41	4.67	4.19	NA	NA	NA	0.00	0.00
12	0.00	0.00	2.84	NA	NA	NA	NA	NA	NA	NA	NA
13	0.00	2.05	4.04	NA	NA	NA	NA	NA	NA	NA	NA
14	0.00	3.19	3.53	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	0.00	0.00	0.00	NA	NA
Median (IQR)	0.00 (0.00-0.00)	0.00 (2.05-0.00)	0.00 (2.84-0.00)	3.77 (5.12-0.00)	0.00 (3.97-0.00)	4.15 (5.75-0.00)	0.24 (1.36-0.00)	0.00 (3.95-0.00)	0.77 (1.81-0.00)	0.54 (2.30-0.00)	0.00 (2.95-0.00)

Definition of abbreviations is listed in page 343

Table A.17 The relative abundance of Bacteroidetes exhaled in different respiratory activities and time periods (percentage)

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	10.52	9.24	10.56	3.13	6.42	10.25	1.16	7.05	5.89	26.42	51.60
2	19.99	30.36	0.00	30.88	31.52	26.62	0.28	9.48	0.77	24.00	25.16
3	3.42	0.00	6.90	14.85	19.62	3.44	10.95	23.92	28.40	10.73	26.90
4	4.73	8.20	0.00	1.46	2.00	7.85	12.08	41.33	10.92	24.07	41.41
5	14.33	42.58	43.60	2.96	5.10	0.00	22.50	30.77	36.45	17.74	37.69
6	11.22	12.77	10.78	4.20	11.81	0.00	NA	NA	NA	54.41	49.74
7	61.60	57.54	56.19	NA	NA	NA	75.56	82.70	71.87	42.05	35.60
8	NA	NA	NA	3.88	9.34	2.95	76.48	77.28	75.67	63.02	55.32
9	NA	NA	NA	77.16	79.49	83.52	63.36	77.50	49.79	55.20	61.71
10	NA	NA	NA	5.32	3.82	4.69	3.37	3.87	4.52	NA	NA
11	NA	NA	NA	28.45	14.75	25.37	NA	NA	NA	35.90	36.86
12	14.04	27.28	4.68	NA	NA	NA	NA	NA	NA	NA	NA
13	58.59	40.08	48.97	NA	NA	NA	NA	NA	NA	NA	NA
14	24.98	55.78	35.62	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	51.12	60.92	57.28	NA	NA
Median	14.19	28.82	10.67	4.76	10.57	6.27	17.29	36.05	32.42	31.16	39.55
(IQR)	(24.98-	(42.58-	(43.60-	(28.45-	(19.62-	(25.37-	(63.36-	(77.28-	(57.28-	(54.41-	(51.60-
	10.52)	9.24)	4.68)	3.13)	5.10)	2.95)	3.37)	9.48)	5.89)	24.00)	35.60)

Definition of abbreviations is listed in page 343

Table A.18 The relative abundance of β-proteobacteria exhaled in different respiratory activities and time periods (percentage)

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	28.91	30.97	31.62	39.92	11.12	42.47	53.87	20.33	27.27	23.77	18.43
2	23.87	24.47	29.61	30.44	28.55	30.14	49.28	22.06	79.38	14.10	25.52
3	41.30	44.01	43.64	27.90	22.08	43.13	24.18	22.99	28.22	24.22	28.46
4	29.90	22.14	31.89	61.28	13.11	50.81	20.30	10.86	22.56	12.38	15.22
5	31.84	16.95	18.42	58.65	35.86	44.80	15.37	10.04	11.18	31.10	13.01
6	31.60	27.31	26.74	43.86	19.44	52.35	NA	NA	NA	8.30	8.96
7	12.73	12.66	14.02	NA	NA	NA	6.28	4.00	7.91	24.83	28.48
8	NA	NA	NA	40.47	39.30	40.96	4.79	3.58	4.06	10.78	10.20
9	NA	NA	NA	10.63	10.16	7.04	8.00	5.15	10.95	7.04	4.67
10	NA	NA	NA	32.34	41.22	41.02	41.17	39.99	40.40	NA	NA
11	NA	NA	NA	32.49	38.71	32.32	NA	NA	NA	12.06	11.85
12	27.20	22.81	30.02	NA	NA	NA	NA	NA	NA	NA	NA
13	14.27	18.84	14.45	NA	NA	NA	NA	NA	NA	NA	NA
14	14.53	11.45	30.96	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	12.54	11.30	11.10	NA	NA
Median	28.05	22.47	29.81	36.20	25.31	41.75	17.84	11.08	16.87	13.24	14.12
(IQR)	(31.60-14.53)	(27.31-16.95)	(31.62-18.42)	(43.86-30.44)	(38.71-13.11)	(44.80-32.32)	(41.17-8.00)	(22.06-5.15)	(28.22-10.95)	(24.22-10.78)	(25.52-10.20)

Definition of abbreviations is listed in page 343

Table A.19 The relative abundance of γ-proteobacteria exhaled in different respiratory activities and time periods (percentage)

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	60.58	59.79	57.82	51.83	78.48	41.81	43.61	68.66	66.84	49.81	29.97
2	56.14	45.17	70.39	34.54	36.00	39.13	49.97	64.29	19.31	61.90	46.37
3	49.45	51.21	49.46	57.26	58.30	47.64	62.80	50.95	41.70	65.05	44.64
4	61.60	69.66	68.11	35.19	84.89	41.33	67.62	47.82	63.06	61.43	37.77
5	53.83	40.47	37.97	32.38	49.28	55.20	62.13	59.19	52.38	44.72	49.30
6	57.18	59.92	62.48	46.58	68.75	47.65	NA	NA	NA	35.00	38.16
7	25.67	29.81	29.79	NA	NA	NA	18.16	13.30	19.23	32.04	35.10
8	NA	NA	NA	50.63	51.36	49.98	18.73	19.13	20.28	23.68	34.48
9	NA	NA	NA	12.21	10.35	9.44	27.40	17.35	37.45	37.75	33.62
10	NA	NA	NA	62.34	54.96	48.54	50.53	50.79	49.20	NA	NA
11	NA	NA	NA	35.66	41.87	38.12	NA	NA	NA	52.04	51.29
12	58.76	49.91	62.46	NA	NA	NA	NA	NA	NA	NA	NA
13	27.14	39.04	32.53	NA	NA	NA	NA	NA	NA	NA	NA
14	60.49	29.58	29.90	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	36.34	27.78	31.62	NA	NA
Median (IQR)	56.66 (60.49-49.45)	47.54 (59.79-39.04)	53.64 (62.48-32.53)	41.12 (51.83-34.54)	53.16 (68.75-41.87)	44.72 (48.54-39.13)	46.79 (62.13-27.40)	49.31 (59.19-19.13)	39.57 (52.38-20.28)	47.26 (61.43-35.00)	37.97 (46.37-34.48)

Definition of abbreviations is listed in page 343

Table A.20 The ratio of the amounts of exhaled albumin to SP-A in pg/mL of different respiratory activities and time periods

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	0.00	0.00	0.84	0.64	2.05	16.80	0.00	0.00	0.30	9.86	8.71
2	0.81	3.53	1.24	8.62	5.86	11.98	13.29	4.87	6.49	21.08	54.36
3	0.45	0.23	2.06	0.18	0.36	2.43	30.95	76.83	156.43	20.77	58.50
4	0.00	0.08	2.23	0.25	0.00	0.00	0.90	1.83	2.76	4.93	13.59
5	1.38	0.00	0.39	2.06	6.18	0.73	21.08	35.71	45.28	3.12	7.22
6	0.71	0.23	0.87	0.79	0.00	12.01	NA	NA	NA	81.15	77.67
7	2.26	0.03	0.14	NA	NA	NA	2.26	3.06	3.45	23.59	61.27
8	NA	NA	NA	0.21	0.00	16.23	16.16	11.61	18.19	17.51	27.86
9	NA	NA	NA	7.17	0.77	2.51	6.82	9.73	110.31	3.74	8.97
10	NA	NA	NA	0.27	13.60	0.31	0.93	3.36	18.65	NA	NA
11	NA	NA	NA	0.00	0.16	0.36	NA	NA	NA	14.49	36.31
12	0.00	0.01	0.48	NA	NA	NA	NA	NA	NA	NA	NA
13	0.00	1.16	0.68	NA	NA	NA	NA	NA	NA	NA	NA
14	5.95	84.92	271.60	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	5.74	16.32	32.20	NA	NA
Median	0.58	0.16	0.86	0.46	0.56	2.47	6.28	7.30	18.42	16.00	32.09
(IQR)	(1.38-	(1.16-	(2.06-	(2.06-	(5.86-	(12.01-	(16.16-	(16.32-	(45.28-	(21.08-	(58.50-
	0.00)	0.01)	0.48)	0.21)	0.00)	0.36)	0.93)	3.06)	3.45)	4.93)	8.97)

Definition of abbreviations is listed in page 343

Table A.21 Arbitrary ratio of the amounts of exhaled α-amylase to SP-A of different respiratory activities and time periods

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	0.16	0.09	1.31	0.51	3.22	5.75	0.34	0.10	<LLD	5.55	3.44
2	0.26	0.41	0.15	0.66	0.93	0.77	4.39	0.89	0.76	4.52	3.95
3	0.38	0.24	<LLD	0.11	0.17	4.13	6.53	6.14	6.55	6.38	7.86
4	0.16	0.15	0.15	0.26	0.19	0.38	3.28	3.23	2.76	4.76	5.82
5	0.19	0.23	0.35	<LLD	0.33	0.23	5.02	8.29	8.16	2.05	4.40
6	0.25	0.68	1.75	0.23	0.27	3.20	NA	NA	NA	6.19	7.17
7	1.27	0.23	0.28	NA	NA	NA	1.57	0.61	2.13	3.01	3.91
8	NA	NA	NA	0.19	0.33	0.25	3.97	3.45	3.92	4.88	4.68
9	NA	NA	NA	5.63	1.93	4.02	3.90	4.64	6.88	4.38	7.18
10	NA	NA	NA	0.66	1.07	0.11	0.80	1.79	4.60	NA	NA
11	NA	NA	NA	0.12	0.39	<LLD	NA	NA	NA	2.39	3.53
12	0.28	0.30	2.30	NA	NA	NA	NA	NA	NA	NA	NA
13	<LLD	0.29	0.12	NA	NA	NA	NA	NA	NA	NA	NA
14	0.35	3.50	7.09	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	4.38	5.46	4.65	NA	NA
Median (IQR)	0.25 (0.33-0.17)	0.26 (0.38-0.23)	0.31 (1.64-0.15)	0.25 (0.62-0.13)	0.36 (1.03-0.28)	0.57 (3.81-0.23)	3.93 (4.39-2.00)	3.34 (5.26-1.12)	4.26 (6.07-2.29)	4.64 (5.38-3.35)	4.54 (6.83-3.92)

Definition of abbreviations is listed in page 343

Table A.22 Arbitrary ratios of the amounts of exhaled lysozyme to SP-A of different respiratory activities and time periods

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	<LLD	<LLD	0.80	0.49	<LLD	0.42	<LLD	<LLD	<LLD	<LLD	0.26
2	0.46	<LLD	<LLD	<LLD	<LLD	0.30	0.56	0.65	0.33	<LLD	0.42
3	<LLD	<LLD	<LLD	0.64	0.71	<LLD	0.65	0.91	1.48	0.53	0.72
4	<LLD	0.58	<LLD	<LLD	0.67	<LLD	<LLD	0.40	0.35	0.48	0.62
5	<LLD	<LLD	0.50	<LLD	<LLD	<LLD	0.57	0.59	0.93	<LLD	<LLD
6	<LLD	0.53	<LLD	<LLD	<LLD	<LLD	NA	NA	NA	0.76	0.85
7	0.59	0.45	<LLD	NA	NA	NA	<LLD	<LLD	<LLD	0.26	0.37
8	NA	NA	NA	<LLD	<LLD	0.41	0.57	0.48	0.60	0.54	0.59
9	NA	NA	NA	<LLD	<LLD	0.37	<LLD	<LLD	0.76	<LLD	0.60
10	NA	NA	NA	<LLD	<LLD	0.54	<LLD	<LLD	0.56	NA	NA
11	NA	NA	NA	<LLD	<LLD	<LLD	NA	NA	NA	<LLD	0.37
12	0.34	<LLD	<LLD	NA	NA	NA	NA	NA	NA	NA	NA
13	0.44	0.38	<LLD	NA	NA	NA	NA	NA	NA	NA	NA
14	<LLD	0.93	1.54	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	0.45	0.39	0.41	NA	NA
Median (IQR)	0.00 (0.42-0.00)	0.19 (0.51-0.00)	0.00 (0.38-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.15 (0.40-0.00)	0.22 (0.57-0.00)	0.39 (0.56-0.00)	0.49 (0.72-0.33)	0.13 (0.52-0.00)	0.50 (0.62-0.37)

Definition of abbreviations is listed in page 343

Table A.23 Arbitrary ratios of the amounts of exhaled protease to SP-A of different respiratory activities and time periods

Subj	NB			IB			IC			RL	
	15	30	60	15	30	60	15	30	60	15	30
1	0.17	0.16	0.53	0.17	0.28	0.25	0.40	0.17	<LLD	2.36	0.12
2	0.31	0.26	0.42	0.23	2.74	0.21	0.22	1.23	0.15	<LLD	<LLD
3	0.38	0.04	0.13	0.20	0.27	1.46	1.56	0.62	0.15	0.39	<LLD
4	1.08	<LLD	0.26	0.47	0.33	0.35	1.78	0.12	0.18	0.08	0.22
5	0.00	0.31	0.24	0.40	0.24	0.19	0.22	<LLD	0.27	<LLD	0.44
6	0.22	0.31	0.21	0.25	<LLD	0.42	NA	NA	NA	0.24	0.21
7	1.44	0.20	0.06	NA	NA	NA	<LLD	0.11	0.24	1.41	0.67
8	NA	NA	NA	0.41	0.27	0.34	0.25	<LLD	0.20	0.13	<LLD
9	NA	NA	NA	2.87	0.39	0.20	0.15	1.78	0.19	0.30	0.03
10	NA	NA	NA	0.18	0.22	0.26	0.13	0.19	<LLD	NA	NA
11	NA	NA	NA	0.16	1.02	0.12	NA	NA	NA	<LLD	0.14
12	0.27	0.26	0.20	NA	NA	NA	NA	NA	NA	NA	NA
13	<LLD	0.16	0.14	NA	NA	NA	NA	NA	NA	NA	NA
14	0.24	2.47	0.37	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	<LLD	0.06	0.23	NA	NA
Median (IQR)	0.26 (0.36-0.18)	0.23 (0.29-0.16)	0.22 (0.35-0.16)	0.24 (0.41-0.19)	0.28 (0.38-0.25)	0.25 (0.34-0.20)	0.22 (0.36-0.14)	0.14 (0.51-0.07)	0.18 (0.22-0.15)	0.18 (0.37-0.02)	0.13 (0.22-0.01)

Definition of abbreviations is listed in page 343

A.2.3. Linear regression results

Note:

D'Agostino and Pearson omnibus K2 was done to test the normality of residuals. The first P value was quoted to describe whether the residuals passed the normality test. Slope was quoted to describe Best-fit values ± SE; R square was quoted to describe Goodness of Fit; The second P value was calculated to determine whether the slope is significantly non-zero.

Table A.24 Linear regression results of exhaled SP-A and albumin over the time.

		Normality of Residuals (P value)	Slope ± SE	R square	P value	Equation
NB	SP-A	No (<0.01)	0.19 ± 0.15	0.62	0.42	Y = 0.19*X + 139
	Albumin	No (<0.01)	0.57 ± 0.00	1.00	<0.01	Y = 0.57*X - 7.16
IB	SP-A	Yes (0.43)	0.81 ± 0.06	0.99	0.04	Y = 0.81*X + 114
	Albumin	No (<0.01)	0.20 ± 0.03	0.98	0.10	Y = 0.20*X - 1.07
IC	SP-A	Yes (0.37)	0.54 ± 0.16	0.92	0.18	Y = 0.54*X + 137
	Albumin	No (<0.01)	1.14 ± 0.12	0.99	0.06	Y = 1.14*X - 4.71

See note in page 364

Table A.25 Linear regression results of exhaled active α-amylase over the time.

	Normality of Residuals (P value)	Slope ± SE	R square	P value	Equation
NB	No (<0.01)	2.70 ± 0.11	1.00	0.03	Y = 2.70*X - 1.09
IB	No (<0.01)	4.42 ± 0.89	0.96	0.13	Y = 4.42*X + 7.50
IC	Yes (0.31)	3.24 ± 0.16	1.00	0.03	Y = 3.24*X + 459

See note in page 364

Table A.26 Linear regression results of exhaled bacteria over the time.

		Normality of Residuals (P value)	Slope ± SE	R square	P value	Equation
NB	Universal *16S*	No (<0.01)	-476.1 ± 341.8	0.66	0.40	Y = -476.1*X + 41292
	Firmicutes	No (<0.01)	-1301 ± 1311	0.50	0.50	Y = -1301*X + 92702
	Actinobacteria	No (<0.01)	-42.58 ± 2.41	1.00	0.04	Y = -42.58*X + 5332
	Bacteroidetes	No (<0.01)	-1785 ± 392.3	0.95	0.14	Y = -1785*X + 146149
	β-proteobacteria	No (<0.01)	-1357 ± 907.9	0.69	0.38	Y = -1357*X + 113171
	γ-proteobacteria	No (<0.01)	-3617 ± 1858	0.79	0.30	Y = -3617*X + 259042
IB	Universal *16S*	No (<0.01)	277.8 ± 372.1	0.36	0.59	Y = 277.8*X + 7611
	Firmicutes	No (<0.01)	904.6 ± 504.9	0.76	0.32	Y = 904.6*X - 10282
	Actinobacteria	Yes (0.11)	-344.2 ± 177	0.79	0.30	Y = -344.2*X + 23588
	Bacteroidetes	No (<0.01)	-1520 ± 390.8	0.94	0.16	Y = -1520*X + 111108
	β-proteobacteria	No (<0.01)	-4483 ± 2826	0.72	0.36	Y = -4483*X + 281074
	γ-proteobacteria	No (<0.01)	-4382 ± 378	0.99	0.05	Y = -4382*X + 301394
IC	Universal *16S*	No (<0.01)	7.001 ± 53.96	0.02	0.92	Y = 7.001*X + 4109
	Firmicutes	No (<0.01)	-138.8 ± 185.1	0.36	0.59	Y = -138.8*X + 14509
	Actinobacteria	No (<0.01)	-222 ± 186.8	0.59	0.45	Y = -222*X + 16249
	Bacteroidetes	No (<0.01)	-2097 ± 1872	0.56	0.46	Y = -2097*X + 188763
	β-proteobacteria	No (<0.01)	-12887 ± 14525	0.44	0.54	Y = -12887*X + 804277
	γ-proteobacteria	No (<0.01)	-15938 ± 14264	0.56	0.46	Y = -15938*X + 943563

See note in page 364

Table A.27 Linear regression results of exhaled active lysozyme over the time.

	Normality of Residuals (P value)	Slope	R square	P value	Equation
NB	Yes (0.10)	-0.02 ± 0.27	0.00	0.96	Y = -0.02*X + 31.9
IB	Yes (0.07)	0.50 ± 0.16	0.90	0.20	Y = 0.50*X + 5.24
IC	No (0.03)	1.12 ± 0.05	1.00	0.03	Y = 1.12*X + 22.26

See note in page 364

A.2.4. Spearman correlation results

Table A.28 Correlation results of exhaled protease vs exhaled SP-A and exhaled albumin

	Prot vs.	NB		IB		IC		RL	
		r	P	r	P	r	P	r	P
15	SP-A	0.15	0.68	-0.33	0.35	0.02	0.95	0.04	0.92
	Alb	0.03	0.94	0.22	0.54	0.34	0.33	0.20	0.58
30	SP-A	-0.01	>0.99	0.64	**0.05**	-0.25	0.49	0.03	0.94
	Alb	0.3	0.39	0.08	0.84	-0.19	0.61	-0.04	0.92
60	SP-A	0.20	0.58	-0.14	0.71	0.52	0.13	NA	
	Alb	0.47	0.18	0.22	0.54	-0.04	0.91	NA	

Definition of abbreviations is listed in page 343

Table A.29 Correlation results of exhaled active protease (nU/mL) vs active α-amylase (mU/mL)

	NB		IB		IC		RL	
	r	P	r	P	r	P	r	P
15	0.70	**0.03**	-0.08	0.84	0.46	0.18	0.23	0.53
30	0.78	**0.01**	0.48	0.17	-0.28	0.43	-0.24	0.51
60	0.13	0.73	0.27	0.45	0.18	0.61	NA	

Definition of abbreviations is listed in page 343

Table A.30 Correlation results of exhaled active α-amylase vs SP-A and albumin exhaled in aerosols

	NB				IB				IC				RL			
	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
	α-A vs SP-A		α-A vs Alb		α-A vs SP-A		α-A vs Alb		α-A vs SP-A		α-A vs Alb		α-A vs SP-A		α-A vs Alb	
15	-0.22	0.54	0.41	0.23	-0.15	0.68	0.55	**0.10**	0.38	0.28	0.88	**<0.01**	-0.20	0.58	0.48	0.17
30	-0.02	0.97	0.66	**0.04**	0.09	0.81	0.55	0.11	0.21	0.56	0.87	**<0.01**	0.09	0.81	0.54	0.12
60	-0.16	0.66	-0.03	0.95	-0.09	0.84	0.62	**0.06**	-0.01	>0.99	0.94	**<0.01**	NA		NA	

Definition of abbreviations is listed in page 343

Table A.31 Correlation results of exhaled lysozyme vs exhaled bacteria in different breathing patterns and time periods

	Lys vs.	NB		IB		IC		RL	
		r	P	r	P	r	P	r	P
15	*16S*	-0.23	0.53	0.10	0.74	-0.31	0.80	0.15	0.67
	Firm	-0.08	0.82	0.50	0.11	-0.28	0.47	-0.21	0.56
	Actin	-0.39	0.46	-0.14	0.71	-0.20	0.59	0.56	**0.10**
	Bacter	0.41	0.24	0.10	0.80	0.12	0.74	0.47	0.17
	Beta	-0.74	**0.02**	-0.14	0.71	-0.39	0.26	0.36	0.32
	Gamma	-0.62	**0.06**	0.03	0.93	-0.37	0.30	0.67	**0.04**
30	*16S*	-0.15	0.68	0.15	0.67	0.10	0.74	0.25	0.48
	Firm	-0.28	0.44	0.27	0.69	0.42	0.20	0.25	0.48
	Actin	0.26	0.49	-0.39	0.46	0.06	0.88	0.62	**0.06**
	Bacter	0.54	0.12	-0.39	0.29	-0.10	0.78	0.59	**0.08**
	Beta	0.36	0.32	-0.32	0.38	-0.01	0.99	0.41	0.25
	Gamma	0.61	**0.07**	0.22	0.56	0.03	0.94	0.58	**0.09**
60	*16S*	0.32	0.37	-0.10	0.80	0.05	0.88	NA	
	Firm	0.32	0.37	-0.16	0.66	-0.41	0.24	NA	
	Actin	0.25	0.53	0.47	0.18	0.14	0.69	NA	
	Bacter	0.40	0.27	0.43	0.21	0.27	0.45	NA	
	Beta	0.10	0.80	0.27	0.46	-0.11	0.77	NA	
	Gamma	0.10	0.80	0.28	0.44	0.07	0.84	NA	

Definition of abbreviations is listed in page 343

Table A.32 Correlation results of exhaled lysozyme vs ratio of exhaled bacteria in different breathing patters and time periods

	Lys vs. ratio of	NB		IB		IC		RL	
		r	P	r	P	r	P	r	P
15	Actin	-0.39	0.46	-0.14	0.71	-0.28	0.44	0.52	0.14
	Bacter	0.62	**0.06**	-0.05	0.89	0.12	0.74	0.30	0.39
	Beta	-0.70	**0.03**	-0.29	0.44	-0.17	0.63	-0.28	0.44
	Gamma	-0.50	0.15	0.53	0.13	0.15	0.69	-0.32	0.37
30	Actin	0.15	0.70	-0.39	0.46	0.03	0.94	0.70	**0.03**
	Bacter	0.24	0.51	-0.23	0.51	-0.17	0.64	-0.21	0.56
	Beta	-0.52	0.13	-0.28	0.47	0.17	0.64	-0.07	0.87
	Gamma	-0.11	0.77	0.54	0.11	0.39	0.27	0.19	0.61
60	Actin	0.14	0.65	0.48	0.16	0.07	0.84	NA	
	Bacter	0.29	0.41	0.24	0.50	0.25	0.48	NA	
	Beta	0.10	0.80	-0.58	**0.09**	-0.13	0.71	NA	
	Gamma	-0.40	0.26	0.10	0.80	0.18	0.61	NA	

Definition of abbreviations is listed in page 343

Table A.33 Correlation results of exhaled lung proteins vs exhaled bacteria (represented by their phyla) in different breathing patterns and time periods

	Phylum	NB				IB				IC				RL			
		r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
		SP-A vs.		Alb vs.		SP-A vs.		Alb vs.		SP-A vs.		Alb vs.		SP-A vs.		Alb vs.	
15	Firm	0.31	0.37	-0.29	0.41	0.24	0.49	0.02	0.98	0.50	0.16	-0.05	0.91	-0.27	0.45	-0.70	**0.03**
	Actin	-0.16	0.67	-0.29	0.43	0.26	0.46	0.44	0.21	-0.73	**0.02**	-0.17	0.63	0.42	0.23	0.25	0.48
	Bacter	-0.21	0.56	0.66	**0.04**	0.21	0.56	0.28	0.43	0.62	**0.06**	0.41	0.25	0.71	**0.03**	0.42	0.23
	Beta	-0.54	0.11	0.28	0.44	0.08	0.84	0.14	0.71	-0.62	**0.06**	-0.32	0.37	0.13	0.73	0.07	0.87
	Gamma	-0.55	**0.10**	0.39	0.27	0.05	0.89	0.18	0.63	-0.56	**0.10**	-0.25	0.49	0.24	0.51	0.39	0.26
30	Firm	0.48	0.16	0.05	0.89	0.34	0.34	-0.25	0.49	-0.41	0.40	-0.06	>0.99	0.06	0.87	0.00	>0.99
	Actin	-0.45	0.19	0.63	**0.05**	0.08	0.82	0.36	0.30	-0.39	0.26	-0.18	0.61	0.29	0.41	0.56	**0.10**
	Bacter	0.35	0.33	0.58	**0.08**	-0.07	0.87	0.53	0.12	0.50	0.14	0.43	0.22	0.53	0.12	0.75	**0.02**
	Beta	-0.30	0.41	0.48	0.17	0.53	0.12	0.53	0.12	-0.30	0.41	0.05	0.89	0.65	**0.05**	0.71	**0.03**
	Gamma	-0.30	0.41	0.47	0.17	0.16	0.66	0.20	0.58	-0.44	0.20	0.07	0.87	0.54	0.11	0.76	**0.01**
60	Firm	-0.17	0.64	-0.38	0.27	0.51	0.14	-0.03	0.95	-0.16	0.67	-0.62	**0.06**	NA			
	Actin	-0.29	0.43	0.26	0.47	0.62	**0.06**	0.61	**0.07**	0.19	0.60	0.18	0.61	NA			
	Bacter	-0.39	0.27	-0.11	0.77	0.68	**0.04**	0.35	0.32	0.36	0.31	0.37	0.30	NA			
	Beta	-0.05	0.89	0.36	0.31	0.52	0.13	0.31	0.39	0.28	0.43	-0.04	0.92	NA			
	Gamma	-0.01	>0.99	0.33	0.35	0.67	**0.04**	0.30	0.41	0.33	0.35	0.13	0.73	NA			

Definition of abbreviations is listed in page 343

Table A.34 Correlation results of exhaled α-amylase vs exhaled bacteria (represented by their phyla) in different breathing patterns and time periods

	Phylum	NB		IB		IC		RL	
		r	P	r	P	r	P	r	P
15	Firm	-0.06	0.87	-0.06	0.87	0.01	>0.99	-0.14	0.69
	Actin	0.03	0.96	-0.23	0.53	-0.37	0.30	0.12	0.74
	Bacter	0.15	0.68	0.18	0.63	0.45	0.19	0.26	0.47
	Beta	0.19	0.61	-0.27	0.45	-0.49	0.15	0.08	0.84
	Gamma	0.36	0.31	-0.15	0.68	-0.41	0.25	0.49	0.15
30	Firm	0.42	0.23	-0.43	0.23	-0.29	0.60	0.05	0.88
	Actin	0.25	0.49	0.52	0.13	-0.27	0.45	0.52	0.13
	Bacter	0.64	**0.05**	0.70	**0.03**	0.14	0.71	0.42	0.23
	Beta	-0.19	0.61	0.50	0.14	-0.12	0.76	0.56	**0.10**
	Gamma	-0.04	0.92	0.19	0.61	-0.07	0.87	0.38	0.28
60	Firm	0.20	0.58	-0.03	0.95	-0.53	0.13	NA	
	Actin	0.35	0.33	0.10	0.78	0.11	0.77	NA	
	Bacter	0.09	0.82	0.30	0.40	0.49	0.15	NA	
	Beta	0.19	0.61	0.04	0.92	-0.16	0.66	NA	
	Gamma	0.25	0.49	-0.12	0.76	0.05	0.89	NA	

Definition of abbreviations is listed in page 343

Table A.35 Correlation results of exhaled protease vs the ratio of albumin to SP-A exhaled in aerosols

	NB		IB		IC		RL	
	r	P	r	P	r	P	r	P
15	0.03	0.94	0.21	0.56	0.26	0.47	0.23	0.53
30	0.30	0.39	0.01	>0.99	-0.15	0.67	-0.09	0.81
60	0.47	0.18	0.15	0.68	-0.13	0.71	NA	

Definition of abbreviations is listed in page 343

Table A.36 Correlation results of exhaled protease vs the ratio of amylase to SP-A exhaled in aerosols

	NB		IB		IC		RL	
	r	P	r	P	r	P	r	P
15	0.50	0.15	-0.03	0.94	0.27	0.44	0.37	0.29
30	0.69	0.03	0.38	0.27	-0.26	0.46	-0.35	0.32
60	0.09	0.80	0.35	0.33	0.09	0.82	NA	

Definition of abbreviations is listed in page 343

Table A.37 Correlation results of exhaled protease vs the ratio of lysozyme to SP-A exhaled in aerosols

	NB		IB		IC		RL	
	r	P	r	P	r	P	r	P
15	0.27	0.45	-0.53	0.13	0.37	0.29	0.20	0.58
30	0.01	>0.99	-0.22	0.56	-0.13	0.72	-0.39	0.26
60	0.16	0.67	-0.01	>0.99	-0.11	0.75	NA	

Definition of abbreviations is listed in page 343

A.3. Face-mask sampling for detection of *Pneumocystis jirovecii* in suspects of *Pneumocystis* pneumonia

A.3.1. Data tables

Table A.38 Review of diagnostic PCR performance

No.	Investigator study	Sample size (overall)	HIV-status	Type of specimen	Type of PCR	Molecular target	Sn %	Sp %	PPV %	NPV %	Gold standard	Cited by*
1	Wakefield *et al.,* 1991	47 pts → 51 epi	+ive	BAL, IS	sPCR	*MtLSU*	95	97	95	97	C, R1	182
2	Schluger *et al.,* 1992	20 pts	+ive	SR	sPCR	*DHFR*	86	100	100	75	C, M1, M3 of IS, BAL	64
3	Lipschik *et al.,* 1992	133 pts	49% +ive	IS (71 sample)	nPCR	*18S* rRNA	100	93	81	100	C, M4, M9, M10 of S, BAL, TBB	191
					sPCR	*MtLSU*	71	94	80	91		
				BAL (113 sample)	nPCR	*18S* rRNA	84	93	70	97		
					sPCR	*MtLSU*	89	94	74	98		
				BL (14 pts)	nPCR	*18S* rRNA	67	91	67	91		
					sPCR	*MtLSU*	0	100	ND	79		
4	Olsson *et al.,* 1993	42 pts	81% +ive	IS	sPCR	*TS*	100	57	70	100	M10	129
		43 pts	-ive	BAL			ND	88	0	100		
5	Ribes *et al.,* 1997	122 pts → 129 smp	≥7%+ive	BAL	sPCR	Mitochondrial *5S*	100	75	62	100	M3, M5	114
6	Tuncer *et al.,* 1998	30 pts	40%+ive	S	sPCR	*5S*	100	77	61	100	M3, M10	25
7	Helweg-Larsen *et al.,* 1998b	76 pts	+ive	BAL	sPCR (touchdown)	*MtLSU*	100	91	90	100	M1, M10 of BAL confirmed by sPCR (*MtSSU*)	136
				OW			89	94	93	91		
		47 pts		BAL	sPCR		96	100	100	100		
				OW			71	100	100	77		
8	Rabodonirina *et al.,* 1999	84 pts	+ive	BL	nPCR	*MtLSU*	21	77	16	83	M1, M3 of BAL	35
9			+ive	BAL	sPCR	*MtLSU*	89	100	100	93		119

№	Study	Sample	Status	Specimen	PCR	Gene					Classification	
	Sing *et al.*, 2000	89 pts → 134 smp			nPCR		100	98	97	100	C, R, L1, R1 (possible PJP) and M1, M3 (proven PJP)	
				IS, ETA	sPCR		0	100	ND	91		
					nPCR		75	100	100	98		
		66 pts → 155 smp	-ive	BAL	sPCR		100	96	60	100		
					nPCR		100	92	43	100		
				IS, ETA	sPCR		0	100	ND	94		
					nPCR		0	81	0	93		
10	Torres *et al.*, 2000	47 pts	+ive	BAL	nPCR	*ITS*	100	86	82	100	C, R1 (probable PJP) and M1, M3 of BAL, PB (proven PJP)	60
11	Oz and Hughes, 2000	31 pts → 45 smp	94%+ive	BAL, IS	sPCR	*MtLSU*	100	100	100	100	M3, M9	34
12	Fischer *et al.*, 2001	175 pts	Unspecified	OW	sPCR (TRF)	*MSG*	91	94	76	98	M3, M9 of BAL, IS	67
						MtLSU	75	96	80	94		
13	Larsen *et al.*, 2002	51 pts → 98 smp	47%+ive	IS, BAL	qPCR (touchdown)	*MSG*	100	67	65	100	M9 of BAL, IS (C, R1 for some)	155
				OW			94	90	85	97		
				IS, BAL	sPCR		95	63	62	95		
				OW			100	81	75	100		
14	Meliani *et al.*, 2003	509 pts → 529 smp	78%+ive	BAL	qPCR	*MtLSU*	100	91	43	100	C, M3, M9	26
15	Flori *et al.*, 2004	150 pts → 173 smp	13% +ive	BAL	sPCR	*MtLSU*	100	87	38	100	C, R, R1	171
					qPCR	*MSG*	100	85	34	100		
16	Larsen *et al.*, 2004	108 pts → 113 epi	+ive	OW	qPCR (touchdown)	*MSG*	88	85	96	61	M7 of IS, BAL	107
17	Linssen *et al.*, 2006	124 pts	25% +ive	BAL	qPCR	*DHPS*	98	91	85	99	M1, M2, M3, M9	52
						MSG-Tamara	100	90	82	100		
						MSG	98	89	80	99		
18		141 pts	78%+ive	BAL, IS	nPCR	*DHPS*	94	81	84	93	M1, M3, M8	101

#	Study	Samples	Positivity	Specimen	PCR type	Target	Sn	Sp	PPV	NPV	Methods	#
	Alvarez-Martinez *et al.,* 2006			(stored)	(touchdown)							
					qPCR		94	96	96	94		
19	Bandt and Monecke, 2007	26 pts → 28 smp + 60 ctrl	11% +ive (unspecified in control group)	BAL	qPCR	*DHFR2*	82	97	92	92	M1, M9	36
						5.8S rRNA	79	100	100	91		
20	Fillaux *et al.,* 2008	400 pts	≥5%+ive (22% in 101 pts sg)	BAL	qPCR	*MSG*	100	90	47	100	M9	57
21	Huggett *et al.,* 2008 (corr. 2009)	132 pts → 136 epi	+ive	BAL	qPCR	*HSP70*	98	92	91	99	C, M3	111
					sPCR	*MtLSU*	98	68	72	98		
22	Fujisawa *et al.,* 2009	86 pts	-ive	IS	sPCR	*MtLSU*	88	81	54	97	C, R, R1	53
					qPCR	*ITS-2 (5.8S* rRNA)	82	99	93	96		
23	Rohner *et al.,* 2009	143 pts → 186 smp	≥13% +ive	BAL	qPCR	Beta-tubulin	100	94	68	100	M4, M5	37
						Kex-1	100	93	64	100		
24	Azoulay *et al.,* 2009	351 pts	-ive	BAL	sPCR	*MtLSU*	84	93	53	98	M10	139
		97 pts		IS			100	90	87	100		
25	Dini *et al.,* 2010	341 pts (equivocal excluded)	unknown	S, ETA, BAL, other (BW, NPA, PF)	qPCR	*MtLSU*	100	83	82	100	M9	37
26	Boondireke *et al.,* 2010	95 smp	unspecified	S, BAL	Multiplex nPCR	*MtLSU*	81	100	100	87	nPCR (Single; *MtLSU*) confirmed by M4, M11	12
27	Gupta *et al.,* 2010	55 pts	4%+ive	BAL	sPCR	*MSG*	100	100	100	100	C	7

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28	Mori *et al.*, 2010b	50 pts	-ive	IS, S	sPCR	*MtLSU*	100	82	10	100	C, R, R1	10
29	Chumpitazi *et al.*, 2011	54 pts → 66 smp	9% +ive	BAL	qPCR (touchdown)	*MSG*	100	98	95	100	C, R, M1, M3, R1	19
30	de Boer *et al.*, 2011	31 pts	-ive	BAL	qPCR	*DHPS*	100	100	100	100	M9, qPCR of BAL	57
31	Alanio *et al.*, 2011	69 pts → 81 smp	+ive	BAL, IS	qPCR	*MtLSU*	100	83	50	100	C, R, M1, M9, M10, R1	106
		169 pts → 197 smp	-ive				100	88	28	100		
32	Chawla *et al.*, 2011	50 pts + 20 ctrl	+ive (71%+ive)	IS, BAL, ETA	sPCR	*MtLSU*	94	94 (96)	88	97 (98)	C, R, R1 (co-trimoxazole)	12
33	Hauser *et al.*, 2011	110 pts	8% +ive	BAL, S, IS, BW, BA, BS, BB	qPCR (cml)	*MtLSU*	93	91	59	99	C, R, M3, M5, M9	76
		83 (Sg)	Unspecified				93 (92)	90 (89)	65 (60)	98 (98)	M9	
34	Mu *et al.*, 2011	60 pts	-ive	S, BAL	sPCR	*MtLSU*	100	100	100	100	M3 (cysts) of BAL	13
35	Oren *et al.*, 2011	214 pts	7%+ive	BAL	nPCR	*MtLSU*	80	83	89	71	C, R, R1	21
			93%-ive		sPCR sPCR	*MSG* *28S*	74	95	83	92		
36	Samuel *et al.*, 2011	202 p. pts → 147 smp	≥65%+ive	NPA	qPCR	*MSG*	86	95	96	85	qPCR of IS, BAL	37
37	Wilson *et al.*, 2011	390 pts	7%+ive (in 27 pts Sg)	BAL, BW, TS, PB, IS and other	qPCR	*cdc2*	100	99	93	100	C, R, A1 (definite, probable and possible PJP); M9, PCR negativity was a PJP-excluder	39

38	Tia *et al.,* 2012	66 pts	+ive	BAL	sPCR	*MtLSU*	96	50	86	80	C, R, R1 (probable PJP) and M1, M9 (definite PJP)	20
					nPCR		98	44	84	87		
					sPCR	*MtLSU* (partial sequence)	100	12	78	100		
					nPCR		100	6	77	100		
		36 pts	-ive		sPCR	*MtLSU*	100	88	77	100		
					nPCR		100	77	62	100		
					sPCR	*MtLSU* (partial sequence)	100	50	43	100		
					nPCR		100	38	38	100		
39	McTaggart *et al.,* 2012	95 smp	Unspecified	BAL	qPCR (cml)	*MtLSU*	100	100	100	100	M9, qPCR (*cdc2*), sPCR (*MtLSU*) with sequencing	34
40	Botterel *et al.,* 2012	287 pts → 353 smp	14%+ive	BAL	qPCR	*MtLSU*	100	81	21	100	M9	70
41	Matsumura *et al.,* 2012	128 pts	4%+ive	BAL, IS	qPCR	*DHPS*	74	73	91	44	M3 (definitive PJP); C, R2 (probable PJP)	49
42	Muhlethaler *et al.,* 2012	242 pts	-ive	BAL	qPCR	*MSG*	100	89	80	100	M9 (definitive PJP)	51
		171 (Sg)					75	91	17	99	C, R (possible PJP)	
43	Orsi *et al.,* 2012	20 pts → 22 smp	10%+ive	BAL (and 1 PB)	qPCR (cml)	*MtLSU*	89	63	67	87	M9	19
44	Seah *et al.,* 2012	278 pts → 411 smp	unspecified	BW, BAL, IS, others	qPCR (cml)	*MtLSU*	93	95	70	99	M5, M9, qPCR (*cdc2*)	7
45	Maillet *et al.,* 2014	35 pts	9% +ive	BAL (31), BA (4)	qPCR	*MSG*	80-100	70-100	100	100	C, R, R1 (probable PJP) and M1,	32

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#	Author	N	Threshold	Sample	Method	Target	Sens	Spec	PPV	NPV	Classification	n
											M3 (definite PJP)	
46	Robert-Gangneux *et al.,* 2014	659 pts (636 analysable Sg)	≥4% +ive (>1% +ive)	BAL	qPCR	*MtLSU*	100	92	69	100	C, R, R1 (possible PJP) and P (retained PJP) or M2, M10 (proven PJP)	40
47	Revathy *et al.,* 2014	75 pts	+ive	IS	qPCR (cml)	*Kex-1*	100	93	83	100	M3, M6	4
48	Gago *et al.,* 2014	40 pts	+ive	BAL and other	Multiplex qPCR	*5.8S (ITS 1&2)*	100	95	95	100	C, M3	23
49	Parian *et al.,* 2015	60 pts	Unknown	BAL	nPCR	*MtLSU*	100	100	100	100	C	3
50	Orsi *et al.,* 2015	41 pts → 44 smp	12%+ive	BAL	qPCR (cml)	*MtLSU*	100	94	80	100	C, R, M9, R1	15
51	Montesinos *et al.,* 2015	120 pts	7%+ive	BAL	qPCR	Beta-tubulin	65	85	63	86	C, R, R1	15
					qPCR (cml)	*MtLSU*	71	83	61	88		
52	Louis *et al.,* 2015	180 pts → 216 smp	+ive	BAL	qPCR	*MtLSU*	100	95	87	100	C, R, M1, M9 or R1 (definitive or probable PJP)	14
		823 pts → 995 smp	-ive				100	88	29	100		
53	Church *et al.,* 2015	87 pts → 92 smp	≥10%+ive	BAL	qPCR	*MtLSU cdc2*	100	91	65	100	M9	7
54	Sasso *et al.,* 2016	148 pts (113 Sg)	27%+ive (unspecified)	BAL, PB	qPCR	*MSG*	89	89	86	91	C, R, R1 (probable and possible PJP) and M2, M3 (proven PJP)	8
					qPCR(cml1)	*MtLSU*	93	80	79	93		
					qPCR(cml2)	*MtLSU*	90	91	89	91		
					qPCR(cml3)	*MtLSU*	75	100	100	83		
				BA, S	qPCR	*MSG*	97	95	97	95		
					qPCR(cml1)	*MtLSU*	97	89	94	94		

					qPCR(cml2)	*MtLSU*	94	95	97	90		
					qPCR(cml3)	*MtLSU*	84	100	100	79		
55	Unnewehr *et al.,* 2016	128 pts	28%+ive	BAL	qPCR	*MtLSU*	100	80	63	100	C, R, A1, M9	3
56	Fauchier *et al.,* 2016	986 pts → 1964 rxn	≥5%+ive	S, IS, BAL	qPCR	*MtLSU*	100	92	32	100	C, L2, R, R1, M3, M4	18
57	Santos *et al.,* 2017	55 pts	-ive	BAL	nPCR	*MtLSU*	100	39	17	100	C	2
58	Moodley *et al.,* 2017	266 pts → 305 smp	+ive	IS	qPCR	*MtLSU*	98	71	80	96	M9	3
59	Montesinos *et al.,* 2017	120 pts	6%+ive	BAL	Multiplex qPCR (cml)	*MtLSU* and two *DHPS* fas g.mut	70	82	60	87	C, R, R1 with A	3
60	Lee *et al.,* 2017b	1,231 pts (169 +ive PCR Sg)	-ive	BAL, BW	nPCR	*MtLSU*	100	93	53	100	C, R, PCR positivity, A2	1
61	Guillaud-Saumur *et al.,* 2017	34 pts	12%+ive	BAL, PB, S	qPCR (cml)	*MtLSU*	96	100	100	86	In-house qPCR (*MtLSU*; Totet *et al.,* 2003)	2
62	Doyle *et al.,* 2017	282 smp	unspecified	BAL, S, IS, TBB	qPCR (touchdown)	*MSG*	100	100	100	100	M4 (cytology), qPCR (*MSG*; Larsen *et al.,* 2002)	1
		874 smp									M4 (histology, qPCR)	

							Sn	Sp	PPV	NPV		
		123 smp									M4 (cytology, histology, qPCR)	
63	Chien *et al.,* 2017	137 pts	8%+ive	S (115) BAL, BW (22)	qPCR (BD MAX)	*MSG*	90	94	76	98	C, R, A2	1
							100	100	100	100		
		11 (Sg)	+ive	S, BAL, BW			78	100	100	50		
		126 (Sg)	-ive				100	94	75	100		
64	Rudramurthy *et al.,* 2018	104 pts + 46 ctrl	21% +ive	BAL, ETA, S (ad); GA (p.)	qPCR	*MSG*	100	100	100	100	C, low Ct (clinically missed PJP), A2 (probable PJP) and M3 (proven PJP)	0

Definitions: Sensitivity (Sn), Specificity (Sp), Positive predictive value (PPV), Negative predictive value (NPV) are calculated per the standard method unless stated otherwise by the relevant study investigators whose familiarity on the local disease prevalence is expected (for PPV and NPV). If the sample size is not representative of the actual prevalence, PPV and NPV should be considered arbitrary values. The diagnostic values of studies with small sample size might be clinically ignorable due to a possible poor statistical power, however they are still mathematically calculable. In case of inconsistence between the number of incidents and the number of patients, both are quoted, otherwise the sample size is the consistent number of patients or incidents. Individuals included as controls are highlighted wherever possible.

Definition of abbreviations: Pts= patients; p.= paediatric; ad= adult; smp= sample; epi= episode; ctrl= control pt.; Sg= subgroup; rxn= qPCR test; →= produce; ND= undefinable; **Type of specimens:** BA= Bronchial aspirates; BAL= Bronchioalveolar lavage; BB= Bronchial biopsy; BL= Blood; BS= Bronchial secretions; BW= Bronchial washing; ETA= Endotracheal aspirate; GA= Gastric aspirate; IS= Induced sputum; NPA= Nasopharyngeal aspirate; OW= Oral wash; PB= Pulmonary biopsy; PF= Pleural fluid; S= Sputum; SR= Serum; TBB= Transbronchial biopsy; TS= Tracheal secretions. **Type of PCR:** nPCR= Nested PCR; qPCR= Quantitative real-time PCR; sPCR= Single-round conventional PCR; TRF= Time-resolved fluorescence; cml= commercial kit; **Molecular target:** cdc2= Cell division cycle 2 (gene encodes cyclin-dependent kinase); *DHFR*= Dihydrofolate reductase; *DHFR*2= Mitochondrial dihydrofolate reductase; *DHPS*= Dihydropteroate synthetase; *DHPS* fas g.mut= Dihydropteroate synthetase fas gene mutations; HSP70= Heat shock protein 70; ITS= Internal transcribed spacer; ITS-2= Internal transcribed spacer region; Kex-1= gene encodes a kexin-like protein homologous to fungal serine endoprotease; *MSG*= Major surface glycoprotein; *MtSSU*= Mitochondrial small subunit rRNA; *MtLSU*= Mitochondrial large subunit rRNA; TS= Thymidylate synthase. **Gold standard:** A= Absence of alternative diagnosis; A1= Anti-pneumocystis therapy initiation/commencement; A2= Receipt of anti-pneumocystis course; C= Clinical findings including risk factors; L1= Elevated serum lactate dehydrogenase; L2= Lymphocyte CD4/CD8 ratio; M1= Microscopic examination (Giemsa stain); M2= Microscopic

examination (May–Grünwald–Giemsa stain); M3= Microscopic examination (Grocott-Gomori's methenamine silver stain); M4= Microscopic examination (Toluidine-blue-O stain); M5= Microscopic examination (Calcofluor white stain); M6= Microscopic examination (Calcoflour stain with KOH); M7= Microscopic examination (Diff-Quik stain); M8= Microscopic examination (Papanicolaou stain); M9= Microscopic examination (Direct immunofluorescence); M10= Microscopic examination (Indirect immunofluorescence); M11= Microscopic examination (unspecified immunofluorescence); p= Post-mortem histological confirmation; R= Radiologic findings; R1= Response to anti-pneumocystis (clinical); R2= Response to anti-pneumocystis (radiological).

(*) Correct at the time of tabling the relevant study.

Table A.39 Quantification results of exhaled *P. jirovecii* presented as *MtLSU* gene copy/mask

Serial	TaqMan	SYBR-Green1	SYBR-Green2
1	3.51E+05	6.60E+04	1.23E+04
2	1.28E+05	1.45E+05	1.11E+05
3	<LLD	8.95E+03	<LLD
4	7.49E+05	2.56E+04	3.75E+03
5	4.34E+04	1.29E+05	7.93E+04
6	1.51E+05	3.05E+04	6.08E+03
7a	1.86E+03	1.02E+05	1.01E+04
7b	<LLD	NA	<LLD
8	<LLD	3.37E+04	<LLD
9	<LLD	3.62E+04	<LLD
10	<LLD	6.70E+03	1.46E+03
11	<LLD	<LLD	<LLD
12	<LLD	<LLD	<LLD
13	<LLD	<LLD	1.05E+04
14	<LLD	<LLD	<LLD
15	<LLD	<LLD	<LLD
16	<LLD	<LLD	<LLD
17	<LLD	6.45E+03	2.63E+03
18	<LLD	8.40E+05	<LLD
19	2.27E+04	4.27E+06	1.13E+03
20	<LLD	1.80E+04	<LLD
21	<LLD	5.15E+04	<LLD
22	<LLD	<LLD	<LLD
23	<LLD	1.27E+04	2.44E+03
24	<LLD	<LLD	<LLD
25	<LLD	<LLD	<LLD
26	<LLD	<LLD	<LLD
27	<LLD	8.55E+04	4.39E+03
28	1.66E+04	3.64E+05	1.66E+05
29	1.28E+07	5.75E+06	NA
30	1.01E+04	NA	NA

31	<LLD	9.40E+04	<LLD
32	<LLD	7.40E+04	1.65E+03
33	<LLD	1.20E+05	2.71E+04
34	<LLD	1.45E+05	1.05E+04
35	<LLD	4.43E+04	<LLD
36	<LLD	3.30E+04	<LLD
37	<LLD	5.65E+05	<LLD
38	<LLD	2.82E+05	6.22E+04
39	<LLD	5.00E+04	<LLD
40	<LLD	3.69E+04	<LLD
41	<LLD	9.25E+05	<LLD
42	<LLD	3.83E+04	<LLD
43	<LLD	3.89E+04	1.50E+03
44	<LLD	3.60E+04	<LLD
45	<LLD	<LLD	1.13E+03
Median	8.57E+04	5.15E+04	6.08E+03
(IQR)	(3.01E+05 - 1.81E+04)	(1.45E+05 - 3.37E+04)	(1.97E+04 - 2.05E+03)

Definition of abbreviation: LLD= Lower limit of detection; Na= No data available

Table A.40 Investigation results of possible PCR inhibition*

Serial	Clinical sample	AC sample	Serial	Clinical sample	AC sample
PC		1.36E+05			
3	<LLD	5.12E+04	35	<LLD	6.54E+04
7b	<LLD	4.75E+04	36	<LLD	4.95E+04
8	<LLD	6.31E+04	37	<LLD	1.31E+04
9	<LLD	6.50E+04	38	<LLD	1.99E+04
10	<LLD	1.29E+04	39	<LLD	6.86E+04
11	<LLD	2.38E+04	40	<LLD	2.36E+04
12	<LLD	2.37E+04	41	<LLD	4.83E+04
31	<LLD	1.03E+04	42	<LLD	3.52E+04
32	<LLD	1.80E+04	43	<LLD	4.28E+04
33	<LLD	2.90E+04	44	<LLD	1.37E+04
34	<LLD	3.40E+04	45	<LLD	4.68E+04

*The results are presented as gene copy/reaction.

Definition of abbreviation: PC= Positive control; AC = Artificially-contaminated; LLD= Lower limit of detection

Table A.41 Spectrophotometer features of DNA extracts

Serial	A260/A280	A260/A230	Serial	A260/A280	A260/A230
1	1.55	0.29	23	1.58	0.15
2	1.49	0.13	24	1.53	0.17
3	1.51	0.16	25	1.42	0.12
4	1.45	0.28	26	1.48	0.30
5	1.36	0.12	27	1.63	0.21
6	1.34	0.13	28	1.51	0.16
7a	1.52	0.18	29	1.53	0.11
7b	1.48	0.10	30	1.44	0.17
8	1.52	0.13	31	1.56	0.22
9	1.49	0.12	32	1.41	0.19
10	1.36	0.27	33	1.53	0.18
11	1.44	0.13	34	1.58	0.19
12	1.35	0.28	35	1.65	0.29
13	1.41	0.18	36	1.55	0.11
14	1.62	0.12	37	0.91	0.19

15	1.55	0.10	38	1.43	0.13
16	1.46	0.14	39	1.48	0.15
17	1.52	0.19	40	1.38	0.18
18	1.67	0.15	41	1.68	0.13
19	1.46	0.27	42	1.57	0.10
20	1.57	0.17	43	1.44	0.17
21	1.67	0.19	44	1.61	0.13
22	1.52	0.15	45	1.52	0.27

Nanodrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, UK) was used as in 1.2.7.3.

The values are presented as average ratio.

Table A.42 Quantification results of exhaled human β-globin presented as gene copy/mask

Serial	*HβG*	Serial	*HβG*
1	8.25E+06	11	9.60E+02
2	5.10E+03	12	7.17E+03
3	5.37E+02	13	7.17E+03
4	1.08E+05	14	<LLD
5	1.19E+04	15	<LLD
6	7.77E+02	16	3.90E+03
7a	<LLD	17	7.56E+05
7b	NA	18	2.01E+03
8	9.21E+03	19	4.74E+03
9	1.23E+04	20	1.25E+03
10	3.96E+05	21	<LLD
Median	7.17E+03		
(IQR)	(1.23E+04 - 2.01E+03)		

Definition of abbreviation: LLD= Lower limit of detection; NA= No data available

Table A.43 Discrepancies between TaqMan and nested PCR results

Serial	TaqMan (copy/mask)	Nested PCR	Serial	TaqMan (copy/mask)	Nested PCR
14	<LLD	-ive	21	<LLD	-ive
15	<LLD	-ive	22	<LLD	-ive
16	<LLD	-ive	23	<LLD	-ive
17	<LLD	-ive	24	<LLD	-ive
18	<LLD	-ive	25	<LLD	+ive
19	2.27E+04	-ive	26	<LLD	+ive
20	<LLD	-ive	27	<LLD	+ive

Definition of abbreviation: LLD= Lower limit of detection

Table A.44 Amount of exhaled active α-amylase in mU/mL

Serial		Serial	
1	250.50	23	56.80
2	109.05	24	9.15
3	34.62	25	18.39
4	238.73	26	<LLD
5	164.60	27	<LLD
6	4.26	28	<LLD
7a	234.42	29	NA
7b	261.00	30	NA
8	70.33	31	50.41
9	NA	32	<LLD
10	207.05	33	1.63
11	<LLD	34	60.75
12	90.56	35	5.75
13	15.46	36	91.63
14	61.99	37	NA
15	<LLD	38	NA
16	232.62	39	NA
17	48.66	40	10.57
18	<LLD	41	32.13
19	4.17	42	229.73
20	<LLD	43	224.27
21	<LLD	44	199.41
22	12.19	45	NA
Median	61.37		
(IQR)	(205.14 - 16.19)		

Definition of abbreviation: LLD= Lower limit of detection; NA= No data available

Table A.45 List of special microorganisms

Microorganism	Value	Notes	Reference
Viral			
Chikungunya virus	L	Pneumonia occurrence has been reported.	Economopoulou *et al.*, 2009
Coxsackieviruses	M	A known etiologic agent of viral pneumonia.	Cesario, 2012
Dengue virus	L	The agent has been isolated from the URT.	Cheng *et al.*, 2017
Enterovirus B (ECHO virus), enterovirus 71 and other enteroviruses	M	A component of the respiratory tract virome. A known etiologic agent of viral pneumonia.	Wylie, 2017; Cesario, 2012
Hantavirus	H	An etiologic agent of viral pneumonia.	Cesario, 2012; Marrie and File, 2018
Hepatitis B & C viruses	H	Opportunistic in immunocompromised pts. A component of the respiratory tract virome.	Wylie, 2017; Goh *et al.*, 2014
Human adenoviruses (B & C)	H	A known etiologic agent of viral pneumonia (CAP). C is component of the respiratory tract virome.	Marrie and File, 2018; Wylie, 2017
Human bocaviruses (Primate bocaparvovirus 1 & 2)	H	A component of the respiratory tract virome.	Wylie, 2017
Human coronaviruses (HKU1, OC43, 229E, NL63)	H	A known etiologic agent of viral pneumonia (CAP). A component of the respiratory tract virome.	Marrie and File, 2018; Wylie, 2017
Human herpesvirus 1 & 2 (herpes simplex virus 1 & 2)	H	Opportunistic in immunocompromised pts. An etiologic agent of viral pneumonia.	Luginbuehl *et al.*, 2017; Mills *et al.*, 2014; Mohan *et al.*, 2006; Short, 2009; Gasparetto *et al.*, 2005; Calore, 2002
Human herpesvirus 3 (varicella-zoster virus)	H	An etiologic agent of viral pneumonia.	Frangides and Pneumatikos, 2004; Marrie and File, 2018
Human herpesvirus 4 (Epstein–Barr virus)	H	An etiologic agent of viral pneumonia. A component of the respiratory tract virome.	Wylie, 2017
Human herpesvirus 5 (human cytomegalovirus)	**H**	Opportunistic in immunocompromised pts. An etiologic agent of viral pneumonia. A component of the respiratory tract virome.	Wylie, 2017
Human herpesvirus 6 (A, B) & 7	H	A component of the respiratory tract virome. HHV7 is an etiologic agent of viral pneumonia.	Wylie, 2017

Human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus)	**H**	Opportunistic in immunocompromised pts. An etiologic agent of viral pneumonia. A component of the respiratory tract virome.	Wylie, 2017
Human T-lymphotropic virus type 1	M	The agent has been associated with interstitial pneumonitis and alveolitis	Mita *et al.*, 1993; Scadden *et al.*, 2018
Human metapneumovirus	H	An etiologic agent of viral pneumonia (CAP). A component of the respiratory tract virome.	Marrie and File, 2018; Wylie, 2017
Human orthopneumovirus	H	A component of the respiratory tract virome.	Wylie, 2017
Human papillomavirus	H	Opportunistic in immunocompromised pts. A component of the respiratory tract virome.	Wylie, 2017
Human parainfluenza viruses (1, 2, 3, 4)	**H**	A known etiologic agent of viral pneumonia (CAP). A component of the respiratory tract virome.	Marrie and File, 2018; Wylie, 2017
Human parvovirus B19	H	The agent was detected in the LRT of pts with different pathological backgrounds.	Costa *et al.*, 2009
Human polyomavirus 1 (BK virus)	M	Opportunistic in immunocompromised pts. An etiologic agent of viral pneumonia. The lungs are a proposed site of infection.	Reploeg *et al.*, 2001
Human polyomavirus 2 (JC virus)	L	Opportunistic in immunocompromised pts. An etiologic agent of viral pneumonia. The agent is acquired via oral or respiratory route.	Beltrami and Gordon, 2014
Human polyomavirus 3 (KI virus)	H	A component of the respiratory tract virome.	Wylie, 2017
Human polyomavirus 4 (WU virus)	H	A component of the respiratory tract virome.	Wylie, 2017
Human respiratory syncytial virus	H	A known etiologic agent of viral pneumonia (CAP). A component of the respiratory tract virome.	Marrie and File, 2018; Wylie, 2017
Human rhinoviruses (A, B, C)	H	A known etiologic agent of viral pneumonia (CAP). A component of the respiratory tract virome.	Marrie and File, 2018; Wylie, 2017
Influenza A, B (and C) viruses	**H**	A known etiologic agent of viral pneumonia (CAP). A component of the respiratory tract virome.	Marrie and File, 2018; Wylie, 2017
Measles virus	H	An etiologic agent of viral pneumonia. A component of the respiratory tract virome.	Wylie, 2017

Middle East respiratory syndrome-related coronavirus (MERS)	H	An etiologic agent of viral pneumonia (CAP).	Marrie and File, 2018
Mimivirus	L	Pneumonia occurrence has been reported.	Cesario, 2012
Parechovirus	H	A component of the respiratory tract virome.	Wylie, 2017
Rotavirus	H	An etiologic agent of viral pneumonia.	
Severe acute respiratory syndrome coronavirus (SARS)		An etiologic agent of viral pneumonia (CAP).	Marrie and File, 2018
Torque teno virus	H	A component of the respiratory tract virome.	Wylie, 2017
Torque teno midi virus	H	A component of the respiratory tract virome.	Wylie, 2017
Torque teno mini virus	H	A component of the respiratory tract virome.	Wylie, 2017
Zika virus	L	The agent has been isolated from the lungs.	Azevedo *et al.*, 2016
Bacterial			
Acinetobacter baumannii, A. calcoaceticus, A. lwoffi and other *spp.*	H	A known etiologic agent of bacterial pneumonia (CAP and HAP). A component of the respiratory tract bacteriome.	Kanafani and Kanj, 2018; Marrie and File, 2018; Klompas, 2018; Dickson *et al.*, 2013
Actinomyces meyeri and other *spp.*	H	An etiologic agent of bacterial pneumonia (non-resolving and ASP). A component of the respiratory tract bacteriome.	Ost *et al.*, 2017; Mabeza and Macfarlane, 2003; Chen *et al.*, 2018
Aggregatibacter (Actinobacillus) actinomycetemcomitans	H	A known component of the respiratory tract bacteriome.	
Bacillus anthracis, B. cereus and other *spp.*	H	An etiologic agent of bacterial pneumonia (CAP).	Shimoyama *et al.*, 2017; Penn and Klotz, 1997; Marrie and File, 2018
Bacteroides fragilis, B. melaninogenicus, B. ureolyticus and other *spp.*	H	A component of the respiratory tract bacteriome. An etiologic agent of bacterial pneumonia (HAP and ASP).	Bartlett, 2018; Marrie and File, 2018; Bartlett *et al.*, 1974
Bartonella (Rochalimaea) henselae, B. quintana	L	Opportunistic in immunocompromised pts. Pulmonary involvement has been reported with bartonellosis.	Caniza *et al.*, 1995
Bifidobacterium animalis, B. breve and *B. longum*	H	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (ASP). A component of the respiratory tract bacteriome.	Esaiassen *et al.*, 2017; Bartlett *et al.*, 1974

Bordetella bronchiseptica, B. pertussis	L	Pneumonia occurrence has been reported.	Monti *et al.*, 2017
Burkholderia cepacia complex, B. pseudomallei and *B. mallei*	H	*B. cepacia* complex is a component of the respiratory tract bacteriome. A known etiologic agent of bacterial pneumonia (melioidosis).	Dickson *et al.*, 2013; Currie and Anstey, 2016
Campylobacter fetus, C. jejuni, C. sputorum	H	Opportunistic in immunocompromised pts. A component of the respiratory tract bacteriome. Pneumonia occurrence has been reported.	Dickson *et al.*, 2013; Sakran *et al.,* 1999
Capnocytophaga canimorsus and other *spp.*	H	A component of the respiratory tract bacteriome. An etiologic agent of bacterial pneumonia.	Chen *et al.*, 2018; Goldberg, 2018
Cardiobacterium hominis and other *spp.*	H	A component of the respiratory tract bacteriome.	Chen *et al.*, 2018
Citrobacter freundii, C. koseri	L	Pneumonia (CAP and ASP) occurrence has been reported.	Ariza-Prota *et al.*, 2015a; Bartlett *et al.*, 1974
Chlamydophila (Chlamydia) pneumoniae, C. psittaci, C. trachomatis	H	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (atypical CAP).	Marrie and File, 2018
Clostridium difficile and other *spp.*	H	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (ASP).	Bayer *et al.,* 1975; Bartlett *et al.,* 1974
Corynebacterium diphtheriae, C. haemolyticum, C. pseudodiphteriticum	L	Pneumonia occurrence has been reported.	Carranza Gonzalez *et al.*, 2006
Coxiella burnetiid	H	An etiologic agent of bacterial pneumonia (CAP).	Raoult, 2018; Marrie and File, 2018
Eikenella corrodens	H	A component of the respiratory tract bacteriome.	
Enterobacter spp.	H	An etiologic agent of bacterial pneumonia (CAP, HAP and ASP)	Marrie and File, 2018; Klompas, 2018; Bartlett *et al.*, 1974
Enterococcus spp.	H	Pneumonia (HAP and ASP) occurrence has been reported.	Berk *et al.*, 1983; Grupper *et al.*, 2009; Savini *et al.*, 2012; Bartlett *et al.*, 1974
Erysipelothrix rhusiopathiae	H	An etiologic agent of bacterial pneumonia	Reboli 2017
Escherichia coli	H	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (CAP, HAP and ASP)	Marrie and File, 2018; Klompas, 2018; Bartlett *et al.*, 1974

Eubacterium spp.	L	An etiologic agent of bacterial pneumonia (ASP).	Bahrani-Mougeot *et al.*, 2007; Bartlett *et al.*, 1974; Lorber and Swenson, 1974
Francisella tularensis	M	An etiologic agent of bacterial pneumonia (pneumonic tularemia, CAP)	Marrie and File, 2018
Fusobacterium nucleatum	H	A component of the respiratory tract bacteriome. An etiologic agent of bacterial pneumonia (CAP and ASP).	Dickson *et al.*, 2013; Marrie and File, 2018; Lorber and Swenson, 1974; Bartlett *et al.*, 1974
Haemophilus influenzae, H. parainfluenzae, H. paraphrophilus	H	Opportunistic in immunocompromised pts. A known etiologic agent of bacterial pneumonia (CAP and ASP). A component of the respiratory tract bacteriome.	Marrie and File, 2018; Bartlett *et al.*, 1974; Dickson *et al.*, 2013; Chen *et al.*, 2018
Helicobacter pylori, H. cineadi and *H. fennelliae*	L	Opportunistic in immunocompromised pts. The agent has been isolated from tracheal secretions	Mitz and Farber, 1993; Roussos *et al.*, 2003 Controversial
Granulicatella adiacens and other *spp.*	H	A component of the respiratory tract bacteriome.	Dewhirst *et al.*, 2010
Klebsiella pneumoniae and other *spp.*	H	A known etiologic agent of bacterial pneumonia (CAP, HAP and ASP)	Marrie and File, 2018; Klompas, 2018; Bartlett *et al.*, 1974
Kingella kingae	H	A component of the respiratory tract bacteriome.	Chen *et al.*, 2018
Lactobacillus spp.	M	An etiologic agent of bacterial pneumonia (ASP).	Bahrani-Mougeot *et al.*, 2007
Lactococcus lactis cremoris	H	Pneumonia occurrence has been reported.	Buchelli-Ramirez *et al.*, 2013
Legionella pneumophila and other *spp. including nonserotype 1*	**H**	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (atypical CAP) Legionellosis	Marrie and File, 2018
Listeria monocytogenes	H	An etiologic agent of bacterial pneumonia	Koufakis *et al.*, 2015; Garcia-Montero *et al.*, 1995; Whitelock-Jones *et al.*, 1989
Megasphaera	H	A component of the respiratory tract bacteriome	Dewhirst *et al.*, 2010
Mesorhizobium	H	A component of the respiratory tract bacteriome	Zakharkina *et al.*, 2013; Segal *et al.*, 2016
Microbacterium	H	A component of the respiratory tract bacteriome	Dewhirst *et al.*, 2010
Micrococcus spp.	H	Pneumonia occurrence has been reported. A component of the respiratory tract bacteriome	Adang *et al.*, 1992; Salar *et al.*, 1997; Wade, 2013; Segal *et al.*, 2016
Moraxella catarrhalis	H	A known etiologic agent of bacterial pneumonia (CAP). A component of the respiratory tract bacteriome.	Marrie and File, 2018
Morganella morganii	H	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (CAP).	Liu *et al.*, 2016; Singla *et al.*, 2010

Mycobacterium avium complex MAC *(M. avium, M. chimaera* and *M. intracellulare), M. fortuitum, M. mucogenicum, M. neoaurum* and other *nontuberculous mycobacteria*	**H**	Opportunistic in immunocompromised pts. The etiologic agent of nontuberculous mycobacteriosis. A component of the respiratory tract bacteriome.	Griffith, 2017; Macovei *et al.*, 2015
Mycobacterium tuberculosis complex MTC *(M. africanum, M. bovis, M. canetti, M. caprae, M. microti, M. mungi, M. orygis, M. pinnipedii, M. suricattae* and *M. tuberculosis)*	**H**	Opportunistic in immunocompromised pts. The etiologic agent of pulmonary tuberculosis.	Marrie and File, 2018
Mycoplasma genitalium, M. hominis, M. pneumoniae, M. orale	**H**	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (atypical CAP) Mycoplasmosis. A component of the respiratory tract bacteriome	Chen *et al.*, 2018; Marrie and File, 2018
Neisseria meningitidis and other *spp.*	H	Opportunistic in immunocompromised pts. A component of the respiratory tract bacteriome. An etiologic agent of bacterial pneumonia (CAP).	Chen *et al.*, 2018; Marrie and File, 2018
Nocardia spp.	H	An etiologic agent of bacterial pneumonia (CAP)	Marrie and File, 2018
Pasteurella multocida and other *spp.*	H	An etiologic agent of bacterial pneumonia. A component of the respiratory tract bacteriome.	Klein and Cunha, 1997; Kofteridis *et al.*, 2009.
Peptostreptococcus spp.	H	A component of the respiratory tract bacteriome. An etiologic agent of bacterial pneumonia (CAP and ASP).	Marrie and File, 2018; Bartlett *et al.*, 1974
Porphyromonas gingivalis and other *spp.*	H	A component of the respiratory tract bacteriome. An etiologic agent of bacterial pneumonia (ASP).	Dickson *et al.*, 2013; Benedyk *et al.*, 2016
Prevotella spp. (Bacteroides melaninogenicus)	H	A component of the respiratory tract bacteriome. An etiologic agent of bacterial pneumonia (CAP).	Dickson *et al.*, 2013; Chen *et al.*, 2018; Dickson *et al.*, 2017; Marrie and File, 2018
Proteus spp.	H	An etiologic agent of bacterial pneumonia (CAP and ASP).	Okimoto *et al.,* 2010; Marrie and File, 2018; Bartlett *et al.*, 1974
Propionibacterium acnes and other *spp.*	H	An etiologic agent of bacterial pneumonia (ASP).	Lorber and Swenson, 1974; Bartlett *et al.*, 1974

Providencia spp.		An etiologic agent of bacterial pneumonia (ASP).	Bartlett *et al.*, 1974
Pseudomonas aeruginosa and other *spp.*	H	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (CAP, HAP and ASP). A component of the respiratory tract bacteriome.	Marrie and File, 2018; Klompas, 2018; Bartlett *et al.*, 1974
Rhizobium radiobacter and other *spp.*	H	Pneumonia occurrence has been reported. A component of the respiratory tract bacteriome.	Lai *et al.,* 2004; Beck *et al.*, 2015
Rhodococcus equi	L	Opportunistic in immunocompromised pts. Pneumonia occurrence has been reported	Kwa *et al.,* 2001
Rickettsia africae, R. conorii, R. rickettsia , R. typhi and other *spp.*	L	Interstitial pneumonia has been reported.	Schulze *et al.,* 2011; Sexton and McClain, 2017
Salmonella enteritidis and other *spp.* (nontyphoid strains)	L	Opportunistic in immunocompromised pts. Pneumonia occurrence has been reported.	Canney *et al.*, 1985; Berkeley and Mangels, 1980; Thompson *et al.*, 2016
Selenomonas sputigena	H	A component of the respiratory tract bacteriome.	Wade, 2013; Segal *et al.*, 2016; Dewhirst *et al.*, 2010; Chen *et al.*, 2018
Serratia spp.	H	An etiologic agent of bacterial pneumonia (CAP and HAP).	Klompas, 2018; Marrie and File, 2018
Shigella sonnei	L	Opportunistic in immunocompromised pts. Pneumonia occurrence has been reported (endemic).	Mancini *et al.,* 2009; Nahid *et al.*, 2017
Sphingomonas	H	A component of the respiratory tract bacteriome	Dewhirst *et al.*, 2010; Segal *et al.*, 2016
Staphylococcus aureus including methicillin-resistant S. aureus and *methicillin-susceptible S. aureus*	H	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (CAP, HAP and ASP). A component of the respiratory tract bacteriome	Marrie and File, 2018; Klompas, 2018; Bartlett *et al.*, 1974
Stenotrophomonas (Xanthomonas) maltophilia	H	An etiologic agent of bacterial pneumonia (HAP). A component of the respiratory tract bacteriome.	Dickson *et al.*, 2013; Klompas, 2018
Stomatococcus spp. (now Rothia mucilaginosa)	H	Pneumonia occurrence has been reported. A component of the respiratory tract bacteriome.	Chen *et al.*, 2018; Gruson *et al.,* 1998; Lambotte *et al.,* 1999; Fanourgiakis *et al.,* 2003; Korsholm *et al.,* 2007; Ramanan *et al.,* 2014
Streptobacillus spp.	H	A component of the respiratory tract bacteriome.	Lau *et al.*, 2016; Chen *et al.*, 2018
Streptococcus pneumoniae, S. pyogenes, S. agalactiae, S. viridans and other *spp.*	**H**	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (CAP, HAP and ASP). A component of the respiratory tract bacteriome.	Chen *et al.*, 2018; Marrie and File, 2018; Klompas, 2018; Bartlett *et al.*, 1974

Streptomyces spp.	L	Pneumonia occurrence has been reported.	Yacoub *et al.,* 2014; Ariza-Prota *et al.,* 2015b
Treponema pallidum and other *spp.*	L	Opportunistic in immunocompromised pts. A component of the respiratory tract bacteriome.	David *et al.,* 2006; Chen *et al.,* 2018; Dewhirst *et al.,* 2010; Wade 2013; Segal *et al.,* 2016
Tropheryma whipplei	H	An etiologic agent of bacterial pneumonia. A component of the respiratory tract bacteriome.	Lagier *et al.,* 2016; Dewhirst *et al.,* 2010
Ureaplasma parvum, U. urealyticum	H	Opportunistic in immunocompromised pts. An etiologic agent of bacterial pneumonia (atypical CAP)	Baum, 2017
Veillonella spp.	H	A component of the respiratory tract bacteriome. An etiologic agent of bacterial pneumonia (HAP and ASP).	Dickson *et al.,* 2013; Chen *et al.,* 2018; Dickson *et al.,* 2017; Beck *et al.,* 2012; Dewhirst *et al.,* 2010; Segal *et al.,* 2016; Wade, 2013; Marik and Careau, 1999; Klompas, 2018; Bartlett *et al.,* 1974
Yersinia enterocolitica; Y. pestis	L	An etiologic agent of bacterial pneumonia (CAP)	Wong *et al.,* 2013; Cleri *et al.,* 1997; Marrie and File, 2018
Archaeal			
Euryarchaeota (phylum)	L	A component of the URT archaeome	Koskinen *et al.,* 2017
Thaumarchaeota (phylum)	L	A component of the URT & LRT archaeome	Koskinen *et al.,* 2017
Woesearchaeota (phylum)	L	A component of the LRT archaeome	Koskinen *et al.,* 2017
Fungal			
Acremonium strictum and other *spp.*	L	Opportunistic in immunocompromised pts. Pneumonia occurrence has been reported.	Niknam *et al.,* 2017; Fakharian *et al.,* 2015
Alternaria alternata and other *spp.*	L	Bronchopulmonary alternariosis (allergic) has been reported	Chowdhary *et al.,* 2012
Aspergillus flavus, A. fumigatus, A. niger, A. terreus and other *spp.*	H	Opportunistic in immunocompromised pts. An etiologic agent of fungal pneumonia. A component of the respiratory tract mycobiome.	Huffnagle and Noverr, 2013; Nguyen *et al.,* 2015; Marrie and File, 2018
Beauveria bassiana	L	Pulmonary involvement has been reported	Gurcan *et al.,* 2006
Blastomyces dermatitidis (asexual), *Ajellomyces dermatitidis* (sexual)	**H**	An etiologic agent of fungal pneumonia (endemic).	Bradsher, 2018; Marrie and File, 2018
Blastoschizomyces capitatus	L	Pneumonia occurrence has been reported	Wills *et al.,* 2004; Gill and Gill, 2011

Bipolaris hawaiisensis, B. maydis and other *spp.*	L	Pneumonia occurrence has been reported	Saenz *et al.*, 2001
Candida albicans and other *spp.*	H	Opportunistic in immunocompromised pts. A component of the respiratory tract mycobiome. Extremely rare to cause fungal pneumonia.	Huffnagle and Noverr, 2013; Nguyen *et al.*, 2015; Marrie and File, 2018
Chaetomium globosum and other *spp.*	L	Pneumonia occurrence has been reported	Yeghen *et al.*, 1996
Cladophialophora bantiana	L	Pneumonia occurrence has been reported	Mansour and Jordan, 2014
Cladosporium cladosporioides and other *spp.*	H	Opportunistic in immunocompromised pts. A component of the respiratory tract mycobiome. Pneumonia occurrence has been reported.	Nguyen *et al.*, 2015; Grava *et al.*, 2016
Coccidioides immitis, C. posadasii	**H**	Opportunistic in immunocompromised pts. An etiologic agent of fungal pneumonia (endemic, CAP).	Kauffman, 2017; Marrie and File, 2018
Cryptococcus gattii, C. laurentii, C. neoformans	**H**	Opportunistic in immunocompromised pts. A component of the respiratory tract mycobiome. An etiologic agent of fungal pneumonia (endemic).	Huffnagle and Noverr, 2013; Shankar *et al.*, 2006; Cox and Perfect, 2017; Marrie and File, 2018
Curvularia lunata and other *spp.*	L	Pneumonia occurrence has been reported	Dharmic *et al.,* 2015
Davidiella tassiana	H	Davidiellaceae is a component of the respiratory tract mycobiome	Nguyen *et al.*, 2015
Emmonsia crescens, E. pasteuriana	H	A component of the respiratory tract mycobiome. The etiologic agent of pulmonary adiaspiromycosis (endemic).	Nguyen *et al.*, 2015
Enterocytozoon bieneusi and other *spp.*	H	Opportunistic in immunocompromised pts. Pneumonitis has been reported with disseminated microsporidiosis. The agent has been isolated from pneumonic sputa.	Leder and Weller, 2018a; Abreu-Acosta *et al.*, 2005
Eremothecium sinecaudum	H	A component of the respiratory tract mycobiome.	Nguyen *et al.*, 2015
Eurotium spp. (Tx. Aspergillus; Hubka et al., 2013)	H	A component of the respiratory tract mycobiome	Nguyen *et al.*, 2015
Exophiala dermatitidis, E. jeanselmei	L	Pneumonia occurrence has been reported	Cohen and Stead, 2015
Exserohilum rostratum and other *spp.*	L	Pulmonary involvement has been reported.	Katragkou *et al.*, 2014

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Fonsecaea (Exophiala) (Wangiella) dermatitidis, F. pedrosoi and other *spp.*	L	Pneumonia occurrence has been reported	Suzuki *et al.,* 2012; Kenney *et al.,* 1992
Fusarium solani, F. oxysporum and *F. moniliforme* and other *spp.*	H	A component of the respiratory tract mycobiome. An etiologic agent of fungal pneumonia in immunocompromised pts.	Huffnagle and Noverr, 2013; Anaissie and Nucci, 2017
Geotrichum candidum and other *spp.*	L	Pneumonia occurrence has been reported in immunocompromised pts.	Fishman, 2017
Hansenula anomala	L	Pneumonia occurrence has been reported	Kane *et al.,* 2002
Histoplasma capsulatum	**H**	Opportunistic in immunocompromised pts. An etiologic agent of fungal pneumonia (endemic).	Kauffman, 2016; Marrie and File, 2018
Hormographiella aspergillata	L	Pneumonia occurrence has been reported	Verweij *et al.,* 1997
Ochroconis (Dactylaria) gallopava	L	Pneumonia occurrence has been reported.	Meriden *et al.*, 2012; Brokalaki *et al.*, 2012; Shoham *et al.*, 2008
Paecilomyces lilacinus, P. variotii and other *spp.*	L	Pneumonia occurrence has been reported	Dharmasena *et al.,* 1985
Paracoccidioides brasiliensis	H	An etiologic agent of fungal pneumonia (endemic).	Silletti *et al.*, 1996
Peniophorella spp.	H	A component of the respiratory tract mycobiome.	Nguyen *et al.,* 2015
Phoma exigua	L	Pneumonitis occurrence has been reported	Balis *et al.,* 2006
Protomyces spp.	H	A component of the respiratory tract mycobiome	Nguyen *et al.,* 2015
Pseudotaeniolina spp.	H	A component of the respiratory tract mycobiome	Nguyen *et al.,* 2015
Malassezia furfur and other *spp.*	L	Pneumonia occurrence has been reported	Baker *et al.,* 2016
Mucor, Rhizomucor, Rhizopus (genera)	H	Opportunistic in immunocompromised pts. An etiologic agent of fungal pneumonia (endemic).	Cox, 2017
Rhodotorula glutinis, R. mucilaginosa and other *spp.*	L	Pneumonia occurrence has been reported	Fischer *et al.,* 2016
Rozella allomycis and other *spp.*	H	A component of the respiratory tract mycobiome	Nguyen *et al.,* 2015
Saccharomyces cerevisiae, S. boulardii	L	Pneumonia occurrence has been reported	Tawfik *et al.,* 1989; Lolis *et al.,* 2008
Vanderwaltozyma polyspora = Saccharomyces polysporus	H	A component of the respiratory tract mycobiome	Nguyen *et al.,* 2015

Sarcinosporon inkin	L	Pneumonia occurrence has been reported	Kenney *et al.,* 1990
Scedosporium apiospermum and *Lomentospora (Scedosporium) prolificans; Pseudallescheria boydii (sexual state)*	H	An etiologic agent of fungal pneumonia.	Costa and Alexander, 2017
Scopulariopsis brevicaulis and other *spp.*	L	Opportunistic in immunocompromised pts. Pneumonia occurrence has been reported	Endo *et al.,* 2002
Sporothrix schenckii	H	An etiologic agent of pulmonary sporotrichosis (endemic).	Kauffman, 2018
Systenostrema alba	H	A component of the respiratory tract mycobiome.	Nguyen *et al.*, 2015
Talaromyces (Penicillium) marneffei	**H**	Opportunistic in immunocompromised pts. A component of the respiratory tract mycobiome. An etiologic agent of fungal pneumonia (endemic).	Nguyen *et al.*, 2015; Deesomchok and Tanprawate, 2006; Chan *et al.*, 2016
Trichoderma longibrachiatum and other *spp.*	L	Opportunistic in immunocompromised pts. Pneumonia occurrence has been reported.	De Miguel *et al.,* 2005
Trichosporon asahii, T. mucoides and other *spp.*	L	Pneumonia occurrence has been reported in immunocompromised pts.	Fishman, 2017
Parasitic			
Ancylostoma duodenale	M	Bronchopneumonia has been reported with ancylostomiasis (diffuse).	Cheepsattayakorn and Cheepsattayakorn, 2014
Ascaris lumbricoides	L	Pulmonary ascariasis (Loffler's syndrome) is known.	Leder and Weller, 2018b
Babesia divergens and *B. microti*	L	Pulmonary babesiosis has been reported	Cheepsattayakorn and Cheepsattayakorn, 2014
Brugia malayi	L	Filariasis (diffuse) has been associated with tropical pulmonary eosinophilia	Cheepsattayakorn and Cheepsattayakorn, 2014
Cryptosporidium hominis, C. parvum and other *spp.*	H	Opportunistic in immunocompromised pts. Pulmonary cryptosporidiosis has been reported.	Meynard *et al.*, 1996; Clavel *et al.*, 1996
Dirofilaria immitis and other *spp.*	L	Pulmonary dirofilariasis is known. Most are asymptomatic.	Cheepsattayakorn and Cheepsattayakorn, 2014; Leder and Weller, 2018c; Haro *et al.,* 2016
Echinococcus multilocularis and *E. granulosus*	M	Pneumonia has been reported with pulmonary echinococcosis.	Cheepsattayakorn and Cheepsattayakorn, 2014
Entamoeba histolytica and *E. dispar*	L	Pulmonary amoebiasis has been reported	Cheepsattayakorn and Cheepsattayakorn, 2014; Leder and Weller, 2017

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Leishmania donovani and *L. chagasi*	M	Opportunistic in immunocompromised pts. Pneumonitis has been reported with visceral leishmaniasis (Kala azar)	Cheepsattayakorn and Cheepsattayakorn, 2014
Necator americanus	M	Bronchopneumonia has been reported with ancylostomiasis (diffuse).	Cheepsattayakorn and Cheepsattayakorn, 2014
Paragonimus westermani and other *spp.*	M	Pneumonitis and bronchopneumonia have been reported with pulmonary paragonimiasis.	Cheepsattayakorn and Cheepsattayakorn, 2014; Leder and Weller, 2018d
Plasmodium vivax and other *spp.*	L	Opportunistic in immunocompromised pts. Pulmonary malaria has been reported.	Cheepsattayakorn and Cheepsattayakorn, 2014
Schistosoma hematobium, S. japonicum and *S. mansoni*	L	Pulmonary schistosomiasis (diffuse) has been reported.	Cheepsattayakorn and Cheepsattayakorn, 2014; Clerinx and Soentjens, 2017
Strongyloides stercoralis	H	Pneumonia has been reported with pulmonary strongyloidiasis (diffuse) in immunocompromised pts.	Cheepsattayakorn and Cheepsattayakorn, 2014; Leder and Weller, 2018e; Fishman, 2017
Toxocara Larval Migrans	M	Pneumonitis has been reported in pulmonary toxocariasis (diffuse)	Cheepsattayakorn and Cheepsattayakorn, 2014; Weller and Leder, 2018a
Toxoplasma gondii	**H**	Opportunistic in immunocompromised pts. Pneumonia is known with pulmonary toxoplasmosis (diffuse)	Cheepsattayakorn and Cheepsattayakorn, 2014; Fishman, 2017; Gandhi, 2018
Trichinella spiralis and other *spp.*	L	Pulmonary trichinellosis has been reported	Cheepsattayakorn and Cheepsattayakorn, 2014; Weller and Leder, 2018b
Trypanosoma brucei gambiense, T. brucei rhodesie and *T. cruzi*	M	Opportunistic in immunocompromised pts. Pneumonitis has been reported in pulmonary trypanosomiasis (Chagas)	Cheepsattayakorn and Cheepsattayakorn, 2014
Wuchereria bancrofti	L	Filariasis (diffuse) has been associated with tropical pulmonary eosinophilia	Cheepsattayakorn and Cheepsattayakorn, 2014

Definition of abbreviations: L= Low; M= Moderate; H= High; **H**= Must be considered (at least) for the differential diagnosis; CAP= Community-acquired pneumonia; HAP=Hospital-acquired pneumonia; ASP= Aspiration pneumonia

A.3.2. Spearman correlation results

Table A.46 Correlation results of exhaled *P. jirovecii* vs

	r	95% CI	P value (two-tailed)
HβG	0.24	-0.22 to 0.62	0.29
a-amylase	0.24	-0.09 to 0.52	0.14
BDG (any)	0.65	0.41 to 0.81	<0.01
BDG (≤ 7 days)	0.64	0.39 to 0.80	<0.01
WBC	-0.10	-0.41 to 0.22	0.52
Neutrophils	-0.11	-0.42 to 0.21	0.49
Lymphocytes	0.02	-0.30 to 0.34	0.88
Monocytes	-0.08	-0.39 to 0.25	0.64
Eosinophils	-0.02	-0.34 to 0.30	0.88
Basophils	-0.18	-0.47 to 0.15	0.27
CRP	-0.09	-0.40 to 0.24	0.59

Definition of abbreviation: *HβG*= Human β-globin; BDG= (1,3)-Beta-D-glucan assay (serum or BAL); WBC= Total White Blood Cell count (serum); CRP= C-reactive protein (serum)

Table A.47 Correlation results of exhaled *P. jirovecii* in patients with likely PJP vs

	r	95% CI	P value (two-tailed)
WBC	-0.27	-0.63 to 0.20	0.24
Neutrophils	-0.30	-0.66 to 0.16	0.18
Lymphocytes	0.04	-0.41 to 0.48	0.85
Monocytes	-0.18	-0.58 to 0.29	0.44
Eosinophils	-0.12	-0.53 to 0.34	0.61
Basophils	-0.33	-0.67 to 0.13	0.15
CRP	-0.22	-0.61 to 0.24	0.33

Definition of abbreviation: WBC= Total White Blood Cell count; CRP= C-reactive protein

Table A.48 Correlation results of exhaled *HβG* vs

	r	95% CI	P value (two-tailed)
α-amylase	0.53	0.10 to 0.79	0.02
WBC	-0.57	-0.82 to -0.14	0.01
Neutrophils	-0.52	-0.79 to -0.07	0.02
Lymphocytes	-0.35	-0.67 to 0.14	0.15
Monocytes	-0.21	-0.61 to 0.29	0.40
Eosinophils	0.24	-0.26 to 0.63	0.33
Basophils	-0.00	-0.47 to 0.46	>0.99
CRP	-0.05	-0.50 to 0.43	0.85

Definition of abbreviation: WBC= Total White Blood Cell count; CRP= C-reactive protein

Table A.49 Correlation results of exhaled α-amylase vs

	r	95% CI	P value (two-tailed)
WBC	-0.06	-0.40 to 0.29	0.72
Neutrophils	-0.15	-0.48 to 0.20	0.39
Lymphocytes	0.08	-0.28 to 0.41	0.66
Monocytes	-0.36	-0.63 to -0.01	0.04
Eosinophils	-0.10	-0.43 to 0.25	0.57
Basophils	-0.15	-0.48 to 0.20	0.38
CRP	-0.37	-0.64 to -0.03	0.03

Definition of abbreviation: WBC= Total White Blood Cell count; CRP= C-reactive protein

A.3.3. Statistical diagnostic tests

MedCalc Version 18.9 (MedCalc Software, Belgium) was used. The software algorithm is based on the following equations:

1. $Sensitivity = \frac{TP}{TP+FN}$

2. $Specificity = \frac{TN}{TN+FP}$

3. $Positive\ Likelihood\ Ratio = \frac{Sensitivity}{1-Specificity}$

4. $Negative\ Likelihood\ Ratio = \frac{1-Sensitivity}{Specificity}$

5. $Positive\ Predictive\ Value = \frac{TP}{TP+FP}$

6. $Negative\ Predictive\ Value = \frac{TN}{TN+FN}$

7. $Accuracy = \frac{TP+TN}{TP+TN+FP+FN}$

For calculating the binomial confidence intervals (CI), Clopper–Pearson interval method was applied for (1), (2) and (7), the Log method explained by Altman (2013) was applied for (3) and (4), and the standard logit confidence intervals were applied for (5) and (6) per Mercaldo *et al.* (2007).

A.4. Exhaled microbiome in health and disease

Table A.50 Number of sequences reads at different filtration steps

Sample	Raw read pairs	Trimmomatic	Joining pair reads	split_library	Chimera removal	Exclude human reads	PHiX reads removed
NB15_3	1.11E+05	1.08E+05	9.27E+04	9.26E+04	9.26E+04		
NB30_3	6.42E+04	6.10E+04	5.21E+04	5.20E+04	5.20E+04		
NB60_3	9.48E+04	9.30E+04	8.04E+04	8.03E+04	8.02E+04		
IB60_3	7.34E+04	7.19E+04	6.29E+04	6.29E+04	6.28E+04		
IC60_3	6.65E+04	6.50E+04	5.51E+04	5.50E+04	5.50E+04		
RL30_3	5.21E+04	4.54E+04	3.92E+04	3.91E+04	3.91E+04		
NB60_1	1.04E+05	1.02E+05	8.88E+04	8.87E+04	8.87E+04		
IB60_1	6.35E+04	6.21E+04	5.51E+04	5.51E+04	5.50E+04		
IC60_1	6.62E+04	6.46E+04	5.89E+04	5.88E+04	5.88E+04		
RL30_1	1.56E+05	1.50E+05	1.32E+05	1.32E+05	1.31E+05		
NB60_4	6.95E+04	6.81E+04	5.80E+04	5.79E+04	5.79E+04		
IB60_4	6.43E+04	6.16E+04	5.26E+04	5.25E+04	5.25E+04		
IC60_4	6.39E+04	6.22E+04	5.30E+04	5.29E+04	5.29E+04		
RL30_4	5.45E+04	5.34E+04	4.71E+04	4.70E+04	4.70E+04		
MASS1	1.52E+04	1.48E+04	1.28E+04	1.27E+04	1.24E+04		
BAL1	1.59E+05	1.56E+05	1.26E+05	1.26E+05	1.17E+05		
MASS3	1.28E+05	1.25E+05	1.10E+05	1.10E+05	1.10E+05		
BAL3	1.05E+05	1.00E+05	8.75E+04	8.74E+04	8.56E+04		
MASS7a	8.80E+03	8.19E+03	7.24E+03	7.23E+03	7.22E+03		
BAL7	9.26E+03	8.35E+03	7.22E+03	7.22E+03	7.22E+03		
NC_I	6.79E+04	6.68E+04	5.68E+04	5.67E+04	5.67E+04		
NC_I_T	4.92E+03	4.73E+03	4.50E+03	4.50E+03	4.50E+03		
NC_II	1.08E+05	1.07E+05	9.59E+04	9.58E+04	9.56E+04		
Total*	1.95E+06	1.89E+06	1.64E+06	1.64E+06	1.63E+06	1.63E+06	1.53E+06
SD	4.08E+04	4.00E+04	3.42E+04	3.42E+04	3.35E+04		
Survived(%)¶		97.07	84.21	84.12	83.51	83.51	78.47

(*) No sample was excluded at this stage

(¶) Percentage of total read pairs survived from raw reads

Table A.51 Relative abundance of genera of different processing methods

Genus	NC_I	NC_I_T	NC_II
Clostridium	5.24E-01	8.66E-03	1.53E-01
Bacillus	3.20E-01	7.11E-03	2.27E-02
Unassigned	6.93E-02	5.13E-02	2.77E-02
Halomonas	2.93E-02	6.50E-01	3.68E-03
Shewanella	1.36E-02	2.51E-01	2.11E-03
Unassigned_Veillonellaceae	1.18E-02	2.22E-04	1.06E-03
Thermomonas	5.68E-03	6.67E-04	5.23E-05
Alicyclobacillus	4.44E-03	1.56E-03	2.09E-05
Acinetobacter	3.77E-03	6.67E-04	5.23E-05
Other_Rhodocyclaceae	2.65E-03	<LLD	1.78E-04
Meiothermus	2.44E-03	<LLD	2.09E-05
Pseudomonas	2.30E-03	<LLD	1.05E-05
Unassigned_Halomonadaceae	2.13E-03	2.40E-02	2.62E-04
Streptococcus	1.03E-03	6.67E-04	7.01E-04
Janthinobacterium	1.00E-03	<LLD	<LLD
Thermoanaerobacterium	5.32E-04	<LLD	<LLD
Rhodanobacter	5.18E-04	<LLD	<LLD
Rhizobium	4.39E-04	2.22E-04	7.30E-01
Unassigned_Xanthomonadaceae	3.37E-04	<LLD	<LLD
Flavobacterium	3.17E-04	<LLD	<LLD
Fervidobacterium	2.98E-04	<LLD	<LLD
Tepidimonas	2.88E-04	<LLD	1.05E-05
Unassigned_Enterobacteriaceae	1.71E-04	<LLD	<LLD
Corynebacterium	1.71E-04	<LLD	<LLD
Chryseobacterium	1.51E-04	<LLD	<LLD
Enhydrobacter	1.37E-04	<LLD	<LLD
Staphylococcus	1.17E-04	1.11E-03	<LLD
Sphingomonas	1.12E-04	<LLD	<LLD
Kyrpidia	1.03E-04	<LLD	<LLD
Delftia	9.77E-05	<LLD	<LLD
Rothia	9.28E-05	2.22E-04	5.23E-05
Micrococcus	9.28E-05	<LLD	<LLD
Lactobacillus	8.30E-05	<LLD	2.09E-05
Thermosinus	8.30E-05	<LLD	<LLD
Haemophilus	7.81E-05	<LLD	<LLD
Paracoccus	7.81E-05	<LLD	<LLD
Actinomyces	7.32E-05	<LLD	1.05E-05
Acidocella	7.32E-05	<LLD	<LLD
Weissella	6.84E-05	<LLD	<LLD
Unassigned_Alcaligenaceae	6.84E-05	<LLD	<LLD

Candidatus Rhodoluna	6.84E-05	<LLD	<LLD
Other_Clostridia	6.84E-05	<LLD	<LLD
Comamonas	6.35E-05	<LLD	<LLD
Other_Clostridiales	6.35E-05	<LLD	<LLD
Unassigned_Cytophagaceae	6.35E-05	<LLD	<LLD
Gemella	5.86E-05	<LLD	1.05E-05
Other_Lactobacillales	5.37E-05	<LLD	2.09E-05
Unassigned_Comamonadaceae	5.37E-05	<LLD	<LLD
Achromobacter	5.37E-05	<LLD	<LLD
Lactococcus	4.88E-05	<LLD	<LLD
Other_Comamonadaceae	4.39E-05	1.33E-03	<LLD
Veillonella	4.39E-05	<LLD	1.05E-05
Sphingobacterium	4.39E-05	<LLD	<LLD
Trabulsiella	4.39E-05	<LLD	<LLD
Providencia	3.91E-05	<LLD	<LLD
Pedobacter	3.91E-05	<LLD	<LLD
Mycobacterium	3.91E-05	<LLD	<LLD
Unassigned_ACK-M1	3.91E-05	<LLD	<LLD
Unassigned_Legionellales	3.91E-05	<LLD	<LLD
Sediminibacterium	3.42E-05	<LLD	2.41E-02
Prevotella	3.42E-05	<LLD	2.09E-05
Unassigned_Hydrogenothermaceae	3.42E-05	<LLD	<LLD
Chitinophaga	3.42E-05	<LLD	<LLD
Bacteroides	2.93E-05	<LLD	<LLD
Unassigned_Sphingobacteriales	2.93E-05	<LLD	<LLD
Dysgonomonas	2.93E-05	<LLD	<LLD
Unassigned_Aeromonadaceae	2.93E-05	<LLD	<LLD
Stenotrophomonas	2.44E-05	8.89E-04	2.09E-05
Kocuria	2.44E-05	<LLD	<LLD
Unassigned_Caulobacteraceae	2.44E-05	<LLD	<LLD
Unassigned_Bacillaceae	2.44E-05	<LLD	<LLD
Cupriavidus	2.44E-05	<LLD	<LLD
Anoxybacillus	2.44E-05	<LLD	<LLD
Unassigned_Thermoanaerobacteraceae	2.44E-05	<LLD	<LLD
Fluviicola	2.44E-05	<LLD	<LLD
Clavibacter	2.44E-05	<LLD	<LLD
Deinococcus	1.95E-05	<LLD	4.19E-05
Fusobacterium	1.95E-05	<LLD	1.05E-05
Enterococcus	1.95E-05	<LLD	<LLD
Unassigned_Oxalobacteraceae	1.95E-05	<LLD	<LLD
Caloramator	1.95E-05	<LLD	<LLD
Brevibacillus	1.95E-05	<LLD	<LLD

Salinispora	1.95E-05	<LLD	<LLD
Brevibacterium	1.95E-05	<LLD	<LLD
Unassigned_Nocardiaceae	1.95E-05	<LLD	<LLD
Unassigned_Acidobacteria-5	1.95E-05	<LLD	<LLD
Unassigned_Cytophagales	1.95E-05	<LLD	<LLD
Unassigned_Deltaproteobacteria	1.95E-05	<LLD	<LLD
Propionibacterium	1.46E-05	4.44E-04	<LLD
Other_Rhizobiaceae	1.46E-05	<LLD	3.87E-04
Unassigned_Streptophyta	1.46E-05	<LLD	<LLD
Unassigned_Chitinophagaceae	1.46E-05	<LLD	<LLD
Moraxella	1.46E-05	<LLD	<LLD
Unassigned_Opitutaceae	1.46E-05	<LLD	<LLD
Arcobacter	1.46E-05	<LLD	<LLD
Myroides	1.46E-05	<LLD	<LLD
Other_Clostridiaceae	1.46E-05	<LLD	<LLD
Unassigned_Rhodospirillaceae	1.46E-05	<LLD	<LLD
Unassigned_[Pedosphaerales]	1.46E-05	<LLD	<LLD
Other_Caulobacteraceae	1.46E-05	<LLD	<LLD
Unassigned_Marinilabiaceae	1.46E-05	<LLD	<LLD
Spirochaeta	1.46E-05	<LLD	<LLD
Bdellovibrio	1.46E-05	<LLD	<LLD
Candidatus Solibacter	1.46E-05	<LLD	<LLD
Rubricoccus	1.46E-05	<LLD	<LLD
Unassigned_MIZ46	1.46E-05	<LLD	<LLD
Ralstonia	9.77E-06	<LLD	1.05E-05
Thermodesulfovibrio	9.77E-06	<LLD	1.05E-05
Neisseria	9.77E-06	<LLD	<LLD
Mycoplasma	9.77E-06	<LLD	<LLD
Unassigned_Bradyrhizobiaceae	9.77E-06	<LLD	<LLD
Leptotrichia	9.77E-06	<LLD	<LLD
Burkholderia	9.77E-06	<LLD	<LLD
Other_Enterobacteriaceae	9.77E-06	<LLD	<LLD
Unassigned_MLE1-12	9.77E-06	<LLD	<LLD
Other_Rhizobiales	9.77E-06	<LLD	<LLD
Unassigned_Acetobacteraceae	9.77E-06	<LLD	<LLD
Unassigned_Microbacteriaceae	9.77E-06	<LLD	<LLD
Hymenobacter	9.77E-06	<LLD	<LLD
Unassigned_Spirobacillales	9.77E-06	<LLD	<LLD
Polaromonas	9.77E-06	<LLD	<LLD
Balneimonas	9.77E-06	<LLD	<LLD
Brevundimonas	9.77E-06	<LLD	<LLD
Unassigned_Rhodocyclaceae	9.77E-06	<LLD	<LLD

Leuconostoc	9.77E-06	<LLD	<LLD
Unassigned_SC3	9.77E-06	<LLD	<LLD
Unassigned_Porphyromonadaceae	9.77E-06	<LLD	<LLD
Coprococcus	9.77E-06	<LLD	<LLD
Thermus	9.77E-06	<LLD	<LLD
Aerococcus	9.77E-06	<LLD	<LLD
Sphingobium	9.77E-06	<LLD	<LLD
Prosthecobacter	9.77E-06	<LLD	<LLD
Unassigned_Methylophilaceae	9.77E-06	<LLD	<LLD
Unassigned_Solibacterales	9.77E-06	<LLD	<LLD
Phycicoccus	9.77E-06	<LLD	<LLD
Unassigned_koll11	9.77E-06	<LLD	<LLD
Polynucleobacter	9.77E-06	<LLD	<LLD
Steroidobacter	9.77E-06	<LLD	<LLD
Other_Cellulomonadaceae	9.77E-06	<LLD	<LLD
Unassigned_Sinobacteraceae	9.77E-06	<LLD	<LLD
Dyadobacter	9.77E-06	<LLD	<LLD
Jeotgalicoccus	9.77E-06	<LLD	<LLD
Unassigned_Gemm-3	9.77E-06	<LLD	<LLD
Unassigned_ZB2	9.77E-06	<LLD	<LLD
Sphingopyxis	9.77E-06	<LLD	<LLD
Unassigned_Ellin6067	9.77E-06	<LLD	<LLD
Unassigned_PHOS-HD29	9.77E-06	<LLD	<LLD
Unassigned_SJA-4	9.77E-06	<LLD	<LLD
Leptospira	4.88E-06	<LLD	3.34E-02
Unassigned_Rhizobiales	4.88E-06	<LLD	1.05E-05
Unassigned_Bacillales	4.88E-06	<LLD	9.42E-05
Other_Aerococcaceae	4.88E-06	<LLD	<LLD
Unassigned_Methylobacteriaceae	4.88E-06	<LLD	<LLD
Treponema	4.88E-06	<LLD	<LLD
Oribacterium	4.88E-06	<LLD	<LLD
Brachybacterium	4.88E-06	<LLD	<LLD
Methylobacterium	4.88E-06	<LLD	<LLD
Kingella	4.88E-06	<LLD	<LLD
Unassigned_Ignavibacteriales	4.88E-06	<LLD	<LLD
Unassigned_Nocardioidaceae	4.88E-06	<LLD	<LLD
Unassigned_Flavobacteriaceae	4.88E-06	<LLD	<LLD
Microbacterium	4.88E-06	<LLD	<LLD
Ochrobactrum	4.88E-06	<LLD	<LLD
Unassigned_Sphingobacteriaceae	4.88E-06	<LLD	<LLD
Vagococcus	4.88E-06	<LLD	<LLD
Unassigned_Sporichthyaceae	4.88E-06	<LLD	<LLD

Thermoanaerovibrio	4.88E-06	<LLD	<LLD
Dechloromonas	4.88E-06	<LLD	<LLD
Coprothermobacter	4.88E-06	<LLD	<LLD
Unassigned_WPS-2	4.88E-06	<LLD	<LLD
Pandoraea	4.88E-06	<LLD	<LLD
Paucibacter	4.88E-06	<LLD	<LLD
Other_Beta-proteobacteria	4.88E-06	<LLD	<LLD
Unassigned_IIb	4.88E-06	<LLD	<LLD
Unassigned_TM7	4.88E-06	<LLD	<LLD
Unassigned_Ignavibacteriaceae	4.88E-06	<LLD	<LLD
Brochothrix	4.88E-06	<LLD	<LLD
Azospirillum	4.88E-06	<LLD	<LLD
Other_Veillonellaceae	4.88E-06	<LLD	<LLD
Unassigned_R4-41B	<LLD	6.67E-04	<LLD
Other_Peptostreptococcaceae	<LLD	<LLD	2.09E-05

Table A.52 Relative abundance of genera over time for NB

Colour key: ■ Background signals excluded ■ Shared signals between three time points ■ Unique signals to certain time points ■ Unique signals after background signals were excluded

Genus	NB15_3	NB30_3	NB60_3
Clostridium	5.45E-01	6.49E-01	5.20E-01
Bacillus	2.63E-01	2.03E-01	2.35E-01
Unassigned	1.03E-01	3.95E-02	4.90E-02
Halomonas	3.75E-02	4.71E-02	4.33E-02
Shewanella	1.26E-02	2.21E-02	1.80E-02
Unassigned_Veillonellaceae	1.10E-02	1.10E-02	1.09E-02
Thermomonas	5.95E-03	4.91E-03	8.16E-03
Alicyclobacillus	4.08E-03	3.68E-03	4.49E-03
Unassigned_Halomonadaceae	3.06E-03	3.62E-03	3.13E-03
Other_Rhodocyclaceae	2.10E-03	1.98E-03	2.67E-03
Meiothermus	1.69E-03	1.79E-03	1.69E-03
Pseudomonas	1.35E-03	1.62E-03	1.02E-02
Acinetobacter	1.19E-03	1.52E-03	1.80E-03
Streptococcus	1.09E-03	1.21E-03	4.06E-02
Rhodanobacter	6.38E-04	3.08E-04	8.61E-04
Rhizobium	5.19E-04	5.39E-04	4.74E-04
Flavobacterium	4.22E-04	5.20E-04	4.12E-04
Thermoanaerobacterium	5.84E-04	3.85E-04	3.25E-04
Sphingomonas	3.24E-05	3.47E-04	5.49E-04
Unassigned_Xanthomonadaceae	3.13E-04	4.24E-04	2.75E-04
Unassigned_Enterobacteriaceae	2.59E-04	3.28E-04	2.00E-04
Fervidobacterium	2.92E-04	1.54E-04	2.12E-04
Candidatus Rhodoluna	2.27E-04	2.12E-04	1.25E-05
Rothia	5.40E-05	1.93E-04	4.76E-03
Unassigned_ACK-M1	2.16E-04	1.93E-04	6.24E-05
Tepidimonas	2.05E-04	7.71E-05	1.62E-04
Unassigned_Comamonadaceae	1.19E-04	2.50E-04	1.37E-04
Stenotrophomonas	2.81E-04	1.16E-04	2.50E-05
Cupriavidus	1.08E-04	<LLD	9.99E-05
Delftia	9.73E-05	1.16E-04	8.74E-05
Kyrpidia	9.73E-05	<LLD	1.87E-04
Acidocella	9.73E-05	1.93E-05	1.12E-04
Haemophilus	7.57E-05	7.71E-05	1.14E-03
Ralstonia	7.57E-05	1.73E-04	2.50E-05
Unassigned_Cytophagaceae	7.57E-05	1.35E-04	2.50E-05
Actinomyces	6.49E-05	3.85E-05	7.76E-03
Gemella	6.49E-05	3.85E-05	1.56E-03
Veillonella	6.49E-05	<LLD	6.98E-03
Chryseobacterium	6.49E-05	1.16E-04	4.99E-05
Unassigned_Sphingobacteriales	4.32E-05	1.93E-04	6.24E-05

Staphylococcus	5.40E-05	5.78E-05	1.12E-04
Other_Lactobacillales	4.32E-05	5.78E-05	1.41E-03
Prevotella	5.40E-05	3.85E-05	9.46E-03
Lactococcus	5.40E-05	7.71E-05	2.50E-05
Pedobacter	5.40E-05	1.16E-04	2.50E-05
Unassigned_Bacillaceae	5.40E-05	5.78E-05	1.25E-05
Comamonas	5.40E-05	3.85E-05	4.99E-05
Unassigned_Chitinophagaceae	6.49E-05	<LLD	4.99E-05
Propionibacterium	4.32E-05	<LLD	1.12E-04
Unassigned_Bradyrhizobiaceae	4.32E-05	5.78E-05	1.25E-05
Unassigned_Rhizobiales	4.32E-05	1.16E-04	<LLD
Dysgonomonas	4.32E-05	5.78E-05	<LLD
Sediminibacterium	2.16E-05	3.85E-05	8.74E-05
Granulicatella	1.08E-05	3.85E-05	6.24E-05
Unassigned_Hydrogenothermaceae	<LLD	3.85E-05	7.49E-05
Weissella	3.24E-05	3.85E-05	7.49E-05
Burkholderia	1.30E-04	3.85E-05	1.25E-05
Enhydrobacter	<LLD	7.71E-05	3.74E-05
Bacteroides	4.32E-05	<LLD	3.74E-05
Unassigned_Caulobacteraceae	3.24E-05	<LLD	7.49E-05
Ochrobactrum	3.24E-05	9.63E-05	<LLD
Unassigned_VC2_1_Bac22	3.24E-05	7.71E-05	<LLD
Other_Comamonadaceae	<LLD	3.85E-05	2.50E-05
Unassigned_TM7-3	3.24E-05	<LLD	2.50E-05
Chitinophaga	4.32E-05	<LLD	2.50E-05
Providencia	3.24E-05	<LLD	2.50E-05
Polaromonas	<LLD	3.85E-05	2.50E-05
Unassigned_SC3	<LLD	3.85E-05	2.50E-05
Micrococcus	2.16E-05	1.93E-05	2.00E-04
Candidatus Rhabdochlamydia	2.16E-05	<LLD	4.99E-05
Fluviicola	2.16E-05	<LLD	2.50E-05
Enterococcus	1.08E-05	1.93E-05	2.50E-05
Lactobacillus	1.08E-05	1.93E-05	6.24E-05
Neisseria	<LLD	1.93E-05	1.57E-03
Leptotrichia	1.08E-05	1.93E-05	9.11E-04
Moryella	1.08E-05	1.93E-05	6.24E-05
Unassigned_Lachnospiraceae	1.08E-05	1.93E-05	1.50E-04
Achromobacter	<LLD	1.93E-05	3.74E-05
Unassigned_Oxalobacteraceae	<LLD	1.93E-05	2.50E-05
Caloramator	8.65E-05	1.93E-05	<LLD
Trabulsiella	1.08E-05	1.93E-05	2.50E-05
Brevibacillus	<LLD	1.93E-05	2.50E-05
Spirochaeta	2.16E-05	1.93E-05	<LLD
Brachybacterium	<LLD	1.54E-04	1.25E-05

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Bifidobacterium	<LLD	1.93E-05	1.25E-05
Thermosinus	1.08E-05	1.93E-05	1.25E-05
Carnobacterium	5.40E-05	<LLD	1.25E-05
Unassigned_Rhodospirillaceae	3.24E-05	<LLD	1.25E-05
Desulfovibrio	2.16E-05	<LLD	1.25E-05
Mycoplasma	1.08E-05	3.85E-05	<LLD
Dialister	1.08E-05	<LLD	6.24E-05
Unassigned_Neisseriaceae	1.08E-05	<LLD	1.25E-05
Unassigned_Alcaligenaceae	1.08E-05	<LLD	4.99E-05
Mycobacterium	1.08E-05	<LLD	3.74E-05
Other_Enterobacteriaceae	1.08E-05	<LLD	1.25E-05
Azospira	1.08E-05	<LLD	1.25E-05
Unassigned_Microbacteriaceae	1.08E-05	<LLD	2.50E-05
Unassigned_Rhodocyclaceae	1.08E-05	3.85E-05	<LLD
Unassigned_Marinilabiaceae	1.08E-05	1.93E-05	<LLD
Other_Aerococcaceae	<LLD	<LLD	3.74E-05
Leptospira	<LLD	<LLD	4.12E-03
[Prevotella]	<LLD	<LLD	8.74E-05
Fusobacterium	<LLD	<LLD	4.74E-04
Porphyromonas	<LLD	<LLD	7.99E-04
Corynebacterium	<LLD	<LLD	3.74E-04
Unassigned_Methylobacteriaceae	<LLD	<LLD	1.25E-05
Treponema	2.16E-05	<LLD	<LLD
Aggregatibacter	<LLD	<LLD	1.25E-05
Capnocytophaga	<LLD	<LLD	1.50E-04
Lautropia	<LLD	<LLD	3.12E-04
Unassigned_[Weeksellaceae]	<LLD	<LLD	4.87E-04
Campylobacter	<LLD	<LLD	4.62E-04
Bulleidia	<LLD	<LLD	8.74E-05
Oribacterium	<LLD	<LLD	2.25E-04
Janthinobacterium	<LLD	<LLD	1.87E-04
Selenomonas	<LLD	<LLD	2.50E-05
Atopobium	<LLD	<LLD	7.49E-05
Unassigned_Clostridiales	<LLD	<LLD	2.25E-04
Methylobacterium	<LLD	<LLD	3.74E-05
Catonella	<LLD	<LLD	1.25E-05
Unassigned_Bacillales	2.16E-05	<LLD	<LLD
Actinobacillus	1.08E-05	<LLD	<LLD
Unassigned_Gemellaceae	<LLD	<LLD	6.24E-05
Unassigned_Coriobacteriaceae	<LLD	<LLD	3.74E-05
Other_Rhizobiaceae	<LLD	<LLD	2.50E-05
Unassigned_Pasteurellaceae	<LLD	<LLD	1.25E-05
Kingella	<LLD	<LLD	4.99E-05
Peptostreptococcus	<LLD	<LLD	3.74E-05

Deinococcus	<LLD	<LLD	2.50E-05
Unassigned_CW040	<LLD	<LLD	1.25E-05
Other_Clostridia	<LLD	<LLD	1.25E-05
Sphingobacterium	<LLD	<LLD	1.62E-04
Paracoccus	<LLD	<LLD	1.25E-05
Other_Clostridiales	5.40E-05	<LLD	<LLD
Unassigned_Ignavibacteriales	<LLD	<LLD	3.74E-05
Thermodesulfovibrio	1.08E-05	<LLD	<LLD
Moraxella	<LLD	<LLD	2.50E-05
Other_Peptostreptococcaceae	<LLD	<LLD	1.25E-05
Salinispora	2.16E-05	<LLD	<LLD
Microbacterium	<LLD	1.93E-05	<LLD
Unassigned_Opitutaceae	<LLD	3.85E-05	<LLD
Arcobacter	<LLD	9.63E-05	<LLD
Other_Gemellaceae	<LLD	<LLD	1.25E-05
Other_Clostridiaceae	1.08E-05	<LLD	<LLD
Pseudoxanthomonas	<LLD	<LLD	1.25E-05
Legionella	<LLD	3.85E-05	<LLD
Unassigned_Aeromonadaceae	<LLD	1.93E-05	<LLD
Unassigned_Acetobacteraceae	4.32E-05	<LLD	<LLD
Peptococcus	<LLD	<LLD	1.25E-05
Hymenobacter	9.73E-05	<LLD	<LLD
Vagococcus	4.32E-05	<LLD	<LLD
Brevundimonas	<LLD	<LLD	1.25E-05
Brevibacterium	2.16E-05	<LLD	<LLD
Other_Planococcaceae	4.32E-05	<LLD	<LLD
Other_Actinomycetales	<LLD	3.85E-05	<LLD
Unassigned_Phyllobacteriaceae	<LLD	<LLD	1.25E-05
Leucobacter	2.16E-05	<LLD	<LLD
Unassigned_Beta-proteobacteria	<LLD	<LLD	1.25E-05
Other_Brucellaceae	1.08E-05	<LLD	<LLD
Rickettsiella	<LLD	9.63E-05	<LLD
Unassigned_[Pedosphaerales]	<LLD	5.78E-05	<LLD
Unassigned_Sphingomonadaceae	<LLD	<LLD	1.25E-05
Unassigned_iii1-15	<LLD	<LLD	2.50E-05
Unassigned_Sphingomonadales	<LLD	7.71E-05	<LLD
Dechloromonas	4.32E-05	<LLD	<LLD
Butyrivibrio	<LLD	<LLD	1.25E-05
Unassigned_Porphyromonadaceae	1.08E-05	<LLD	<LLD
Unassigned_Myxococcales	<LLD	<LLD	2.50E-05
Unassigned_Aurantimonadaceae	<LLD	<LLD	6.24E-05
Agromyces	5.40E-05	<LLD	<LLD
Unassigned_TM7-1	<LLD	3.85E-05	<LLD
Dermacoccus	<LLD	<LLD	3.74E-05

	Column1	Column2	Column3
Unassigned_OP11-3	<LLD	<LLD	2.50E-05
Unassigned_envOPS12	<LLD	5.78E-05	<LLD
Unassigned_GKS2-174	<LLD	5.78E-05	<LLD
Unassigned_Pseudomonadaceae	<LLD	<LLD	2.50E-05
Unassigned_SM2F11	<LLD	3.85E-05	<LLD
Gordonia	<LLD	<LLD	1.25E-05
Microvirgula	<LLD	<LLD	1.25E-05
Unassigned_Methylophilaceae	<LLD	<LLD	1.25E-05
Unassigned_Endomicrobia	4.32E-05	<LLD	<LLD
Unassigned_Haliangiaceae	4.32E-05	<LLD	<LLD
Paucibacter	2.16E-05	<LLD	<LLD
Unassigned_KD8-87	<LLD	<LLD	1.25E-05
Proteus	<LLD	<LLD	2.50E-05
Unassigned_Saprospiraceae	<LLD	<LLD	1.25E-05
Unassigned_Solibacterales	<LLD	<LLD	2.50E-05
Phycicoccus	<LLD	<LLD	2.50E-05
Kineococcus	3.24E-05	<LLD	<LLD
Candidatus Aquiluna	3.24E-05	<LLD	<LLD
Unassigned_SM2F09	3.24E-05	<LLD	<LLD
Aneurinibacillus	3.24E-05	<LLD	<LLD
Phaeospirillum	3.24E-05	<LLD	<LLD
Unassigned_IIb	<LLD	<LLD	2.50E-05
Unassigned_TM7	<LLD	<LLD	2.50E-05
Polynucleobacter	<LLD	1.93E-05	<LLD
Desulfosporosinus	<LLD	<LLD	2.50E-05
Hyphomicrobium	<LLD	<LLD	2.50E-05
Unassigned_Rickettsiaceae	<LLD	<LLD	2.50E-05
Brochothrix	<LLD	1.93E-05	<LLD
Armatimonas	2.16E-05	<LLD	<LLD
Unassigned_Stramenopiles	2.16E-05	<LLD	<LLD
Rhodobacter	2.16E-05	<LLD	<LLD
Unassigned_Coxiellaceae	2.16E-05	<LLD	<LLD
Unassigned_Erythrobacteraceae	<LLD	<LLD	1.25E-05

Table A.53 Relative abundance of genera of different breathing patterns

Colour key: ■ Background signals excluded ■ Shared signals between different breathing patterns ■ Shared signals after background signals were excluded ■ Unique signals to a certain respiratory pattern ■ Unique signals after background signals were excluded

Genus	NB60_1	NB60_3	NB60_4	IB60_1	IB60_3	IB60_4	IC60_1	IC60_3	IC60_4	RL30_1	RL30_3	RL30_4
Clostridium	4.51E-01	5.20E-01	4.97E-01	4.54E-01	3.99E-01	6.57E-01	2.77E-01	4.39E-01	5.52E-01	4.70E-01	5.20E-01	2.82E-01
Bacillus	3.82E-01	2.35E-01	3.27E-01	3.37E-01	4.72E-01	1.18E-01	5.48E-01	1.03E-01	2.81E-01	2.72E-01	7.93E-02	3.49E-01
Unassigned	5.51E-02	4.90E-02	4.42E-02	8.91E-02	3.16E-02	1.07E-01	3.20E-02	5.08E-02	4.31E-02	3.26E-02	1.10E-01	5.47E-02
Halomonas	4.22E-02	4.33E-02	5.44E-02	2.68E-02	2.76E-02	6.21E-02	4.52E-02	1.36E-02	3.39E-02	2.97E-02	1.38E-01	5.29E-02
Shewanella	1.91E-02	1.80E-02	2.04E-02	8.44E-03	1.18E-02	2.23E-02	1.64E-02	5.76E-03	1.40E-02	1.31E-02	5.11E-02	2.45E-02
Unassigned_Veillonellaceae	1.38E-02	1.09E-02	1.62E-02	1.56E-02	1.82E-02	4.94E-03	2.45E-02	5.03E-03	1.09E-02	1.17E-02	3.64E-03	1.66E-02
Thermomonas	8.64E-03	8.16E-03	9.35E-03	6.57E-03	1.08E-02	1.55E-03	1.56E-02	2.30E-03	5.89E-03	7.05E-03	1.51E-03	9.83E-03
Streptococcus	1.32E-03	4.06E-02	1.78E-03	5.22E-03	2.09E-03	1.89E-03	9.87E-04	1.48E-01	5.32E-03	4.71E-02	2.31E-02	5.56E-02
Alicyclobacillus	5.66E-03	4.49E-03	5.12E-03	6.42E-03	6.74E-03	2.12E-03	9.54E-03	2.62E-03	4.54E-03	4.82E-03	1.56E-03	6.14E-03
Unassigned_Halomonadaceae	3.56E-03	3.13E-03	5.10E-03	2.11E-03	2.13E-03	5.09E-03	3.79E-03	1.04E-03	3.08E-03	2.44E-03	1.29E-02	3.43E-03
Other_Rhodocyclaceae	2.43E-03	2.67E-03	2.58E-03	2.55E-03	3.87E-03	8.78E-04	5.09E-03	1.00E-03	2.08E-03	2.38E-03	9.22E-04	3.88E-03
Pseudomonas	2.47E-03	1.02E-02	2.39E-03	5.46E-03	8.76E-04	6.22E-03	8.91E-03	7.05E-03	9.84E-04	2.24E-03	3.33E-04	8.74E-04
Meiothermus	2.73E-03	1.69E-03	2.66E-03	2.42E-03	3.49E-03	6.30E-04	3.44E-03	5.10E-04	1.93E-03	2.24E-03	3.07E-04	3.54E-03
Acinetobacter	1.12E-03	1.80E-03	2.84E-03	2.76E-03	1.23E-03	1.56E-03	7.31E-04	4.61E-03	8.21E-03	1.53E-03	1.20E-03	6.72E-03
Rothia	9.03E-05	4.76E-03	1.21E-04	1.91E-03	6.37E-05	7.63E-05	5.10E-05	1.22E-02	5.49E-04	2.66E-03	6.61E-03	5.27E-03
Neisseria	<LLD	1.57E-03	8.65E-05	3.60E-03	<LLD	7.82E-04	<LLD	3.08E-02	4.16E-04	1.39E-02	9.76E-03	2.33E-02
Haemophilus	7.90E-05	1.14E-03	1.21E-04	1.55E-03	1.11E-04	2.29E-04	5.10E-05	5.40E-02	1.08E-03	9.53E-03	8.96E-03	1.89E-02
Prevotella	1.13E-05	9.46E-03	3.46E-05	8.73E-04	1.27E-04	9.54E-05	5.10E-05	1.58E-02	5.11E-04	1.40E-02	2.87E-03	1.49E-02
Rhodanobacter	5.98E-04	8.61E-04	7.95E-04	4.55E-04	6.85E-04	2.10E-04	1.04E-03	2.73E-04	9.08E-04	9.16E-04	1.28E-04	5.54E-04
Veillonella	7.90E-05	6.98E-03	5.19E-05	8.00E-04	4.78E-05	1.91E-05	1.70E-05	3.95E-03	4.73E-04	7.34E-03	8.96E-04	8.59E-03
Porphyromonas	<LLD	7.99E-04	1.21E-04	8.18E-04	<LLD	5.72E-05	<LLD	1.01E-02	2.27E-04	5.17E-03	2.61E-03	6.82E-03
Gemella	1.35E-04	1.56E-03	1.21E-04	5.64E-04	6.37E-05	2.86E-04	1.70E-05	2.40E-02	3.60E-04	4.47E-03	3.23E-03	2.24E-03
Rhizobium	3.16E-04	4.74E-04	3.98E-04	4.18E-04	3.66E-04	7.25E-04	1.53E-04	7.11E-04	5.30E-04	7.18E-04	1.31E-03	3.20E-04
Thermoanaerobacterium	4.29E-04	3.25E-04	6.57E-04	3.82E-04	8.60E-04	5.72E-05	9.70E-04	1.28E-04	4.54E-04	3.51E-04	1.02E-04	3.84E-04
Actinomyces	4.51E-05	7.76E-03	1.21E-04	2.00E-04	4.78E-05	<LLD	<LLD	3.01E-03	5.49E-04	1.55E-03	1.28E-03	5.24E-03
Corynebacterium	2.26E-05	3.74E-04	3.29E-04	4.00E-04	7.96E-05	1.91E-05	1.70E-05	1.33E-03	6.62E-04	3.82E-04	2.05E-04	2.22E-03
Fusobacterium	<LLD	4.74E-04	<LLD	9.51E-03	<LLD	2.10E-04	<LLD	6.43E-03	1.14E-04	1.19E-03	2.23E-03	3.11E-03
Other_Lactobacillales	3.38E-05	1.41E-03	8.65E-05	1.45E-04	6.37E-05	3.43E-04	3.40E-05	3.50E-03	2.27E-04	1.87E-03	1.54E-03	4.43E-03
Unassigned_Xanthomonadaceae	1.92E-04	2.75E-04	2.77E-04	2.73E-04	3.19E-04	2.67E-04	5.27E-04	2.91E-04	4.54E-04	4.58E-04	1.79E-04	4.90E-04
Lautropia	<LLD	3.12E-04	<LLD	9.46E-04	<LLD	1.34E-04	<LLD	2.37E-03	1.51E-04	9.54E-04	7.43E-04	1.98E-03
Capnocytophaga	<LLD	1.50E-04	6.92E-05	3.27E-04	<LLD	1.34E-04	<LLD	5.52E-03	3.03E-04	7.40E-04	1.38E-03	1.43E-03
Tepidimonas	4.40E-04	1.62E-04	2.77E-04	2.36E-04	3.34E-04	7.63E-05	2.38E-04	1.82E-05	1.51E-04	2.90E-04	<LLD	2.13E-04
Unassigned_Enterobacteriaceae	2.93E-04	2.00E-04	3.46E-04	1.82E-04	2.39E-04	2.29E-04	3.23E-04	1.82E-04	2.46E-04	1.91E-04	5.12E-05	2.13E-04

Staphylococcus	6.77E-04	1.12E-04	1.73E-04	3.64E-04	2.39E-04	9.54E-05	5.10E-05	2.00E-04	5.15E-03	2.29E-04	2.05E-04	2.28E-03
Fervidobacterium	3.38E-04	2.12E-04	8.65E-05	2.00E-04	3.34E-04	7.63E-05	3.06E-04	7.29E-05	2.46E-04	2.29E-04	<LLD	1.92E-04
Propionibacterium	3.72E-04	1.12E-04	5.01E-04	9.64E-04	2.12E-03	5.72E-05	1.36E-04	5.47E-05	3.22E-04	8.40E-05	5.12E-05	2.35E-04
Kyrpidia	1.24E-04	1.87E-04	2.42E-04	1.82E-04	3.50E-04	<LLD	1.70E-04	7.29E-05	2.46E-04	1.22E-04	<LLD	3.20E-04
Janthinobacterium	1.13E-05	1.87E-04	1.90E-04	9.09E-05	1.59E-05	7.63E-05	1.53E-04	1.46E-04	5.49E-04	6.87E-05	1.02E-04	5.97E-04
Aggregatibacter	<LLD	1.25E-05	<LLD	3.64E-05	<LLD	1.34E-03	<LLD	3.90E-03	2.08E-04	1.24E-03	2.18E-03	3.94E-03
Chryseobacterium	1.24E-04	4.99E-05	3.46E-04	1.09E-04	1.59E-05	1.14E-04	2.21E-04	9.11E-05	2.46E-04	1.76E-04	1.02E-04	8.53E-05
[Prevotella]	2.26E-05	8.74E-05	<LLD	2.71E-03	<LLD	<LLD	1.70E-05	6.94E-03	1.14E-04	1.49E-02	1.56E-03	3.84E-03
Acidocella	1.35E-04	1.12E-04	1.73E-04	1.09E-04	1.43E-04	<LLD	1.19E-04	3.64E-05	1.89E-05	8.40E-05	<LLD	<LLD
Stenotrophomonas	1.02E-04	2.50E-05	6.92E-05	2.00E-04	7.96E-05	2.29E-04	8.51E-05	1.46E-04	3.78E-05	1.22E-04	5.12E-05	1.07E-04
Delftia	1.02E-04	8.74E-05	1.56E-04	7.27E-05	3.19E-05	1.72E-04	<LLD	3.64E-05	9.46E-05	6.87E-05	2.56E-04	8.53E-05
Unassigned_Caulobacteraceae	1.24E-04	7.49E-05	3.46E-05	3.64E-05	1.27E-04	<LLD	2.04E-04	<LLD	3.78E-05	2.14E-04	7.68E-05	2.13E-04
Campylobacter	<LLD	4.62E-04	<LLD	1.27E-04	<LLD	<LLD	<LLD	7.65E-04	1.89E-05	2.67E-04	3.33E-04	4.48E-04
Chitinophaga	9.03E-05	2.50E-05	8.65E-05	3.64E-05	1.11E-04	9.54E-05	1.36E-04	1.82E-05	1.89E-05	8.40E-05	5.12E-05	<LLD
Sphingomonas	4.85E-04	5.49E-04	<LLD	7.27E-05	2.39E-04	<LLD	6.12E-04	5.47E-05	1.32E-04	4.58E-05	<LLD	4.26E-05
Unassigned_Lachnospiraceae	1.13E-05	1.50E-04	1.73E-05	2.18E-04	<LLD	9.54E-05	<LLD	2.91E-04	<LLD	1.30E-04	<LLD	5.12E-04
Leptospira	<LLD	4.12E-03	2.59E-04	1.82E-05	1.59E-05	<LLD	<LLD	2.19E-04	9.33E-03	6.11E-05	5.12E-05	4.24E-03
Granulicatella	1.13E-05	6.24E-05	<LLD	6.73E-04	<LLD	1.91E-05	<LLD	1.00E-02	5.68E-05	4.53E-03	5.12E-05	6.82E-04
Unassigned_Hydrogenothermaceae	1.13E-04	7.49E-05	1.38E-04	3.64E-05	3.19E-05	<LLD	5.10E-05	1.82E-05	5.68E-05	6.87E-05	<LLD	1.49E-04
Comamonas	9.03E-05	4.99E-05	1.21E-04	3.64E-05	4.78E-05	5.72E-05	1.70E-05	1.09E-04	9.46E-05	2.29E-05	3.07E-04	<LLD
Unassigned_Comamonadaceae	5.64E-05	1.37E-04	5.19E-05	<LLD	4.78E-05	3.82E-05	5.10E-05	1.64E-04	1.89E-05	3.05E-05	1.79E-04	<LLD
Treponema	4.51E-05	<LLD	1.73E-05	4.00E-04	<LLD	1.91E-05	5.10E-05	2.33E-03	<LLD	4.43E-04	2.05E-04	2.56E-04
Unassigned_[Weeksellaceae]	<LLD	4.87E-04	1.73E-05	1.82E-05	<LLD	<LLD	<LLD	2.82E-03	7.57E-05	3.22E-03	1.54E-04	3.20E-04
Sediminibacterium	6.77E-05	8.74E-05	<LLD	<LLD	1.59E-05	<LLD	5.10E-05	<LLD	4.54E-04	6.11E-05	2.56E-04	4.26E-05
Leptotrichia	3.38E-05	9.11E-04	<LLD	3.64E-05	<LLD	1.91E-05	<LLD	1.04E-03	5.68E-05	6.87E-04	2.82E-04	2.52E-03
Providencia	6.77E-05	2.50E-05	1.73E-05	9.09E-05	<LLD	3.82E-05	8.51E-05	<LLD	7.57E-05	5.34E-05	2.30E-04	<LLD
Lactococcus	1.13E-05	2.50E-05	1.04E-04	9.09E-05	6.37E-05	<LLD	1.87E-04	1.82E-05	1.14E-04	1.53E-05	2.56E-05	1.49E-04
Flavobacterium	3.38E-04	4.12E-04	<LLD	<LLD	7.96E-05	5.72E-05	5.10E-05	<LLD	5.68E-05	3.82E-05	<LLD	<LLD
Unassigned_Streptophyta	4.40E-04	<LLD	1.04E-04	3.64E-05	<LLD	<LLD	5.10E-05	3.64E-04	6.25E-04	1.53E-05	<LLD	1.28E-04
Unassigned_Neisseriaceae	2.26E-05	1.25E-05	<LLD	5.46E-05	<LLD	<LLD	<LLD	4.55E-04	2.08E-04	1.09E-03	3.33E-04	9.38E-04
Enhydrobacter	4.51E-05	3.74E-05	4.15E-04	3.64E-05	<LLD	5.72E-05	8.51E-05	1.82E-05	2.08E-04	1.53E-05	<LLD	<LLD
Unassigned_Alcaligenaceae	1.13E-05	4.99E-05	3.46E-05	<LLD	4.78E-05	2.48E-04	1.70E-05	3.64E-05	<LLD	4.58E-05	7.68E-05	<LLD
Achromobacter	5.64E-05	3.74E-05	<LLD	3.64E-05	7.96E-05	1.91E-05	8.51E-05	1.82E-05	3.78E-05	3.05E-05	<LLD	<LLD
Weissella	9.03E-05	7.49E-05	1.73E-05	<LLD	4.78E-05	<LLD	1.53E-04	1.82E-05	5.68E-05	2.29E-05	<LLD	4.26E-05
Ralstonia	2.26E-05	2.50E-05	5.19E-05	3.64E-05	1.59E-05	1.53E-04	3.40E-05	1.82E-05	<LLD	5.34E-05	1.54E-04	<LLD
Pedobacter	5.64E-05	2.50E-05	6.92E-05	3.64E-05	<LLD	<LLD	3.40E-05	1.82E-05	1.14E-04	4.58E-05	<LLD	<LLD
Unassigned_Bradyrhizobiaceae	<LLD	1.25E-05	1.73E-04	1.82E-05	1.11E-04	<LLD	5.10E-05	3.64E-05	3.78E-05	7.63E-06	1.02E-04	2.13E-05

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Brevibacillus	4.51E-05	2.50E-05	5.19E-05	<LLD	4.78E-05	<LLD	<LLD	3.64E-05	3.78E-05	3.05E-05	<LLD	2.13E-05
Cupriavidus	6.77E-05	9.99E-05	1.73E-05	7.27E-05	3.19E-05	<LLD	1.70E-05	1.82E-05	7.57E-05	6.11E-05	<LLD	2.13E-05
Methylobacterium	3.38E-05	3.74E-05	<LLD	7.27E-04	3.19E-05	<LLD	<LLD	1.82E-05	3.78E-05	<LLD	5.12E-05	<LLD
Unassigned_Bacillaceae	5.64E-05	1.25E-05	1.73E-05	<LLD	1.59E-05	5.72E-05	3.40E-05	3.64E-05	5.68E-05	3.05E-05	<LLD	<LLD
Unassigned_Oxalobacteraceae	2.26E-05	2.50E-05	<LLD	3.64E-05	<LLD	7.63E-05	<LLD	3.64E-05	3.78E-05	7.63E-06	<LLD	4.26E-05
Enterococcus	<LLD	2.50E-05	1.73E-05	1.82E-05	1.59E-05	1.91E-05	3.40E-05	5.47E-05	9.46E-05	3.05E-05	2.56E-05	2.13E-05
Unassigned_Ignavibacteriales	4.51E-05	3.74E-05	3.46E-05	3.64E-05	<LLD	<LLD	6.80E-05	1.82E-05	1.89E-05	2.29E-05	<LLD	2.13E-05
Lactobacillus	2.26E-05	6.24E-05	1.73E-05	<LLD	6.37E-05	1.91E-05	<LLD	1.82E-05	4.16E-04	2.29E-05	<LLD	1.28E-04
Sphingobacterium	2.26E-05	1.62E-04	<LLD	<LLD	<LLD	1.91E-05	<LLD	5.47E-05	<LLD	4.58E-05	5.12E-05	6.40E-05
Burkholderia	2.26E-05	1.25E-05	3.46E-05	1.82E-05	6.37E-05	<LLD	3.40E-05	1.82E-05	1.89E-05	4.58E-05	<LLD	1.07E-04
Trabulsiella	3.38E-05	2.50E-05	1.04E-04	<LLD	<LLD	<LLD	6.80E-05	1.82E-05	1.89E-05	8.40E-05	<LLD	2.13E-05
Peptostreptococcus	<LLD	3.74E-05	<LLD	<LLD	<LLD	<LLD	<LLD	3.28E-04	3.78E-05	6.87E-05	1.02E-04	1.49E-04
Other_Clostridia	3.38E-05	1.25E-05	<LLD	1.82E-05	3.19E-05	<LLD	3.40E-05	1.82E-05	1.89E-05	1.30E-04	<LLD	2.13E-05
Unassigned_Gemellaceae	<LLD	6.24E-05	1.73E-05	<LLD	<LLD	<LLD	<LLD	6.92E-04	1.89E-05	1.15E-04	2.30E-04	4.26E-05
Bacteroides	3.38E-05	3.74E-05	1.73E-05	7.27E-05	<LLD	<LLD	<LLD	1.82E-05	1.89E-05	7.63E-06	1.79E-04	<LLD
Atopobium	<LLD	7.49E-05	1.73E-05	1.82E-05	1.59E-05	<LLD	<LLD	1.82E-05	3.78E-05	1.22E-04	<LLD	2.13E-04
Dysgonomonas	<LLD	<LLD	3.46E-05	1.82E-05	1.59E-05	3.82E-05	1.70E-05	<LLD	3.78E-05	3.05E-05	<LLD	6.40E-05
Mycobacterium	6.77E-05	3.74E-05	1.73E-05	<LLD	1.59E-05	<LLD	1.70E-05	<LLD	1.14E-04	6.87E-05	1.54E-04	<LLD
Moraxella	<LLD	2.50E-05	<LLD	1.82E-05	1.59E-05	1.91E-05	<LLD	<LLD	<LLD	3.05E-05	2.56E-05	2.13E-05
Unassigned_TM7-3	1.13E-05	2.50E-05	<LLD	<LLD	<LLD	1.91E-05	<LLD	2.55E-04	<LLD	4.58E-05	1.02E-04	8.53E-05
Selenomonas	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	3.10E-04	3.78E-05	5.34E-05	5.12E-05	4.48E-04
Caloramator	5.64E-05	<LLD	<LLD	3.64E-05	6.37E-05	9.54E-05	5.10E-05	<LLD	<LLD	2.29E-05	<LLD	<LLD
Micrococcus	2.26E-05	2.00E-04	<LLD	<LLD	<LLD	5.72E-05	<LLD	<LLD	7.19E-04	4.58E-05	<LLD	4.26E-04
Oribacterium	<LLD	2.25E-04	<LLD	<LLD	<LLD	<LLD	<LLD	9.84E-04	1.89E-05	1.68E-04	4.35E-04	4.05E-04
Paracoccus	1.13E-05	1.25E-05	3.46E-05	<LLD	<LLD	<LLD	5.10E-05	<LLD	1.89E-05	7.63E-06	<LLD	6.40E-05
Other_Aerococcaceae	<LLD	3.74E-05	<LLD	<LLD	<LLD	1.91E-05	<LLD	1.82E-05	5.68E-05	6.11E-05	<LLD	4.26E-04
Deinococcus	<LLD	2.50E-05	<LLD	1.82E-05	<LLD	<LLD	<LLD	3.64E-05	1.14E-04	<LLD	1.54E-04	1.49E-04
Caulobacter	<LLD	<LLD	1.73E-05	9.09E-05	<LLD	5.72E-05	6.80E-05	5.47E-05	1.32E-04	<LLD	<LLD	<LLD
Other_Clostridiales	3.38E-05	<LLD	1.73E-05	<LLD	<LLD	1.91E-05	1.70E-05	<LLD	<LLD	5.34E-05	<LLD	2.13E-05
Catonella	<LLD	1.25E-05	<LLD	3.64E-05	<LLD	<LLD	<LLD	3.64E-04	<LLD	6.11E-05	1.79E-04	1.71E-04
Unassigned_Pasteurellaceae	<LLD	1.25E-05	<LLD	<LLD	<LLD	1.91E-05	<LLD	3.64E-04	<LLD	7.63E-05	5.12E-05	3.41E-04
Thermosinus	6.77E-05	1.25E-05	<LLD	1.82E-05	<LLD	<LLD	3.40E-05	<LLD	<LLD	8.40E-05	<LLD	2.13E-05
Pseudoxanthomonas	<LLD	1.25E-05	5.19E-05	<LLD	1.59E-05	<LLD	1.70E-05	<LLD	1.89E-05	5.34E-05	<LLD	<LLD
Kingella	1.13E-05	4.99E-05	<LLD	<LLD	<LLD	<LLD	<LLD	2.73E-04	<LLD	6.11E-05	2.56E-05	2.77E-04
Rhodococcus	1.13E-05	<LLD	1.73E-05	9.09E-05	<LLD	<LLD	<LLD	3.64E-05	1.89E-05	<LLD	<LLD	2.13E-05
Other_Carnobacteriaceae	1.13E-05	<LLD	1.73E-05	5.46E-05	<LLD	<LLD	<LLD	1.82E-05	<LLD	3.05E-05	<LLD	4.26E-05
Other_Rhizobiaceae	1.13E-05	2.50E-05	<LLD	<LLD	3.19E-05	<LLD	<LLD	<LLD	1.89E-05	7.63E-06	2.56E-05	<LLD

Azospira	3.38E-05	1.25E-05	<LLD	<LLD	1.59E-05	3.82E-05	<LLD	<LLD	1.89E-05	7.63E-06	<LLD	<LLD
Parvimonas	<LLD	<LLD	<LLD	2.18E-04	<LLD	<LLD	<LLD	2.55E-04	<LLD	7.63E-05	<LLD	<LLD
Unassigned_Methylobacteriaceae	<LLD	1.25E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	4.26E-05
Mycoplasma	<LLD	<LLD	<LLD	2.18E-04	<LLD	1.91E-05	<LLD	3.10E-04	<LLD	2.21E-04	<LLD	<LLD
Dialister	<LLD	6.24E-05	<LLD	<LLD	<LLD	<LLD	<LLD	1.82E-04	3.78E-05	8.40E-05	<LLD	2.98E-04
Kocuria	<LLD	<LLD	1.73E-05	1.82E-05	<LLD	<LLD	<LLD	<LLD	5.49E-04	7.63E-06	<LLD	4.26E-04
Bulleidia	<LLD	8.74E-05	<LLD	<LLD	<LLD	<LLD	<LLD	9.11E-05	<LLD	1.60E-04	<LLD	1.92E-04
Megasphaera	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.64E-05	<LLD	3.82E-05	<LLD	<LLD
Tannerella	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	5.10E-04	<LLD	3.82E-05	2.82E-04	1.92E-04
Moryella	<LLD	6.24E-05	<LLD	<LLD	<LLD	<LLD	<LLD	5.47E-05	<LLD	3.82E-05	2.56E-05	2.56E-04
Unassigned_Rhizobiales	<LLD	<LLD	5.19E-05	<LLD	1.59E-05	<LLD	<LLD	1.82E-05	1.89E-05	<LLD	<LLD	<LLD
Brachybacterium	<LLD	1.25E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.46E-04	<LLD	1.02E-04	3.20E-04
Paludibacter	<LLD	<LLD	<LLD	4.18E-04	<LLD	<LLD	<LLD	5.47E-04	<LLD	2.29E-05	<LLD	1.71E-04
Unassigned_Clostridiales	<LLD	2.25E-04	<LLD	5.46E-05	<LLD	<LLD	<LLD	3.83E-04	<LLD	1.53E-04	2.05E-04	<LLD
Unassigned_Propionibacteriaceae	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.19E-04	<LLD	7.63E-06	5.12E-05	<LLD
Agrobacterium	<LLD	<LLD	1.73E-05	3.64E-05	<LLD	<LLD	3.40E-05	<LLD	<LLD	<LLD	<LLD	6.40E-05
Unassigned_Bacteroidales	5.64E-05	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	2.19E-04	<LLD	6.87E-05	1.28E-04	<LLD
Schwartzia	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	2.13E-05
Unassigned_Bacillales	<LLD	<LLD	<LLD	<LLD	1.59E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.13E-05
Other_Comamonadaceae	7.90E-05	2.50E-05	<LLD	5.46E-05	<LLD	7.63E-05	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Unassigned_[Mogibacteriaceae]	<LLD	<LLD	<LLD	4.55E-04	<LLD	<LLD	<LLD	5.47E-04	<LLD	2.06E-04	1.54E-04	4.26E-05
Anaerococcus	1.13E-05	<LLD	<LLD	7.27E-05	<LLD	<LLD	<LLD	<LLD	4.54E-04	<LLD	<LLD	3.20E-04
Actinobacillus	<LLD	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	7.83E-04	<LLD	1.68E-04	7.68E-05	1.28E-04
Unassigned_Coriobacteriaceae	<LLD	3.74E-05	<LLD	<LLD	<LLD	<LLD	6.80E-05	5.47E-05	<LLD	<LLD	<LLD	<LLD
Unassigned_Leptotrichiaceae	<LLD	<LLD	<LLD	1.09E-04	<LLD	<LLD	<LLD	<LLD	<LLD	1.04E-03	<LLD	<LLD
Bilophila	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	4.26E-05
Unassigned_BD1-5	<LLD	<LLD	<LLD	5.46E-05	<LLD	<LLD	<LLD	5.65E-04	1.89E-05	2.37E-04	<LLD	<LLD
Other_Enterococcaceae	<LLD	<LLD	<LLD	<LLD	1.59E-05	<LLD	3.40E-05	<LLD	<LLD	<LLD	<LLD	2.13E-05
Mogibacterium	<LLD	<LLD	<LLD	<LLD	<LLD	1.91E-05	<LLD	2.37E-04	<LLD	3.05E-05	2.56E-05	1.07E-04
Bifidobacterium	<LLD	1.25E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	5.68E-05	7.63E-06	<LLD	4.26E-05
Finegoldia	6.77E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.41E-04	7.63E-06	<LLD	<LLD
TG5	<LLD	<LLD	<LLD	1.64E-04	<LLD	<LLD	<LLD	3.46E-04	<LLD	5.34E-05	<LLD	8.53E-05
Candidatus Rhodoluna	9.03E-05	1.25E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_CW040	<LLD	1.25E-05	<LLD	<LLD	<LLD	3.05E-04	<LLD	1.46E-04	<LLD	4.58E-05	7.68E-05	<LLD
Cardiobacterium	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.00E-04	1.89E-05	8.40E-05	<LLD	1.28E-04
Unassigned_ACK-M1	<LLD	6.24E-05	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	2.13E-05
Peptoniphilus	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.57E-05	<LLD	<LLD	8.53E-05

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Unassigned_Sphingobacteriales	<LLD	6.24E-05	<LLD	1.82E-05	<LLD	5.72E-05	<LLD	<LLD	1.89E-05	1.53E-05	<LLD	<LLD
Unassigned_Moraxellaceae	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.07E-04	<LLD
Unassigned_Peptostreptococcaceae	<LLD	<LLD	<LLD	9.09E-05	<LLD	<LLD	<LLD	2.91E-04	<LLD	2.29E-05	<LLD	<LLD
Filifactor	3.38E-05	<LLD	<LLD	9.09E-05	<LLD	<LLD	<LLD	2.19E-04	<LLD	3.05E-05	<LLD	<LLD
Eikenella	<LLD	<LLD	<LLD	2.73E-04	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	6.40E-05
Other_Enterobacteriaceae	<LLD	1.25E-05	<LLD	5.46E-05	1.59E-05	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	2.13E-05
Unassigned_MLE1-12	<LLD	<LLD	<LLD	<LLD	1.59E-05	<LLD	1.02E-04	<LLD	<LLD	7.63E-06	<LLD	<LLD
Thermodesulfovibrio	<LLD	<LLD	<LLD	<LLD	1.27E-04	1.91E-05	5.10E-05	<LLD	7.57E-05	<LLD	<LLD	<LLD
Unassigned_Cytophagaceae	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Carnobacterium	<LLD	1.25E-05	5.19E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.29E-05	<LLD	<LLD
Unassigned_Chitinophagaceae	2.26E-05	4.99E-05	<LLD	<LLD	1.59E-05	1.91E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Nocardioidaceae	3.38E-05	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Anoxybacillus	<LLD	<LLD	3.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.29E-05	<LLD	<LLD
Other_Peptostreptococcaceae	<LLD	1.25E-05	1.90E-04	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Flavobacteriaceae	6.77E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.82E-05	5.68E-05	1.53E-05	<LLD	<LLD
Unassigned_Thermoanaerobacteraceae	<LLD	<LLD	<LLD	<LLD	<LLD	5.72E-05	5.10E-05	<LLD	3.78E-05	7.63E-06	<LLD	4.26E-05
Salinispora	<LLD	<LLD	<LLD	<LLD	1.59E-05	1.91E-05	3.40E-05	<LLD	3.78E-05	2.29E-05	<LLD	<LLD
Microbacterium	<LLD	<LLD	<LLD	1.82E-05	1.59E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	4.26E-05
Unassigned_Opitutaceae	<LLD	<LLD	<LLD	5.46E-05	<LLD	<LLD	<LLD	<LLD	9.46E-05	<LLD	<LLD	<LLD
Scardovia	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.82E-05	<LLD	4.58E-05	<LLD	2.13E-05
Arcobacter	1.13E-05	<LLD	<LLD	<LLD	1.59E-05	1.91E-05	<LLD	<LLD	<LLD	3.82E-05	<LLD	<LLD
Other_Rhizobiales	<LLD	<LLD	<LLD	<LLD	<LLD	1.34E-04	<LLD	<LLD	<LLD	4.58E-05	<LLD	<LLD
Other_Gemellaceae	<LLD	1.25E-05	<LLD	1.82E-05	<LLD	<LLD	<LLD	1.82E-05	<LLD	1.07E-04	2.56E-05	<LLD
Myroides	<LLD	<LLD	1.73E-05	<LLD	<LLD	<LLD	<LLD	1.82E-05	<LLD	<LLD	1.28E-04	<LLD
Other_Clostridiaceae	<LLD	<LLD	1.73E-05	<LLD	<LLD	<LLD	<LLD	<LLD	1.89E-05	<LLD	<LLD	<LLD
Ochrobactrum	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.89E-05	1.53E-05	<LLD	<LLD
Legionella	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.28E-04	<LLD
Unassigned_Aeromonadaceae	3.38E-05	<LLD	<LLD	<LLD	<LLD	<LLD	6.80E-05	<LLD	<LLD	7.63E-06	<LLD	<LLD
Unassigned_Microbacteriaceae	9.03E-05	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Rhodospirillaceae	<LLD	1.25E-05	3.46E-05	1.82E-05	1.59E-05	<LLD	<LLD	<LLD	<LLD	2.29E-05	<LLD	<LLD
Unassigned_Sphingobacteriaceae	1.13E-05	<LLD	<LLD	1.82E-05	<LLD	9.54E-05	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD
Peptococcus	<LLD	1.25E-05	<LLD	<LLD	<LLD	<LLD	<LLD	3.64E-05	<LLD	3.05E-05	<LLD	6.40E-05
Hymenobacter	<LLD	<LLD	<LLD	<LLD	3.19E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Spirobacillales	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.28E-04	<LLD
Polaromonas	2.26E-05	2.50E-05	<LLD	<LLD	<LLD	1.91E-05	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD
Vagococcus	1.13E-05	<LLD	1.73E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.13E-05
Balneimonas	<LLD	<LLD	8.65E-05	3.64E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD

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Paenibacillus	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	3.40E-05	<LLD	7.57E-05	<LLD	<LLD	<LLD
Unassigned_Ruminococcaceae	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.78E-05	<LLD	<LLD	6.40E-05
Brevundimonas	<LLD	1.25E-05	<LLD	<LLD	4.78E-05	<LLD	<LLD	<LLD	<LLD	6.11E-05	<LLD	<LLD
Brevibacterium	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Unassigned_VC2_1_Bac22	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.89E-05	<LLD	<LLD	<LLD
Other_Planococcaceae	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	8.51E-05	<LLD	<LLD	<LLD	<LLD	<LLD
Luteimonas	<LLD	<LLD	<LLD	1.27E-04	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Anaerovorax	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.09E-04	<LLD	1.53E-05	<LLD	<LLD
Unassigned_Rhodocyclaceae	1.13E-05	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD
Blautia	<LLD	<LLD	<LLD	<LLD	<LLD	1.91E-05	<LLD	<LLD	<LLD	<LLD	1.02E-04	<LLD
Devosia	<LLD	<LLD	1.21E-04	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Other_Xanthomonadaceae	<LLD	<LLD	<LLD	<LLD	3.19E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Other_Actinomycetales	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.68E-05	<LLD
Unassigned_Phyllobacteriaceae	<LLD	1.25E-05	<LLD	<LLD	<LLD	1.91E-05	3.40E-05	1.82E-05	<LLD	<LLD	<LLD	<LLD
Desulfobulbus	1.13E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD
Candidatus Rhabdochlamydia	<LLD	4.99E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.78E-05	<LLD	<LLD	<LLD
Leucobacter	<LLD	<LLD	<LLD	5.46E-05	3.19E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Novosphingobium	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.89E-05	<LLD	<LLD	<LLD
Janibacter	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	5.68E-05	<LLD	<LLD	2.13E-05
Leuconostoc	1.13E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	8.53E-05
Unassigned_Beta-proteobacteria	<LLD	1.25E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD
Rhodoplanes	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.02E-04	<LLD
Planomicrobium	<LLD	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Other_Brucellaceae	<LLD	<LLD	8.65E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Desulfovibrio	<LLD	1.25E-05	<LLD	<LLD	<LLD	5.72E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Other_Geodermatophilaceae	<LLD	<LLD	<LLD	5.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Cloacibacterium	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.57E-05	1.53E-05	<LLD	<LLD
Unassigned_[Pedosphaerales]	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD
Other_Caulobacteraceae	<LLD	<LLD	3.46E-05	<LLD	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	<LLD
Unassigned_Sphingomonadaceae	<LLD	1.25E-05	<LLD	1.82E-05	<LLD	<LLD	<LLD	5.47E-05	<LLD	<LLD	<LLD	<LLD
Amaricoccus	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	5.47E-05	<LLD	<LLD	<LLD	<LLD
Labrys	4.51E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_iii1-15	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	5.68E-05	<LLD	<LLD	<LLD
Unassigned_SC3	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Other_Pasteurellaceae	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	4.26E-05
Unassigned_Sporichthyaceae	<LLD	<LLD	3.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	3.78E-05	<LLD	<LLD	<LLD
Thermoanaerovibrio	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	5.68E-05	1.53E-05	<LLD	<LLD

Unassigned_SM1D11	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.68E-05	<LLD
Alloiococcus	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.57E-05	<LLD	<LLD	<LLD
Pimelobacter	<LLD	<LLD	<LLD	<LLD	<LLD	5.72E-05	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD
Dechloromonas	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.82E-05	<LLD	7.63E-06	<LLD	<LLD
Unassigned_Clostridiaceae	<LLD	<LLD	<LLD	7.27E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Thermoanaerobacter	<LLD	<LLD	3.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	2.13E-05
Fluviicola	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Bacteriovoracaceae	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	1.82E-05	<LLD	1.53E-05	<LLD	<LLD
Unassigned_F16	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.64E-05	<LLD	7.63E-06	2.56E-05	<LLD
Chroococcidiopsis	<LLD	<LLD	6.92E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Butyrivibrio	<LLD	1.25E-05	1.73E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	2.13E-05
Unassigned_Patulibacteraceae	1.13E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.64E-05	1.89E-05	<LLD	<LLD	<LLD
Dokdonella	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	5.10E-05	<LLD	<LLD	1.53E-05	<LLD	<LLD
Lysinibacillus	<LLD	<LLD	<LLD	<LLD	6.37E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Porphyromonadaceae	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	4.26E-05
Unassigned_Myxococcales	<LLD	2.50E-05	<LLD	<LLD	<LLD	3.82E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Aurantimonadaceae	<LLD	6.24E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Coprococcus	<LLD	<LLD	<LLD	3.64E-05	1.59E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Agromyces	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Unassigned_TM7-1	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Sharpea	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	6.11E-05	<LLD	<LLD
Friedmanniella	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	4.26E-05
Coprothermobacter	1.13E-05	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Dermacoccus	2.26E-05	3.74E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_WPS-2	<LLD	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	<LLD
Thermus	1.13E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_OP11-3	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	3.40E-05	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Pseudomonadaceae	<LLD	2.50E-05	<LLD	<LLD	3.19E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Elusimicrobiales	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	5.68E-05	<LLD	<LLD	<LLD
Unassigned_Marinilabiaceae	1.13E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Rubellimicrobium	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.64E-05	1.89E-05	<LLD	<LLD	<LLD
Vibrio	3.38E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.13E-05
Aerococcus	4.51E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Geodermatophilus	<LLD	<LLD	<LLD	5.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Pseudonocardia	<LLD	<LLD	<LLD	5.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Ureibacillus	<LLD	<LLD	<LLD	5.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_BD7-3	2.26E-05	<LLD	<LLD	<LLD	3.19E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD

Unassigned_SM2F11	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD
Facklamia	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.78E-05	1.53E-05	<LLD	<LLD
Unassigned_Rhodobacteraceae	<LLD	<LLD	3.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Gordonia	<LLD	1.25E-05	1.73E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.29E-05	<LLD	<LLD
Psychrobacter	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.13E-05
Microvirgula	<LLD	1.25E-05	<LLD	<LLD	<LLD	1.91E-05	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD
Pandoraea	<LLD	<LLD	1.73E-05	<LLD	<LLD	1.91E-05	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Other_Neisseriaceae	<LLD	<LLD	<LLD	<LLD	4.78E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Sphingobium	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.78E-05	<LLD	<LLD	<LLD
Unassigned_Legionellales	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Prosthecobacter	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Euglenozoa	4.51E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Methylophilaceae	2.26E-05	1.25E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Wautersiella	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	4.26E-05
[Ruminococcus]	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	4.26E-05
Unassigned_[Chthoniobacteraceae]	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	4.26E-05
Paucibacter	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD
Unassigned_Acidimicrobiales	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.29E-05	<LLD	<LLD
Unassigned_Burkholderiaceae	1.13E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	2.13E-05
Unassigned_KD8-87	<LLD	1.25E-05	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Unassigned_Bacteroidaceae	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.78E-05	<LLD	<LLD	<LLD
Candidatus Xiphinematobacter	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.78E-05	<LLD	<LLD	<LLD
Bdellovibrio	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Other_Chloroflexaceae	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.64E-05	<LLD	<LLD	<LLD	<LLD
Proteus	1.13E-05	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Exiguobacterium	<LLD	<LLD	<LLD	<LLD	<LLD	1.91E-05	1.70E-05	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Saprospiraceae	2.26E-05	1.25E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Solibacterales	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Phycicoccus	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Symbiobacterium	<LLD	<LLD	3.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Sutterella	<LLD	<LLD	3.46E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Other_Beta-proteobacteria	1.13E-05	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Erwinia	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.40E-05	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_koll11	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_JG30-KF-CM45	<LLD	<LLD	<LLD	<LLD	3.19E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Actinomycetaceae	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	3.05E-05	<LLD	<LLD
Unassigned_IIb	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD

Unassigned_TM7	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Steroidobacter	<LLD	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Ignavibacteriaceae	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Other_Cellulomonadaceae	<LLD	<LLD	1.73E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Streptococcaceae	<LLD	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Rathayibacter	<LLD	<LLD	<LLD	<LLD	1.59E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Sinobacteraceae	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD
Desulfosporosinus	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Hyphomicrobium	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Rickettsiaceae	<LLD	2.50E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Alcaligenes	<LLD	<LLD	1.73E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	7.63E-06	<LLD	<LLD
Azospirillum	<LLD	<LLD	<LLD	<LLD	<LLD	1.91E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Other_Veillonellaceae	<LLD	<LLD	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_RF39	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	2.29E-05	<LLD	<LLD
Unassigned_Ellin6075	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_RF16	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Phenylobacterium	2.26E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Erythrobacteraceae	<LLD	1.25E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Dyadobacter	1.13E-05	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Ellin329	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD
Methyloversatilis	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD
Unassigned_0319-6G20	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD
Other_Oceanospirillales	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	<LLD	1.53E-05	<LLD	<LLD

Table A.54 Relative abundance of genera of paired mask and BAL samples

Color key: ■ Background signals excluded ■ Shared signals between masks and BALs ■ Shared signals after background signals were excluded ■ Unique sigals to a either masks or BALs ■ Unique signals after background signals excluded

	MASS1	MASS3	MASS7a	BAL1	BAL3	BAL7
Unassigned	7.82E-02	9.84E-02	8.34E-02	5.70E-02	6.48E-02	8.43E-02
Rhizobium	1.52E-01	7.20E-01	7.39E-01	4.28E-04	7.49E-04	1.41E-04
Clostridium	1.25E-01	6.78E-02	1.19E-01	1.20E-04	1.05E-04	<LLD
Bacillus	1.94E-01	2.90E-02	2.26E-02	3.34E-04	8.89E-04	1.41E-04
Staphylococcus	3.27E-02	1.22E-03	<LLD	3.70E-03	7.05E-01	1.77E-02
Halomonas	2.73E-02	4.43E-03	6.93E-04	4.48E-03	1.21E-02	2.58E-01
Streptococcus	3.23E-03	3.51E-03	3.74E-03	8.74E-03	1.95E-01	1.56E-02
Sediminibacterium	8.82E-02	2.49E-02	1.01E-02	8.56E-06	1.17E-05	2.25E-03
Leptospira	8.55E-02	3.85E-02	1.21E-02	1.71E-05	1.17E-05	<LLD
Shewanella	1.48E-02	2.13E-03	<LLD	1.43E-03	2.70E-03	6.19E-02
Rothia	8.71E-03	1.19E-04	1.25E-03	3.97E-01	1.17E-04	1.55E-03
Unassigned_Halomonadaceae	2.18E-03	2.46E-04	1.39E-04	9.33E-04	1.24E-03	1.48E-02
Actinomyces	1.69E-03	3.65E-05	<LLD	1.15E-02	4.68E-05	5.35E-03
Lactobacillus	1.37E-01	5.47E-05	<LLD	2.81E-02	2.34E-05	1.41E-03
Unassigned_Veillonellaceae	1.80E-02	1.14E-03	4.16E-03	<LLD	<LLD	<LLD
Enterococcus	9.68E-04	<LLD	<LLD	2.25E-01	1.54E-02	1.41E-04
Corynebacterium	8.87E-04	3.26E-03	<LLD	1.71E-05	<LLD	1.17E-02
Prevotella	2.42E-04	9.21E-04	4.16E-04	5.99E-05	3.51E-05	2.19E-02
Other_Lactobacillales	1.61E-04	8.21E-05	2.77E-04	1.45E-04	2.57E-04	6.05E-03
Acinetobacter	3.23E-04	5.47E-05	2.77E-04	5.99E-05	<LLD	3.66E-03
Veillonella	1.61E-04	1.77E-03	<LLD	2.90E-03	1.17E-04	1.41E-04
Other_Rhodocyclaceae	2.42E-03	2.46E-04	8.32E-04	<LLD	<LLD	<LLD
Other_Enterococcaceae	8.07E-05	<LLD	<LLD	5.13E-04	2.34E-05	1.41E-04
Alicyclobacillus	2.74E-03	1.00E-04	1.39E-04	<LLD	<LLD	<LLD
Haemophilus	3.23E-04	5.47E-05	<LLD	9.41E-05	3.51E-05	<LLD
Thermomonas	5.65E-04	6.38E-05	5.54E-04	<LLD	1.17E-05	<LLD
Gemella	8.07E-05	9.12E-06	<LLD	3.42E-05	3.51E-05	5.63E-04
Mycoplasma	1.94E-03	9.12E-06	<LLD	4.28E-05	1.17E-05	1.70E-02
Meiothermus	7.26E-04	4.56E-05	1.39E-04	<LLD	<LLD	<LLD
Unassigned_Streptophyta	2.34E-03	1.82E-05	<LLD	<LLD	2.34E-05	1.27E-02
Thermoanaerobacterium	3.23E-04	3.65E-05	1.39E-04	<LLD	<LLD	<LLD
Stenotrophomonas	8.07E-05	2.74E-05	<LLD	<LLD	<LLD	3.52E-03
Achromobacter	1.61E-04	2.74E-05	<LLD	<LLD	<LLD	4.22E-04
Kocuria	<LLD	1.82E-05	2.77E-04	8.56E-06	<LLD	5.49E-03
Micrococcus	8.07E-05	1.82E-05	<LLD	<LLD	<LLD	1.51E-02
Fusobacterium	6.37E-03	9.12E-06	<LLD	8.56E-06	<LLD	5.91E-03
Unassigned_Enterobacteriaceae	8.07E-05	<LLD	<LLD	2.56E-01	1.17E-05	<LLD

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Delftia	1.61E-04	<LLD	<LLD	<LLD	1.17E-05	9.85E-04
Parvimonas	1.61E-04	9.12E-06	<LLD	<LLD	<LLD	2.87E-02
Sphingomonas	<LLD	9.12E-06	<LLD	<LLD	2.34E-05	1.28E-02
Propionibacterium	9.68E-04	9.12E-06	<LLD	<LLD	<LLD	4.92E-03
Brachybacterium	<LLD	9.12E-06	<LLD	<LLD	1.17E-05	1.27E-03
Moraxella	<LLD	9.12E-06	<LLD	2.57E-05	3.51E-05	<LLD
Other_Aerococcaceae	9.68E-04	<LLD	<LLD	8.56E-06	<LLD	2.49E-01
Unassigned_Coriobacteriaceae	1.61E-04	<LLD	<LLD	8.56E-06	<LLD	8.44E-04
Pseudomonas	8.07E-04	<LLD	<LLD	<LLD	<LLD	4.22E-04
Neisseria	<LLD	9.12E-06	<LLD	1.71E-05	<LLD	<LLD
[Prevotella]	<LLD	<LLD	1.39E-04	<LLD	<LLD	1.93E-02
Porphyromonas	8.07E-05	<LLD	<LLD	<LLD	1.17E-05	<LLD
Unassigned_Methylobacteriaceae	8.07E-05	<LLD	<LLD	<LLD	<LLD	2.18E-02
Granulicatella	<LLD	<LLD	<LLD	9.41E-05	1.52E-04	<LLD
Treponema	3.23E-04	<LLD	<LLD	<LLD	<LLD	1.10E-02
Dialister	<LLD	<LLD	<LLD	1.71E-05	<LLD	1.36E-02
Capnocytophaga	1.61E-04	<LLD	<LLD	<LLD	<LLD	1.13E-03
Unassigned_Bradyrhizobiaceae	<LLD	<LLD	<LLD	<LLD	<LLD	7.18E-03
Campylobacter	2.42E-04	<LLD	<LLD	<LLD	<LLD	4.36E-03
Bulleidia	<LLD	<LLD	<LLD	1.71E-05	<LLD	5.63E-03
Leptotrichia	<LLD	1.82E-05	<LLD	<LLD	3.51E-05	<LLD
Flavobacterium	<LLD	<LLD	<LLD	<LLD	<LLD	2.67E-03
Megasphaera	<LLD	<LLD	<LLD	8.56E-06	<LLD	4.92E-03
Oribacterium	<LLD	<LLD	<LLD	<LLD	<LLD	2.67E-03
Fervidobacterium	<LLD	<LLD	<LLD	<LLD	<LLD	4.22E-04
Tannerella	1.61E-04	<LLD	<LLD	<LLD	<LLD	1.83E-03
Selenomonas	<LLD	2.74E-05	<LLD	<LLD	<LLD	1.97E-03
Moryella	<LLD	<LLD	<LLD	<LLD	<LLD	2.39E-03
Atopobium	<LLD	9.12E-06	<LLD	<LLD	<LLD	2.25E-03
Unassigned_Rhizobiales	<LLD	9.12E-06	<LLD	<LLD	<LLD	2.39E-03
Paludibacter	<LLD	<LLD	<LLD	<LLD	<LLD	1.13E-03
Slackia	<LLD	<LLD	<LLD	<LLD	<LLD	2.25E-03
Unassigned_Clostridiales	8.07E-05	<LLD	<LLD	<LLD	<LLD	1.13E-03
Unassigned_Caulobacteraceae	<LLD	<LLD	<LLD	<LLD	<LLD	4.22E-04
Sphaerochaeta	2.10E-03	<LLD	<LLD	<LLD	<LLD	<LLD
Methylobacterium	<LLD	2.74E-05	<LLD	<LLD	<LLD	9.85E-04
Catonella	<LLD	<LLD	<LLD	<LLD	<LLD	1.13E-03
Unassigned_Propionibacteriaceae	<LLD	<LLD	<LLD	<LLD	<LLD	1.55E-03
Unassigned_Comamonadaceae	<LLD	<LLD	<LLD	8.56E-06	<LLD	5.63E-04
Agrobacterium	<LLD	<LLD	<LLD	<LLD	<LLD	1.55E-03
Unassigned_Bacteroidales	1.61E-04	<LLD	<LLD	<LLD	<LLD	1.13E-03
Schwartzia	<LLD	<LLD	<LLD	<LLD	<LLD	1.69E-03

Unassigned_Bacillales	1.29E-03	2.28E-04	<LLD	<LLD	<LLD	<LLD
Unassigned_[Mogibacteriaceae]	2.42E-04	9.12E-06	<LLD	<LLD	<LLD	<LLD
Curtobacterium	<LLD	<LLD	<LLD	<LLD	<LLD	1.55E-03
Enhydrobacter	<LLD	1.19E-04	<LLD	<LLD	<LLD	<LLD
Anaerococcus	<LLD	9.12E-06	<LLD	<LLD	<LLD	<LLD
Lactococcus	1.61E-04	<LLD	<LLD	<LLD	<LLD	<LLD
Comamonas	<LLD	9.12E-06	<LLD	<LLD	<LLD	<LLD
Unassigned_Gemellaceae	<LLD	9.12E-06	<LLD	<LLD	<LLD	<LLD
Other_Rhizobiaceae	<LLD	3.37E-04	2.77E-04	<LLD	<LLD	<LLD
Roseomonas	<LLD	<LLD	<LLD	<LLD	<LLD	1.13E-03
Rubrobacter	<LLD	<LLD	<LLD	<LLD	<LLD	1.13E-03
Unassigned_TM7-3	<LLD	<LLD	<LLD	<LLD	<LLD	4.22E-04
Dermatophilus	<LLD	<LLD	<LLD	<LLD	<LLD	9.85E-04
Ralstonia	<LLD	<LLD	<LLD	8.56E-06	<LLD	<LLD
Bilophila	<LLD	<LLD	<LLD	4.28E-05	<LLD	8.44E-04
Unassigned_Pasteurellaceae	<LLD	<LLD	<LLD	1.71E-05	<LLD	<LLD
Unassigned_Lactobacillales	<LLD	<LLD	<LLD	7.62E-04	1.17E-05	<LLD
Mogibacterium	3.23E-04	<LLD	<LLD	<LLD	<LLD	<LLD
Deinococcus	1.61E-04	<LLD	<LLD	<LLD	<LLD	<LLD
Saccharopolyspora	<LLD	<LLD	<LLD	<LLD	<LLD	7.03E-04
Escherichia	<LLD	<LLD	<LLD	7.02E-04	<LLD	<LLD
Bifidobacterium	<LLD	<LLD	<LLD	8.56E-05	<LLD	4.22E-04
Unassigned_Bacillaceae	<LLD	<LLD	<LLD	<LLD	1.17E-05	<LLD
Thermosinus	2.42E-04	9.12E-06	<LLD	<LLD	<LLD	<LLD
Other_Clostridia	8.07E-05	9.12E-06	<LLD	<LLD	<LLD	<LLD
Unassigned_Oxalobacteraceae	<LLD	4.56E-05	<LLD	<LLD	<LLD	<LLD
Peptoniphilus	8.07E-05	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Moraxellaceae	<LLD	2.74E-05	<LLD	<LLD	<LLD	<LLD
Paracoccus	<LLD	5.47E-05	<LLD	8.56E-06	<LLD	<LLD
Other_Enterobacteriaceae	<LLD	<LLD	<LLD	7.70E-05	<LLD	<LLD
Other_Clostridiales	8.07E-05	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Xenococcaceae	3.23E-04	<LLD	<LLD	<LLD	<LLD	<LLD
Nocardioides	<LLD	2.28E-04	<LLD	<LLD	<LLD	<LLD
Carnobacterium	8.07E-05	<LLD	<LLD	2.57E-05	<LLD	<LLD
Unassigned_Nocardioidaceae	1.61E-04	2.74E-05	<LLD	<LLD	<LLD	<LLD
Anoxybacillus	1.61E-04	<LLD	<LLD	<LLD	<LLD	<LLD
Scardovia	<LLD	<LLD	<LLD	<LLD	1.17E-04	<LLD
Other_Carnobacteriaceae	<LLD	9.12E-06	<LLD	<LLD	<LLD	<LLD
Other_Clostridiaceae	8.07E-05	<LLD	<LLD	<LLD	<LLD	<LLD
Pontibacter	1.61E-04	<LLD	<LLD	<LLD	<LLD	<LLD
Unassigned_Acetobacteraceae	<LLD	4.56E-05	<LLD	<LLD	<LLD	<LLD
Unassigned_Microbacteriaceae	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD

Vagococcus	<LLD	<LLD	<LLD	<LLD	3.51E-05	<LLD
Desulfobulbus	8.07E-05	<LLD	<LLD	<LLD	<LLD	<LLD
Planomicrobium	8.07E-05	<LLD	<LLD	<LLD	<LLD	<LLD
Other_Geodermatophilaceae	<LLD	3.65E-05	<LLD	<LLD	<LLD	<LLD
Unassigned_Planococcaceae	<LLD	<LLD	<LLD	<LLD	8.19E-05	<LLD
Other_Bacillales	<LLD	<LLD	<LLD	<LLD	7.02E-05	<LLD
Unassigned_[Tissierellaceae]	<LLD	9.12E-06	<LLD	<LLD	<LLD	<LLD
Unassigned_Enterococcaceae	<LLD	<LLD	<LLD	6.85E-05	<LLD	<LLD
Butyrivibrio	<LLD	<LLD	<LLD	8.56E-06	<LLD	<LLD
Unassigned_Nocardiaceae	<LLD	4.56E-05	<LLD	<LLD	<LLD	<LLD
Friedmanniella	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD
Unassigned_Rhodobacteraceae	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD
Unassigned_Bacteria	<LLD	<LLD	<LLD	3.42E-05	1.17E-05	<LLD
Unassigned_Acidimicrobiales	<LLD	1.82E-05	<LLD	<LLD	<LLD	<LLD
Kaistobacter	<LLD	2.74E-05	<LLD	<LLD	<LLD	<LLD
Jonquetella	<LLD	<LLD	<LLD	2.57E-05	<LLD	<LLD
Rathayibacter	<LLD	9.12E-06	<LLD	<LLD	<LLD	<LLD
Other_Staphylococcaceae	<LLD	<LLD	<LLD	<LLD	2.34E-05	<LLD
Other_Shewanellaceae	<LLD	<LLD	<LLD	<LLD	2.34E-05	<LLD
Unassigned_Erythrobacteraceae	<LLD	9.12E-06	<LLD	<LLD	<LLD	<LLD

Table A.55 Comparison of richness, diversity and evenness indices at phylum level between different respiratory activities

α diversity	S_{Chao1}	H	1-D	E_H
NB60_1	120.00	0.54	0.26	0.20
NB60_3	153.00	0.69	0.31	0.24
NB60_4	66.00	0.55	0.27	0.23
NB (median)	**120.00**	**0.55**	**0.27**	**0.23**
IB60_1	171.00	0.67	0.31	0.23
IB60_3	78.00	0.42	0.19	0.17
IB60_4	78.00	0.68	0.36	0.28
IB (median)	**78.00**	**0.67**	**0.31**	**0.23**
IC60_1	91.00	0.50	0.24	0.20
IC60_3	171.00	0.92	0.42	0.32
IC60_4	190.00	0.56	0.25	0.19
IC (median)	**171.00**	**0.56**	**0.25**	**0.20**
RL30_1	190.00	0.68	0.31	0.23
RL30_3	55.00	0.98	0.53	0.43
RL30_4	120.00	0.92	0.44	0.34
RL (median)	**120.00**	**0.92**	**0.44**	**0.34**
P value*	0.91	0.15	0.19	0.20

β diversity	S_S		S_J	
NB60_1 vs IB60_1	0.73		0.57	
NB60_3 vs IB60_3	0.62		0.45	
NB60_4 vs IB60_4	0.70		0.53	
NB vs IB (median)	**0.70**		**0.53**	
NB60_1 vs IC60_1	0.79		0.65	
NB60_3 vs IC60_3	0.69		0.52	
NB60_4 vs IC60_4	0.73		0.58	
NB vs IC (median)	**0.73**		**0.58**	
NB60_1 vs RL30_1	0.76		0.62	
NB60_3 vs RL30_3	0.67		0.50	
NB60_4 vs RL30_4	0.85		0.73	
NB vs RL (median)	**0.76**		**0.62**	
IB60_1 vs IC60_1	0.71		0.55	
IB60_3 vs IC60_3	0.73		0.58	
IB60_4 vs IC60_4	0.65		0.48	
IB vs IC (median)	**0.71**		**0.55**	
IB60_1 vs RL30_1	0.92		0.85	
IB60_3 vs RL30_3	0.73		0.57	
IB60_4 vs RL30_4	0.74		0.59	
IB vs RL (median)	**0.74**		**0.59**	
IC60_1 vs RL30_1	0.69		0.52	

IC60_3 vs RL30_3	0.71	0.56
IC60_4 vs RL30_4	0.82	0.70
IC vs RL (median)	**0.71**	**0.56**

(*) Friedman test (non-parametric ANOVA) was done

Table A.56 Comparison of richness, diversity and evenness indices at genus level between different respiratory activities

α diversity	S_{Chao1}	H	1-D	E_H
NB60_1	8385.00	1.39	0.65	0.29
NB60_3	11628.00	1.69	0.67	0.34
NB60_4	5253.00	1.41	0.64	0.30
NB (median)	**8385.00**	**1.41**	**0.65**	**0.30**
IB60_1	8911.00	1.56	0.67	0.32
IB60_3	4095.00	1.29	0.62	0.29
IB60_4	4095.00	1.24	0.54	0.28
IB (median)	**4095.00**	**1.29**	**0.62**	**0.29**
IC60_1	3655.00	1.39	0.62	0.31
IC60_3	10011.00	2.19	0.77	0.44
IC60_4	8646.00	1.44	0.61	0.29
IC (median)	**8646.00**	**1.44**	**0.62**	**0.31**
RL30_1	16653.00	1.84	0.70	0.35
RL30_3	4465.00	1.74	0.69	0.38
RL30_4	8778.00	2.21	0.79	0.45
RL (median)	**8778.00**	**1.84**	**0.70**	**0.38**
P value*	0.52	0.21	0.34	0.30

β diversity	S_S		S_J	
NB60_1 vs IB60_1	0.58		0.41	
NB60_3 vs IB60_3	0.59		0.42	
NB60_4 vs IB60_4	0.55		0.38	
NB vs IB (median)	**0.58**		**0.41**	
NB60_1 vs IC60_1	0.63		0.46	
NB60_3 vs IC60_3	0.68		0.52	
NB60_4 vs IC60_4	0.64		0.47	
NB vs IC (median)	**0.64**		**0.47**	
NB60_1 vs RL30_1	0.60		0.43	
NB60_3 vs RL30_3	0.63		0.46	
NB60_4 vs RL30_4	0.58		0.41	
NB vs RL (median)	**0.60**		**0.43**	
IB60_1 vs IC60_1	0.55		0.38	
IB60_3 vs IC60_3	0.51		0.34	
IB60_4 vs IC60_4	0.56		0.39	

IB vs IC (median)	**0.55**	**0.38**
IB60_1 vs RL30_1	0.66	0.49
IB60_3 vs RL30_3	0.52	0.35
IB60_4 vs RL30_4	0.51	0.35
IB vs RL (median)	**0.52**	**0.35**
IC60_1 vs RL30_1	0.55	0.38
IC60_3 vs RL30_3	0.68	0.52
IC60_4 vs RL30_4	0.67	0.50
IC vs RL (median)	**0.67**	**0.50**
(*) Friedman test (non-parametric ANOVA) was done		

Table A.57 Eigenvalues and squared cosines for PCA and PCoA

Figure 5.6B, c and D

Eigenvalues

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
Eigenvalue	7.79	4.45	3.71	2.52	2.19	1.84	1.62	1.40	0.72	0.54	0.22
Variability (%)	28.84	16.47	13.74	9.33	8.10	6.83	6.01	5.19	2.66	2.01	0.83
Cumulative %	28.84	45.31	59.05	68.38	76.47	83.31	89.31	94.50	97.16	99.17	100.00

Squared cosines

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
NB60_1	0.29	0.02	0.08	0.01	0.36	0.13	0.03	0.04	0.03	0.01	0.00
IB60_1	0.07	0.02	0.08	0.16	0.28	0.33	0.00	0.05	0.00	0.00	0.00
IC60_1	0.37	0.08	0.12	0.00	0.00	0.05	0.13	0.04	0.20	0.01	0.00
RL30_1	0.08	0.00	0.27	0.08	0.05	0.09	0.11	0.32	0.00	0.01	0.00
NB60_3	0.10	0.15	0.11	0.15	0.03	0.20	0.19	0.00	0.06	0.00	0.00
IB60_3	0.16	0.03	0.12	0.49	0.08	0.01	0.01	0.03	0.05	0.01	0.00
IC60_3	0.76	0.11	0.03	0.04	0.03	0.00	0.02	0.00	0.00	0.00	0.00
RL30_3	0.24	0.26	0.45	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.02
NB60_4	0.18	0.24	0.01	0.01	0.06	0.00	0.05	0.00	0.01	0.40	0.03
IB60_4	0.06	0.37	0.24	0.07	0.05	0.03	0.02	0.07	0.00	0.02	0.06
IC60_4	0.24	0.51	0.15	0.04	0.00	0.02	0.03	0.01	0.01	0.00	0.00
RL30_4	0.02	0.00	0.00	0.15	0.12	0.06	0.35	0.26	0.01	0.00	0.03

Figure 5.6E

Eigenvalues

	F1	F2	F3	F4	F5	F6
Eigenvalue	0.01	0.00	0.00	0.00	0.00	0.00
Variability (%)	89.41	6.83	3.40	0.25	0.07	0.04
Cumulative %	89.41	96.25	99.65	99.89	99.96	100.00

Squared cosines

	F1	F2	F3	F4	F5	F6
NB60_1	0.95	0.03	0.02	0.00	0.00	0.00
IB60_1	0.25	0.57	0.17	0.00	0.01	0.00
IC60_1	0.80	0.06	0.14	0.00	0.00	0.00
RL30_1	0.39	0.36	0.20	0.04	0.00	0.00
NB60_3	0.78	0.05	0.04	0.10	0.00	0.03
IB60_3	0.99	0.00	0.01	0.00	0.00	0.00
IC60_3	0.75	0.12	0.12	0.00	0.00	0.00
RL30_3	0.99	0.00	0.01	0.00	0.00	0.00
NB60_4	0.82	0.01	0.17	0.00	0.00	0.00
IB60_4	0.23	0.75	0.01	0.01	0.00	0.00
IC60_4	0.98	0.00	0.00	0.01	0.01	0.00
RL30_4	0.92	0.07	0.00	0.00	0.00	0.00

Figure 5.6F and G

Eigenvalues

	F1	F2	F3	F4	F5	F6	F7
Eigenvalue	0.06	0.01	0.00	0.00	0.00	0.00	0.00
Inertia (eig>0)(%)	78.01	15.18	3.68	2.24	0.65	0.22	0.03
Cumulative %	78.01	93.19	96.87	99.11	99.75	99.97	100.00

Squared cosines

	F1	F2	F3	F4	F5	F6	F7
NB60_1	0.85	0.05	0.02	0.02	0.01	0.06	0.00

IB60_1	0.10	0.48	0.42	0.01	0.00	0.00	0.00				
IC60_1	0.77	0.00	0.18	0.02	0.01	0.00	0.00				
RL30_1	0.39	0.38	0.15	0.06	0.02	0.00	0.00				
NB60_3	0.39	0.29	0.13	0.10	0.05	0.05	0.00				
IB60_3	0.95	0.02	0.00	0.01	0.02	0.00	0.00				
IC60_3	0.43	0.52	0.01	0.04	0.00	0.00	0.00				
RL30_3	0.96	0.04	0.00	0.00	0.00	0.00	0.00				
NB60_4	0.78	0.01	0.18	0.02	0.01	0.00	0.01				
IB60_4	0.25	0.57	0.01	0.17	0.00	0.00	0.00				
IC60_4	0.90	0.00	0.00	0.05	0.04	0.00	0.00				
RL30_4	0.66	0.28	0.01	0.04	0.00	0.00	0.00				

Figure 5.7B and C

Eigenvalues

	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**
Eigenvalue	55.72	40.33	33.98	29.96	29.83	27.10	24.80	22.99	20.16	17.25	13.89
Variability (%)	17.63	12.76	10.75	9.48	9.44	8.58	7.85	7.28	6.38	5.46	4.39
Cumulative %	17.63	30.39	41.15	50.63	60.07	68.65	76.49	83.77	90.15	95.61	100.00

Squared cosines

NB60_1	0.19	0.03	0.03	0.02	0.20	0.21	0.15	0.12	0.04	0.00	0.00
IB60_1	0.00	0.06	0.05	0.51	0.18	0.00	0.16	0.01	0.03	0.00	0.00
IC60_1	0.20	0.02	0.03	0.01	0.00	0.00	0.04	0.10	0.29	0.01	0.30
RL30_1	0.04	0.01	0.39	0.37	0.08	0.02	0.03	0.07	0.00	0.00	0.00
NB60_3	0.01	0.01	0.00	0.03	0.44	0.01	0.34	0.11	0.04	0.00	0.00
IB60_3	0.18	0.00	0.02	0.02	0.00	0.02	0.01	0.03	0.17	0.12	0.42
IC60_3	0.75	0.06	0.00	0.00	0.00	0.08	0.03	0.08	0.00	0.00	0.00
RL30_3	0.03	0.19	0.25	0.04	0.00	0.18	0.01	0.14	0.00	0.14	0.02
NB60_4	0.11	0.01	0.00	0.03	0.13	0.08	0.16	0.17	0.29	0.01	0.00
IB60_4	0.02	0.18	0.06	0.01	0.00	0.04	0.00	0.02	0.10	0.55	0.01
IC60_4	0.05	0.22	0.40	0.02	0.05	0.21	0.04	0.00	0.00	0.00	0.00
RL30_4	0.19	0.56	0.00	0.05	0.05	0.09	0.01	0.04	0.00	0.01	0.00

Figure 5.7D

Eigenvalues

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	
Eigenvalue	0.03	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
Variability (%)	77.45	16.82	4.64	0.76	0.15	0.08	0.06	0.02	0.01	0.01	
Cumulative %	77.45	94.27	98.92	99.68	99.83	99.91	99.96	99.98	99.99	100.00	

Squared cosines

NB60_1	0.76	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
IB60_1	0.40	0.18	0.00	0.40	0.00	0.00	0.00	0.00	0.00	0.00	
IC60_1	0.99	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
RL30_1	0.17	0.08	0.53	0.10	0.06	0.00	0.05	0.00	0.00	0.00	
NB60_3	0.84	0.03	0.07	0.02	0.00	0.03	0.00	0.00	0.00	0.00	
IB60_3	0.94	0.05	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
IC60_3	0.43	0.49	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
RL30_3	0.81	0.03	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
NB60_4	0.05	0.87	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	
IB60_4	0.88	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
IC60_4	0.29	0.59	0.09	0.01	0.00	0.01	0.01	0.00	0.00	0.00	

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| RL30_4 | 0.53 | 0.44 | 0.03 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | |

Figure 5.7E and F

Eigenvalues

	F1	F2	F3	F4	F5	F6	F7				
Eigenvalue	0.27	0.11	0.03	0.02	0.01	0.01	0.00				
Inertia (eig>0)(%)	59.91	23.89	6.99	4.75	2.87	1.44	0.16				
Cumulative %	59.91	83.80	90.78	95.53	98.40	99.84	100.00				

Squared cosines

	F1	F2	F3	F4	F5	F6	F7				
NB60_3	0.35	0.00	0.08	0.33	0.13	0.09	0.01				
IB60_3	0.77	0.01	0.15	0.00	0.03	0.03	0.00				
IC60_3	0.41	0.53	0.04	0.01	0.00	0.00	0.00				
RL30_3	0.79	0.04	0.11	0.01	0.04	0.01	0.00				
NB60_1	0.74	0.11	0.04	0.08	0.00	0.00	0.03				
IB60_1	0.14	0.02	0.10	0.10	0.28	0.36	0.01				
IC60_1	0.96	0.01	0.00	0.02	0.01	0.00	0.00				
RL30_1	0.02	0.41	0.02	0.48	0.07	0.00	0.00				
NB60_4	0.18	0.68	0.07	0.01	0.00	0.00	0.05				
IB60_4	0.58	0.33	0.02	0.03	0.05	0.00	0.00				
IC60_4	0.02	0.42	0.04	0.46	0.02	0.04	0.00				
RL30_4	0.35	0.40	0.22	0.00	0.01	0.01	0.00				

Figure 5.9B

Eigenvalues

	F1	F2	F3	F4	F5						
Eigenvalue	5.75	3.66	2.79	1.67	0.12						
Variability (%)	41.07	26.17	19.93	11.94	0.89						
Cumulative %	41.07	67.24	87.17	99.11	100.00						

Squared cosines

	F1	F2	F3	F4	F5						
MASS1	0.23	0.32	0.41	0.04	0.00						
BAL1	0.69	0.17	0.03	0.12	0.00						
MASS3	0.00	0.34	0.59	0.02	0.04						
BAL3	0.25	0.00	0.09	0.65	0.00						
MASS7a	0.04	0.23	0.66	0.00	0.08						
BAL7	0.60	0.40	0.01	0.00	0.00						

Figure 5.9C

Eigenvalues

	F1	F2	F3	F4	F5						
Eigenvalue	0.14	0.03	0.00	0.00	0.00						
Variability (%)	80.87	17.06	1.93	0.13	0.01						
Cumulative %	80.87	97.93	99.86	99.99	100.00						

Squared cosines

	F1	F2	F3	F4	F5						
MASS1	0.78	0.03	0.19	0.00	0.00						
BAL1	0.00	1.00	0.00	0.00	0.00						
MASS3	0.96	0.04	0.00	0.00	0.00						
BAL3	0.96	0.03	0.01	0.00	0.00						
MASS7a	0.92	0.06	0.01	0.00	0.00						
BAL7	0.35	0.08	0.17	0.40	0.00						

Figure 5.9D and E

Eigenvalues				
	F1	**F2**	**F3**	**F4**
Eigenvalue	0.46	0.15	0.05	0.01
Inertia (eig>0)(%)	68.02	22.67	7.41	1.90
Cumulative %	68.02	90.69	98.10	100.00
Squared cosines				
MASS1	0.55	0.02	0.40	0.03
BAL1	0.00	0.97	0.02	0.00
MASS3	0.89	0.10	0.00	0.01
BAL3	0.89	0.07	0.04	0.00
MASS7a	0.86	0.08	0.06	0.00
BAL7	0.06	0.06	0.32	0.56

Figure 5.10B

Eigenvalues					
	F1	**F2**	**F3**	**F4**	**F5**
Eigenvalue	62.56	33.64	24.27	15.31	6.21
Variability (%)	44.06	23.69	17.09	10.78	4.37
Cumulative %	44.06	67.75	84.84	95.63	100.00
Squared cosines					
MASS1	0.09	0.90	0.00	0.00	0.01
BAL1	0.10	0.15	0.40	0.34	0.01
MASS3	0.15	0.15	0.67	0.02	0.01
BAL3	0.11	0.13	0.15	0.57	0.05
MASS7a	0.20	0.01	0.00	0.05	0.74
BAL7	1.00	0.00	0.00	0.00	0.00

Figure 5.10C and D

Eigenvalues					
	F1	**F2**	**F3**	**F4**	**F5**
Eigenvalue	0.14	0.07	0.03	0.01	0.00
Variability (%)	54.15	26.92	13.16	5.65	0.12
Cumulative %	54.15	81.07	94.23	99.88	100.00
Squared cosines					
MASS1	0.01	0.07	0.14	0.78	0.00
BAL1	0.22	0.52	0.26	0.00	0.00
MASS3	0.95	0.03	0.01	0.01	0.00
BAL3	0.53	0.45	0.01	0.00	0.00
MASS7a	0.95	0.03	0.01	0.01	0.00
BAL7	0.22	0.15	0.54	0.08	0.00

Figure 5.10E and F

Eigenvalues					
	F1	**F2**	**F3**	**F4**	**F5**
Eigenvalue	0.72	0.42	0.39	0.21	0.00
Inertia (eig>0)(%)	41.28	24.18	22.36	12.00	0.16
Cumulative %	41.28	65.47	87.83	99.84	100.00
Squared cosines					
MASS1	0.19	0.00	0.01	0.80	0.00

BAL1	0.33	0.66	0.01	0.00	0.00						
MASS3	0.87	0.00	0.01	0.11	0.01						
BAL3	0.34	0.12	0.53	0.00	0.00						
MASS7a	0.88	0.00	0.01	0.10	0.01						
BAL7	0.22	0.29	0.47	0.02	0.00						

Bibliography

AARON, S. D. 2016. Changes in the Cystic Fibrosis Airway Microbiome after Lung Transplant: The More Things Change, the More They Stay the Same. *Ann Am Thorac Soc,* 13**,** 2109-2110.

AAS, J. A., PASTER, B. J., STOKES, L. N., OLSEN, I. & DEWHIRST, F. E. 2005. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol,* 43**,** 5721-32.

ABREU-ACOSTA, N., LORENZO-MORALES, J., LEAL-GUIO, Y., CORONADO-ALVAREZ, N., FORONDA, P., ALCOBA-FLOREZ, J., IZQUIERDO, F., BATISTA-DIAZ, N., DEL AGUILA, C. & VALLADARES, B. 2005. Enterocytozoon bieneusi (microsporidia) in clinical samples from immunocompetent individuals in Tenerife, Canary Islands, Spain. *Trans R Soc Trop Med Hyg,* 99**,** 848-55.

ABU-HASAN, M., ELMALLAH, M., NEAL, D. & BROOKES, J. 2014. Salivary amylase level in bronchoalveolar fluid as a marker of chronic pulmonary aspiration in children. *Pediatr Allergy Immunol Pulmonol,* 27**,** 115-119.

ADAL, K. A., ANGLIM, A. M., PALUMBO, C. L., TITUS, M. G., COYNER, B. J. & FARR, B. M. 1994. The use of high-efficiency particulate air-filter respirators to protect hospital workers from tuberculosis. A cost-effectiveness analysis. *N Engl J Med,* 331**,** 169-73.

ADAMS, R. I., BHANGAR, S., PASUT, W., ARENS, E. A., TAYLOR, J. W., LINDOW, S. E., NAZAROFF, W. W. & BRUNS, T. D. 2015. Chamber bioaerosol study: outdoor air and human occupants as sources of indoor airborne microbes. *PLOS ONE,* 10**,** e0128022.

ADANG, R. P., SCHOUTEN, H. C., VAN TIEL, F. H. & BLIJHAM, G. H. 1992. Pneumonia due to Micrococcus spp. in a patient with acute myeloid leukaemia. *Leukemia,* 6**,** 224-6.

ADERAYE, G., WOLDEAMANUEL, Y., ASRAT, D., LEBBAD, M., BESER, J., WORKU, A., FERNANDEZ, V. & LINDQUIST, L. 2008. Evaluation of Toluidine Blue O staining for the diagnosis of Pneumocystis jiroveci in expectorated sputum sample and bronchoalveolar lavage from HIV-infected patients in a tertiary care referral center in Ethiopia. *Infection,* 36**,** 237-43.

AFELTRA, J., MEIS, J. F., VITALE, R. G., MOUTON, J. W., VERWEIJ, P. E. & EUROFUNG, N. 2002. In vitro activities of pentamidine, pyrimethamine, trimethoprim, and sulfonamides against Aspergillus species. *Antimicrob Agents Chemother,* 46**,** 2029-31.

AFONINA, I., ZIVARTS, M., KUTYAVIN, I., LUKHTANOV, E., GAMPER, H. & MEYER, R. B. 1997. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res,* 25**,** 2657-60.

AHN, J. H., KIM, B. Y., SONG, J. & WEON, H. Y. 2012. Effects of PCR cycle number and DNA polymerase type on the 16S rRNA gene pyrosequencing analysis of bacterial communities. *J Microbiol,* 50**,** 1071-4.

AKKERMAN, O. W., VAN DER WERF, T. S., DE BOER, M., DE BEER, J. L., RAHIM, Z., ROSSEN, J. W., VAN SOOLINGEN, D., KERSTJENS, H. A. & VAN DER ZANDEN, A. G. 2013. Comparison of 14 molecular assays for detection of Mycobacterium tuberculosis complex in bronchoalveolar lavage fluid. *J Clin Microbiol,* 51**,** 3505-11.

ALANIO, A. & BRETAGNE, S. 2017. Pneumocystis jirovecii detection in asymptomatic patients: what does its natural history tell us? *F1000Res,* 6**,** 739.

ALANIO, A., DESOUBEAUX, G., SARFATI, C., HAMANE, S., BERGERON, A., AZOULAY, E., MOLINA, J. M., DEROUIN, F. & MENOTTI, J. 2011. Real-time PCR assay-based strategy for differentiation between active Pneumocystis jirovecii pneumonia and colonization in immunocompromised patients. *Clin Microbiol Infect,* 17**,** 1531-7.

ALANIO, A., HAUSER, P. M., LAGROU, K., MELCHERS, W. J., HELWEG-LARSEN, J., MATOS, O., CESARO, S., MASCHMEYER, G., EINSELE, H., DONNELLY, J. P., CORDONNIER, C., MAERTENS, J. & BRETAGNE, S. 2016. ECIL guidelines for the diagnosis of Pneumocystis jirovecii pneumonia in patients with haematological malignancies and stem cell transplant recipients. *J Antimicrob Chemother,* 71**,** 2386-96.

ALBERTSEN, M., KARST, S. M., ZIEGLER, A. S., KIRKEGAARD, R. H. & NIELSEN, P. H. 2015. Back to Basics – The Influence of DNA Extraction and Primer Choice on Phylogenetic Analysis of Activated Sludge Communities. *PLOS ONE,* 10**,** e0132783.

ALDOUS, W. K., POUNDER, J. I., CLOUD, J. L. & WOODS, G. L. 2005. Comparison of six methods of extracting Mycobacterium tuberculosis DNA from processed sputum for testing by quantitative real-time PCR. *J Clin Microbiol,* 43**,** 2471-3.

ALIOUAT-DENIS, C. M., CHABE, M., DEMANCHE, C., ALIOUAT EL, M., VISCOGLIOSI, E., GUILLOT, J., DELHAES, L. & DEI-CAS, E. 2008. Pneumocystis species, co-evolution and pathogenic power. *Infect Genet Evol,* 8**,** 708-26.

ALMEIDA, E. P., ALMEIDA, A. C., ALMEIDA, F. F., MONTESSI, J., GOMES, C. A. & FERREIRA, L. E. 2015a. Transtracheal puncture: a forgotten procedure. *Braz J Med Biol Res,* 48**,** 725-7.

ALMEIDA, J. M., CISSE, O. H., FONSECA, A., PAGNI, M. & HAUSER, P. M. 2015b. Comparative genomics suggests primary homothallism of Pneumocystis species. *MBio,* 6.

ALMSTRAND, A. C., BAKE, B., LJUNGSTROM, E., LARSSON, P., BREDBERG, A., MIRGORODSKAYA, E. & OLIN, A. C. 2010. Effect of airway opening on production of exhaled particles. *J Appl Physiol (1985),* 108**,** 584-8.

ALMSTRAND, A. C., LJUNGSTROM, E., LAUSMAA, J., BAKE, B., SJOVALL, P. & OLIN, A. C. 2009. Airway monitoring by collection and mass spectrometric analysis of exhaled particles. *Anal Chem,* 81**,** 662-8.

ALONSO-ECHANOVE, J., GRANICH, R. M., LASZLO, A., CHU, G., BORJA, N., BLAS, R., OLORTEGUI, A., BINKIN, N. J. & JARVIS, W. R. 2001. Occupational transmission of Mycobacterium tuberculosis to health care workers in a university hospital in Lima, Peru. *Clin Infect Dis,* 33**,** 589-96.

ALTMAN, D. 2013. *Statistics with Confidence Confidence Intervals and Statistical Guidelines,* Hoboken, Wiley.

ALVAREZ-MARTINEZ, M. J., MIRO, J. M., VALLS, M. E., MORENO, A., RIVAS, P. V., SOLE, M., BENITO, N., DOMINGO, P., MUNOZ, C., RIVERA, E., ZAR, H. J., WISSMANN, G., DIEHL, A. R., PROLLA, J. C., DE ANTA, M. T., GATELL, J. M., WILSON, P. E. & MESHNICK, S. R. 2006. Sensitivity and specificity of nested and real-time PCR for the detection of Pneumocystis jiroveci in clinical specimens. *Diagn Microbiol Infect Dis,* 56**,** 153-60.

AMARO, A., DUARTE, E., AMADO, A., FERRONHA, H. & BOTELHO, A. 2008. Comparison of three DNA extraction methods for Mycobacterium bovis, Mycobacterium tuberculosis and Mycobacterium avium subsp. avium. *Lett Appl Microbiol,* 47**,** 8-11.

AMRHEIN, V., GREENLAND, S. & MCSHANE, B. 2019. Scientists rise up against statistical significance. *Nature,* 567**,** 305-307.

ANAISSIE, E. & NUCCI, M. 2017. Clinical manifestations and diagnosis of Fusarium infection. *In:* THORNER, A. R. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

ANDERSEN, A. A. 1958. New sampler for the collection, sizing, and enumeration of viable airborne particles. *J Bacteriol,* 76**,** 471-84.

ANGLY, F. E., DENNIS, P. G., SKARSHEWSKI, A., VANWONTERGHEM, I., HUGENHOLTZ, P. & TYSON, G. W. 2014. CopyRighter: a rapid tool for improving the accuracy of microbial community profiles through lineage-specific gene copy number correction. *Microbiome,* 2**,** 11.

APPRILL, A., MILLER, C. A., MOORE, M. J., DURBAN, J. W., FEARNBACH, H. & BARRETT-LENNARD, L. G. 2017. Extensive Core Microbiome in Drone-Captured Whale Blow Supports a Framework for Health Monitoring. *mSystems,* 2.

ARIZA-PROTA, M. A., PANDO-SANDOVAL, A., FOLE-VÁZQUEZ, D., GARCÍA-CLEMENTE, M., BUDIÑO, T. & CASAN, P. 2015b. Community-acquired bacteremic Streptomyces atratus pneumonia in an immunocompetent adult: a case report. *Journal of Medical Case Reports,* 9**,** 262.

ARIZA-PROTA, M. A., PANDO-SANDOVAL, A., GARCIA-CLEMENTE, M., FERNANDEZ, R. & CASAN, P. 2015a. Community-Acquired Pneumonia and Empyema Caused by Citrobacter koseri in an Immunocompetent Patient. *Case Rep Pulmonol,* 2015**,** 670373.

ARMBRUSTER, C., HASSL, A. & KRIWANEK, S. 1997. Pneumocystis carinii colonization in the absence of immunosuppression. *Scand J Infect Dis,* 29**,** 591-3.

ARMBRUSTER, D. A. & PRY, T. 2008. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev,* 29 Suppl 1**,** S49-52.

ASHELFORD, K. E., WEIGHTMAN, A. J. & FRY, J. C. 2002. PRIMROSE: a computer program for generating and estimating the phylogenetic range of 16S rRNA oligonucleotide probes and primers in conjunction with the RDP-II database. *Nucleic Acids Res,* 30**,** 3481-9.

ASLANZADEH, J., DE LA VIUDA, M., FILLE, M., SMITH, W. B. & NAMDARI, H. 1998. Comparison of culture and acid-fast bacilli stain to PCR for detection of Mycobacterium tuberculosis in clinical samples. *Mol Cell Probes,* 12**,** 207-11.

ATKINSON, M. P. & WEIN, L. M. 2008. Quantifying the routes of transmission for pandemic influenza. *Bull Math Biol,* 70**,** 820-67.

AZEVEDO, R. S. S., ARAUJO, M. T., MARTINS FILHO, A. J., OLIVEIRA, C. S., NUNES, B. T. D., CRUZ, A. C. R., NASCIMENTO, A., MEDEIROS, R. C., CALDAS, C. A. M., ARAUJO, F. C., QUARESMA, J. A. S., VASCONCELOS, B. C. B., QUEIROZ, M. G. L., TRAVASSOS DA ROSA, E. S., HENRIQUES, D. F., SILVA, E. V. P., CHIANG, J. O., MARTINS, L. C., MEDEIROS, D. B. A., LIMA, J. A., NUNES, M. R. T., CARDOSO, J. F., SILVA, S. P., SHI, P. Y., TESH, R. B., RODRIGUES, S. G. & VASCONCELOS, P. F. C. 2016. Zika virus epidemic in Brazil. I. Fatal disease in adults: Clinical andlaboratorial aspects. *J Clin Virol,* 85**,** 56-64.

AZOULAY, E., BERGERON, A., CHEVRET, S., BELE, N., SCHLEMMER, B. & MENOTTI, J. 2009. Polymerase chain reaction for diagnosing pneumocystis pneumonia in non-HIV immunocompromised patients with pulmonary infiltrates. *Chest,* 135**,** 655-661.

BACCHETTI DE GREGORIS, T., ALDRED, N., CLARE, A. S. & BURGESS, J. G. 2011. Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. *J Microbiol Methods,* 86**,** 351-6.

BAHRANI-MOUGEOT, F. K., PASTER, B. J., COLEMAN, S., BARBUTO, S., BRENNAN, M. T., NOLL, J., KENNEDY, T., FOX, P. C. & LOCKHART, P. B. 2007. Molecular analysis of oral and respiratory bacterial species associated with ventilator-associated pneumonia. *J Clin Microbiol,* 45**,** 1588-93.

BAI, C., LIU, J. & SONG, Y. 2013. Amylase in bronchoalveolar lavage fluid: a new marker for pulmonary aspiration. *Crit Care Med,* 41**,** 916-7.

BAKER, R. M., STEGINK, R. J., MANALOOR, J. J., SCHMITT, B. H., STEVENS, J. C. & CHRISTENSON, J. C. 2016. Malassezia Pneumonia: A Rare Complication of Parenteral Nutrition Therapy. *JPEN J Parenter Enteral Nutr,* 40**,** 1194-1196.

BALIS, E., VELEGRAKI, A., FRAGOU, A., PEFANIS, A., KALABOKAS, T. & MOUNTOKALAKIS, T. 2006. Lung mass caused by Phoma exigua. *Scand J Infect Dis,* 38**,** 552-5.

BANDT, D. & MONECKE, S. 2007. Development and evaluation of a real-time PCR assay for detection of Pneumocystis jiroveci. *Transpl Infect Dis,* 9**,** 196-202.

BANDYOPADHYAY, T., GERARDI, D. A. & METERSKY, M. L. 2000. A comparison of induced and expectorated sputum for the microbiological diagnosis of community acquired pneumonia. *Respiration,* 67**,** 173-6.

BANNIER, M., ROSIAS, P. P. R., JOBSIS, Q. & DOMPELING, E. 2019. Exhaled Breath Condensate in Childhood Asthma: A Review and Current Perspective. *Front Pediatr,* 7**,** 150.

BARRON, T. F., BIRNBAUM, N. S., SHANE, L. B., GOLDSMITH, S. J. & ROSEN, M. J. 1985. Pneumocystis carinii pneumonia studied by gallium-67 scanning. *Radiology,* 154**,** 791-3.

BARTLETT, J. A. & HULETTE, C. 1997. Central nervous system pneumocystosis in a patient with AIDS. *Clin Infect Dis,* 25**,** 82-5.

BARTLETT, J. G. 2018. Aspiration pneumonia in adults. *In:* BOND, S. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

BARTLETT, J. G., DOWELL, S. F., MANDELL, L. A., FILE, T. M., JR., MUSHER, D. M. & FINE, M. J. 2000. Practice guidelines for the management of community-acquired pneumonia in adults. Infectious Diseases Society of America. *Clin Infect Dis,* 31**,** 347-82.

BARTLETT, J. G., GORBACH, S. L. & FINEGOLD, S. M. 1974. The bacteriology of aspiration pneumonia. *Am J Med,* 56**,** 202-7.

BARTLETT, J. M. & STIRLING, D. 2003. A short history of the polymerase chain reaction. *Methods Mol Biol,* 226**,** 3-6.

BARTLETT, M. S., ANGUS, W. C., SHAW, M. M., DURANT, P. J., LEE, C. H., PASCALE, J. M. & SMITH, J. W. 1998. Antibody to Pneumocystis carinii protects rats and mice from developing pneumonia. *Clin Diagn Lab Immunol,* 5**,** 74-7.

BARTLETT, M. S., QUEENER, S. F., SHAW, M. M., RICHARDSON, J. D. & SMITH, J. W. 1994. Pneumocystis carinii is resistant to imidazole antifungal agents. *Antimicrob Agents Chemother,* 38**,** 1859-61.

BARTLETT, M. S., VERMUND, S. H., JACOBS, R., DURANT, P. J., SHAW, M. M., SMITH, J. W., TANG, X., LU, J. J., LI, B., JIN, S. & LEE, C. H. 1997. Detection of Pneumocystis carinii DNA in air samples: likely environmental risk to susceptible persons. *J Clin Microbiol,* 35**,** 2511-3.

BASSIS, C. M., ERB-DOWNWARD, J. R., DICKSON, R. P., FREEMAN, C. M., SCHMIDT, T. M., YOUNG, V. B., BECK, J. M., CURTIS, J. L. & HUFFNAGLE, G. B. 2015. Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. *MBio,* 6**,** e00037.

BASSIS, C. M., TANG, A. L., YOUNG, V. B. & PYNNONEN, M. A. 2014. The nasal cavity microbiota of healthy adults. *Microbiome,* 2**,** 27.

BASU, S., ANDREWS, J. R., POOLMAN, E. M., GANDHI, N. R., SHAH, N. S., MOLL, A., MOODLEY, P., GALVANI, A. P. & FRIEDLAND, G. H. 2007. Prevention of nosocomial transmission of extensively drug-resistant tuberculosis in rural South African district hospitals: an epidemiological modelling study. *Lancet,* 370**,** 1500-7.

BAUM, S. G. 2017. Mycoplasma hominis and Ureaplasma urealyticum infections. *In:* BLOOM, A. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

BAYER, A. S., NELSON, S. C., GALPIN, J. E., CHOW, A. W. & GUZE, L. B. 1975. Necrotizing pneumonia and empyema due to clostridium perfringens: Report of a case and review of the literature. *The American Journal of Medicine,* 59**,** 851-856.

BAZAZ, G. R., MANFREDI, O. L., HOWARD, R. G. & CLAPS, A. A. 1970. Pneumocystis carinii pneumonia in three full-term siblings. *J Pediatr,* 76**,** 767-9.

BEARD, C. B., CARTER, J. L., KEELY, S. P., HUANG, L., PIENIAZEK, N. J., MOURA, I. N., ROBERTS, J. M., HIGHTOWER, A. W., BENS, M. S., FREEMAN, A. R., LEE, S., STRINGER, J. R., DUCHIN, J. S., DEL RIO, C., RIMLAND, D., BAUGHMAN, R. P., LEVY, D. A., DIETZ, V. J., SIMON, P. & NAVIN, T. R. 2000. Genetic variation in Pneumocystis carinii isolates from different geographic regions: implications for transmission. *Emerg Infect Dis,* 6**,** 265-72.

BECK, J. M., SCHLOSS, P. D., VENKATARAMAN, A., TWIGG, H., 3RD, JABLONSKI, K. A., BUSHMAN, F. D., CAMPBELL, T. B., CHARLSON, E. S., COLLMAN, R. G., CROTHERS, K., CURTIS, J. L., DREWS, K. L., FLORES, S. C., FONTENOT, A. P., FOULKES, M. A., FRANK, I., GHEDIN, E., HUANG, L., LYNCH, S. V., MORRIS, A., PALMER, B. E., SCHMIDT, T. M., SODERGREN, E., WEINSTOCK, G. M., YOUNG, V. B. & LUNG, H. I. V. M. P. 2015. Multicenter Comparison of Lung and Oral Microbiomes of HIV-infected and HIV-uninfected Individuals. *Am J Respir Crit Care Med,* 192**,** 1335-44.

BECK, J. M., YOUNG, V. B. & HUFFNAGLE, G. B. 2012. The Microbiome of the Lung. *Transl Res,* 160**,** 258-66.

BECK-SAGUE, C., DOOLEY, S. W., HUTTON, M. D., OTTEN, J., BREEDEN, A., CRAWFORD, J. T., PITCHENIK, A. E., WOODLEY, C., CAUTHEN, G. & JARVIS, W. R. 1992. Hospital outbreak of multidrug-resistant Mycobacterium tuberculosis infections. Factors in transmission to staff and HIV-infected patients. *JAMA,* 268**,** 1280-6.

BEHR, M. A., WARREN, S. A., SALAMON, H., HOPEWELL, P. C., PONCE DE LEON, A., DALEY, C. L. & SMALL, P. M. 1999. Transmission of Mycobacterium tuberculosis from patients smear-negative for acid-fast bacilli. *Lancet,* 353**,** 444-9.

BELFIELD, G. P., ROSS-SMITH, N. J. & TUITE, M. F. 1995. Translation elongation factor-3 (EF-3): an evolving eukaryotic ribosomal protein? *J Mol Evol,* 41**,** 376-87.

BELTRAMI, S. & GORDON, J. 2014. Immune surveillance and response to JC virus infection and PML. *J Neurovirol,* 20**,** 137-49.

BEMER-MELCHIOR, P. & DRUGEON, H. B. 1999. Inactivation of Mycobacterium tuberculosis for DNA typing analysis. *J Clin Microbiol,* 37**,** 2350-1.

BENEDETTI, G., RASTELLI, F., DAMIANI, S., CALANDRI, C. & CRINO, L. 2004. Challenging problems in malignancy: case 1. Presentation of small-cell lung cancer with marked hyperamylasemia. *J Clin Oncol,* 22**,** 3826-8.

BENEDYK, M., MYDEL, P. M., DELALEU, N., PLAZA, K., GAWRON, K., MILEWSKA, A., MARESZ, K., KOZIEL, J., PYRC, K. & POTEMPA, J. 2016. Gingipains: Critical Factors in the Development of Aspiration Pneumonia Caused by Porphyromonas gingivalis. *J Innate Immun,* 8**,** 185-98.

BENSOUSAN, T., GARO, B., ISLAM, S., BOURBIGOT, B., CLEDES, J. & GARRE, M. 1990. Possible transfer of Pneumocystis carinii between kidney transplant recipients. *Lancet,* 336**,** 1066-7.

BERK, J. E., SHIMAMURA, J. & FRIDHANDLER, L. 1978. Amylase changes in disorders of the lung. *Gastroenterology,* 74**,** 1313-17.

BERK, S. L., VERGHESE, A., HOLTSCLAW, S. A. & SMITH, J. K. 1983. Enterococcal pneumonia. Occurrence in patients receiving broad-spectrum antibiotic regimens and enteral feeding. *Am J Med,* 74**,** 153-4.

BERKELEY, D. & MANGELS, J. 1980. Salmonella pneumonia in a patient with carcinoma of the lung. *Am J Clin Pathol,* 74**,** 476-8.

BIGBY, T. D., MARGOLSKEE, D., CURTIS, J. L., MICHAEL, P. F., SHEPPARD, D., HADLEY, W. K. & HOPEWELL, P. C. 1986. The usefulness of induced sputum in the diagnosis of Pneumocystis carinii pneumonia in patients with the acquired immunodeficiency syndrome. *Am Rev Respir Dis,* 133**,** 515-8.

BIRRING, S. S., FLEMING, T., MATOS, S., RAJ, A. A., EVANS, D. H. & PAVORD, I. D. 2008. The Leicester Cough Monitor: preliminary validation of an automated cough detection system in chronic cough. *Eur Respir J,* 31**,** 1013-8.

BLAHD, M., LESLIE, E. I. & ROSENTHAL, S. R. 1946. Infectiousness of the closed case of tuberculosis. *Am J Public Health Nations Health,* 36**,** 723-6.

BLAXHULT, A., KIRK, O., PEDERSEN, C., DIETRICH, M., BARTON, S. E., GATELL, J. M., MULCAHY, F., HIRSCHEL, B., MOCROFT, A. & LUNDGREN, J. D. 2000. Regional differences in presentation of AIDS in Europe. *Epidemiol Infect,* 125**,** 143-51.

BLOUNT, R. J., DJAWE, K., DALY, K. R., JARLSBERG, L. G., FONG, S., BALMES, J., MILLER, R. F., WALZER, P. D. & HUANG, L. 2013. Ambient air pollution associated with suppressed serologic responses to Pneumocystis jirovecii in a prospective cohort of HIV-infected patients with Pneumocystis pneumonia. *PLoS One,* 8**,** e80795.

BOLGER, A. M., LOHSE, M. & USADEL, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics,* 30**,** 2114-20.

BOONDIREKE, S., MUNGTHIN, M., TAN-ARIYA, P., BOONYONGSUNCHAI, P., NAAGLOR, T., WATTANATHUM, A., TREEWATCHAREEKORN, S. & LEELAYOOVA, S. 2010. Evaluation of sensitivity of multiplex PCR for Detection of Mycobacterium tuberculosis and Pneumocystis jirovecii in clinical samples. *J Clin Microbiol,* 48**,** 3165-8.

BOSTON, L. N. 1901. THE SPREAD OF TUBERCULOSIS BY COUGHING. *Journal of the American Medical Association,* XXXVII, 685-688.

BOTERO, L. E., DELGADO-SERRANO, L., CEPEDA, M. L., BUSTOS, J. R., ANZOLA, J. M., DEL PORTILLO, P., ROBLEDO, J. & ZAMBRANO, M. M. 2014. Respiratory tract clinical sample selection for microbiota analysis in patients with pulmonary tuberculosis. *Microbiome,* 2, 29.

BOTTEREL, F., CABARET, O., FOULET, F., CORDONNIER, C., COSTA, J. M. & BRETAGNE, S. 2012. Clinical significance of quantifying Pneumocystis jirovecii DNA by using real-time PCR in bronchoalveolar lavage fluid from immunocompromised patients. *J Clin Microbiol,* 50, 227-31.

BOZEC, L. & ODLYHA, M. 2011. Thermal denaturation studies of collagen by microthermal analysis and atomic force microscopy. *Biophys J,* 101, 228-36.

BRADSHER, R. W. 2018. Clinical manifestations and diagnosis of blastomycosis. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

BRAY, J. R. & CURTIS, J. T. 1957. An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecological Monographs,* 27, 325-349.

BRAZINSKY, J. H. & PHILLIPS, J. E. 1969. Pneumocystis pneumonia transmission between patients with lymphoma. *JAMA,* 209, 1527.

BREDBERG, A., GOBOM, J., ALMSTRAND, A. C., LARSSON, P., BLENNOW, K., OLIN, A. C. & MIRGORODSKAYA, E. 2012. Exhaled endogenous particles contain lung proteins. *Clin Chem,* 58, 431-40.

BREDBERG, A., JOSEFSON, M., ALMSTRAND, A. C., LAUSMAA, J., SJOVALL, P., LEVINSSON, A., LARSSON, P. & OLIN, A. C. 2013. Comparison of exhaled endogenous particles from smokers and non-smokers using multivariate analysis. *Respiration,* 86, 135-42.

BRINCKMANN, J. 2005. Collagens at a Glance. *In:* BRINCKMANN, J., NOTBOHM, H. & MÜLLER, P. K. (eds.) *Collagen: Primer in Structure, Processing and Assembly.* Berlin, Heidelberg: Springer Berlin Heidelberg.

BROAD, T. E., FORREST, J. W. & PUGH, P. A. 1988. Effect of NaOH on hybridization efficiency of DNA. *Trends Genet,* 4, 146.

BROKALAKI, E. I., SOMMERWERCK, U., VON HEINEGG, E. H. & HILLEN, U. 2012. Ochroconis gallopavum infection in a lung transplant recipient: report of a case. *Transplant Proc,* 44, 2778-80.

BROWN, G. D., DENNING, D. W., GOW, N. A., LEVITZ, S. M., NETEA, M. G. & WHITE, T. C. 2012. Hidden killers: human fungal infections. *Sci Transl Med,* 4, 165rv13.

BRYANT, J. M., GROGONO, D. M., RODRIGUEZ-RINCON, D., EVERALL, I., BROWN, K. P., MORENO, P., VERMA, D., HILL, E., DRIJKONINGEN, J., GILLIGAN, P., ESTHER, C. R., NOONE, P. G., GIDDINGS, O., BELL, S. C., THOMSON, R., WAINWRIGHT, C. E., COULTER, C., PANDEY, S., WOOD, M. E., STOCKWELL, R. E., RAMSAY, K. A., SHERRARD, L. J., KIDD, T. J., JABBOUR, N., JOHNSON, G. R., KNIBBS, L. D., MORAWSKA, L., SLY, P. D., JONES, A., BILTON, D., LAURENSON, I., RUDDY, M., BOURKE, S., BOWLER, I. C., CHAPMAN, S. J., CLAYTON, A., CULLEN, M., DANIELS, T., DEMPSEY, O., DENTON, M., DESAI, M., DREW, R. J., EDENBOROUGH, F., EVANS, J., FOLB, J., HUMPHREY, H., ISALSKA, B., JENSEN-FANGEL, S., JONSSON, B., JONES, A. M., KATZENSTEIN, T. L., LILLEBAEK, T., MACGREGOR, G., MAYELL, S., MILLAR, M., MODHA, D., NASH, E. F., O'BRIEN, C., O'BRIEN, D., OHRI, C., PAO, C. S., PECKHAM, D., PERRIN, F., PERRY, A., PRESSLER, T., PRTAK, L., QVIST, T., ROBB, A., RODGERS, H., SCHAFFER, K., SHAFI, N., VAN INGEN, J., WALSHAW, M., WATSON, D., WEST, N., WHITEHOUSE, J., HAWORTH, C. S., HARRIS, S. R., ORDWAY, D., PARKHILL, J. & FLOTO, R. A. 2016. Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. *Science,* 354, 751-757.

BUCHELLI-RAMIREZ, H. L., ALVAREZ-ALVAREZ, C., ROJO-ALBA, S., GARCIA-CLEMENTE, M., CIMADEVILLA-SUAREZ, R., PANDO-SANDOVAL, A. & CASAN-CLARA, P. 2013. Necrotising pneumonia caused by Lactococcus lactis cremoris. *Int J Tuberc Lung Dis,* 17, 565-7.

BUCK, G. E., O'HARA, L. C. & SUMMERSGILL, J. T. 1992. Rapid, simple method for treating clinical specimens containing Mycobacterium tuberculosis to remove DNA for polymerase chain reaction. *J Clin Microbiol,* 30, 1331-4.

BURDZ, T. V., WOLFE, J. & KABANI, A. 2003. Evaluation of sputum decontamination methods for Mycobacterium tuberculosis using viable colony counts and flow cytometry. *Diagn Microbiol Infect Dis,* 47, 503-9.

BURTON, N. C., ADHIKARI, A., GRINSHPUN, S. A., HORNUNG, R. & REPONEN, T. 2005. The effect of filter material on bioaerosol collection of Bacillus subtilis spores used as a Bacillus anthracis simulant. *J Environ Monit,* 7, 475-80.

BURTON, N. C., GRINSHPUN, S. A. & REPONEN, T. 2007. Physical collection efficiency of filter materials for bacteria and viruses. *Ann Occup Hyg,* 51, 143-51.

CALDERON, E. J., REGORDAN, C., MEDRANO, F. J., OLLERO, M. & VARELA, J. M. 1996. Pneumocystis carinii infection in patients with chronic bronchial disease. *Lancet,* 347**,** 977.

CALORE, E. E. 2002. Herpes simplex type 2 pneumonia. *Braz J Infect Dis,* 6**,** 305-8.

CAMANOCHA, A. & DEWHIRST, F. E. 2014. Host-associated bacterial taxa from Chlorobi, Chloroflexi, GN02, Synergistetes, SR1, TM7, and WPS-2 Phyla/candidate divisions. *J Oral Microbiol,* 6.

CANIZA, M. A., GRANGER, D. L., WILSON, K. H., WASHINGTON, M. K., KORDICK, D. L., FRUSH, D. P. & BLITCHINGTON, R. B. 1995. Bartonella henselae: etiology of pulmonary nodules in a patient with depressed cell-mediated immunity. *Clin Infect Dis,* 20**,** 1505-11.

CANNEY, P. A., LARSSON, S. N., HAY, J. H. & YUSSUF, M. A. 1985. Case report: Salmonella pneumonia associated with chemotherapy for non-Hodgkin's lymphoma. *Clin Radiol,* 36**,** 459-60.

CANO, S., CAPOTE, F., PEREIRA, A., CALDERON, E. & CASTILLO, J. 1993. Pneumocystis carinii pneumonia in patients without predisposing illnesses. Acute episode and follow-up of five cases. *Chest,* 104**,** 376-81.

CAPORASO, J. G., BITTINGER, K., BUSHMAN, F. D., DESANTIS, T. Z., ANDERSEN, G. L. & KNIGHT, R. 2010. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics,* 26**,** 266-7.

CAPORASO, J. G., LAUBER, C. L., WALTERS, W. A., BERG-LYONS, D., HUNTLEY, J., FIERER, N., OWENS, S. M., BETLEY, J., FRASER, L., BAUER, M., GORMLEY, N., GILBERT, J. A., SMITH, G. & KNIGHT, R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *Isme j,* 6**,** 1621-4.

CAPORASO, J. G., LAUBER, C. L., WALTERS, W. A., BERG-LYONS, D., LOZUPONE, C. A., TURNBAUGH, P. J., FIERER, N. & KNIGHT, R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America,* 108 Suppl 1**,** 4516-4522.

CARPAGNANO, G. E., LACEDONIA, D., COTUGNO, G., DEPALO, A., PALMIOTTI, G. A., GIUFFREDA, E. & FOSCHINO BARBARO, M. P. 2015. Exhaled fungal microbiome in asthma: Possible association with severity and control. *European Respiratory Journal,* 46**,** PA4004.

CARPAGNANO, G. E., LACEDONIA, D., PALLADINO, G. P., LOGRIECO, G., CRISETTI, E., SUSCA, A., LOGRIECO, A. & FOSCHINO-BARBARO, M. P. 2014. Aspergillus spp. colonization in exhaled breath condensate of lung cancer patients from Puglia Region of Italy. *BMC Pulmonary Medicine,* 14**,** 22.

CARPAGNANO, G. E., MALERBA, M., LACEDONIA, D., SUSCA, A., LOGRIECO, A., CARONE, M., COTUGNO, G., PALMIOTTI, G. A. & FOSCHINO-BARBARO, M. P. 2016. Analysis of the fungal microbiome in exhaled breath condensate of patients with asthma. *Allergy Asthma Proc,* 37**,** 41-6.

CARPAGNANO, G. E., SUSCA, A., SCIOSCIA, G., LACEDONIA, D., COTUGNO, G., SOCCIO, P., SANTAMARIA, S., RESTA, O., LOGRIECO, G. & FOSCHINO BARBARO, M. P. 2019. A survey of fungal microbiota in airways of healthy volunteer subjects from Puglia (Apulia), Italy. *BMC Infectious Diseases,* 19**,** 78.

CARRANZA GONZALEZ, R., TENA GOMEZ, D., PRIETO GOMEZ, E., BARBERA FARRE, J. R. & FERNANDEZ CENJOR, J. 2006. [Pneumonia by Corynebacterium pseudodiphteriticum: an infection to consider]. *An Med Interna,* 23**,** 124-6.

CASANOVA-CARDIEL, L. & LEIBOWITZ, M. J. 1997. Presence of Pneumocystis carinii DNA in pond water. *J Eukaryot Microbiol,* 44**,** 28S.

CASE, R. J., BOUCHER, Y., DAHLLOF, I., HOLMSTROM, C., DOOLITTLE, W. F. & KJELLEBERG, S. 2007. Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. *Appl Environ Microbiol,* 73**,** 278-88.

CATANZARO, A. 1982. Nosocomial tuberculosis. *Am Rev Respir Dis,* 125**,** 559-62.

CAVERLY, L. J., ZHAO, J. & LIPUMA, J. J. 2015. Cystic fibrosis lung microbiome: opportunities to reconsider management of airway infection. *Pediatr Pulmonol,* 50 Suppl 40**,** S31-8.

CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC). 2017. Pneumocystis pneumonia. Available at: https://www.cdc.gov (Accessed on October 02, 2018.).

CESARIO, T. C. 2012. Viruses associated with pneumonia in adults. *Clin Infect Dis,* 55**,** 107-13.

CHABE, M., KHALIFE, S., GANTOIS, N., EVEN, G. & AUDEBERT, C. 2014. An improved single-round PCR leads to rapid and highly sensitive detection of Pneumocystis spp. *Med Mycol,* 52**,** 841-6.

CHAFFEY, M. H., KLEIN, J. S., GAMSU, G., BLANC, P. & GOLDEN, J. A. 1990. Radiographic distribution of Pneumocystis carinii pneumonia in patients with AIDS treated with prophylactic inhaled pentamidine. *Radiology,* 175**,** 715-9.

CHAKRAVORTY, S., SIMMONS, A. M., ROWNEKI, M., PARMAR, H., CAO, Y., RYAN, J., BANADA, P. P., DESHPANDE, S., SHENAI, S., GALL, A., GLASS, J., KRIESWIRTH, B., SCHUMACHER, S. G., NABETA, P., TUKVADZE, N., RODRIGUES, C., SKRAHINA, A., TAGLIANI, E., CIRILLO, D. M., DAVIDOW, A., DENKINGER, C. M., PERSING, D., KWIATKOWSKI, R., JONES, M. & ALLAND, D. 2017. The New Xpert MTB/RIF Ultra: Improving Detection of Mycobacterium tuberculosis and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing. *mBio,* 8.

CHAN, J. F. W., LAU, S. K. P., YUEN, K. Y. & WOO, P. C. Y. 2016. Talaromyces (Penicillium) marneffei infection in non-HIV-infected patients. *Emerg Microbes Infect,* 5**,** e19-.

CHAO, A. 1984. Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics,* 11**,** 265-270.

CHAPIN, C. V. 1910. The Spread of Scarlet Fever and Diphtheria in Schools. *Am J Public Hygiene,* 20**,** 813-7.

CHARLSON, E. S., BITTINGER, K., HAAS, A. R., FITZGERALD, A. S., FRANK, I., YADAV, A., BUSHMAN, F. D. & COLLMAN, R. G. 2011. Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am J Respir Crit Care Med,* 184**,** 957-63.

CHATTERJEE, M., BHATTACHARYA, S., KARAK, K. & DASTIDAR, S. G. 2013. Effects of different methods of decontamination for successful cultivation of Mycobacterium tuberculosis. *Indian J Med Res,* 138**,** 541-8.

CHAWLA, K., MARTENA, S., GURUNG, B., MUKHOPADHYAY, C., VARGHESE, G. K. & BAIRY, I. 2011. Role of PCR for diagnosing Pneumocystis jirovecii pneumonia in HIV-infected individuals in a tertiary care hospital in India. *Indian J Pathol Microbiol,* 54**,** 326-9.

CHEAH, E. S., MALKIN, J., FREE, R. C., LEE, S. M., PERERA, N., WOLTMANN, G., PATEL, H., KIMMITT, P. T., SMITH, R. J., RAJAKUMAR, K. & BARER, M. R. 2010. A two-tube combined TaqMan/SYBR Green assay to identify mycobacteria and detect single global lineage-defining polymorphisms in Mycobacterium tuberculosis. *J Mol Diagn,* 12**,** 250-6.

CHEAH, E. S. G. 2010. *DEVELOPMENT AND EVALUATION OF A RAPID MOLECULAR ASSAY AND A MASK SAMPLING METHOD ENABLING THE STUDY OF TUBERCULOSIS TRANSMISSION.* Doctor of Philosophy, University of Leicester.

CHEEPSATTAYAKORN, A. & CHEEPSATTAYAKORN, R. 2014. Parasitic pneumonia and lung involvement. *Biomed Res Int,* 2014**,** 874021.

CHEN, C., HEMME, C., BELENO, J., SHI, Z. J., NING, D., QIN, Y., TU, Q., JORGENSEN, M., HE, Z., WU, L. & ZHOU, J. 2018. Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. *The ISME Journal,* 12**,** 1210-1224.

CHEN, W., GIGLIOTTI, F. & HARMSEN, A. G. 1993. Latency is not an inevitable outcome of infection with Pneumocystis carinii. *Infect Immun,* 61**,** 5406-9.

CHENG, N. M., SY, C. L., CHEN, B. C., HUANG, T. S., LEE, S. S. & CHEN, Y. S. 2017. Isolation of dengue virus from the upper respiratory tract of four patients with dengue fever. *PLoS Negl Trop Dis,* 11**,** e0005520.

CHIEN, J. Y., LIU, C. J., CHUANG, P. C., LEE, T. F., HUANG, Y. T., LIAO, C. H., HUNG, C. C., SHENG, W. H., YU, C. J. & HSUEH, P. R. 2017. Evaluation of the automated Becton Dickinson MAX real-time PCR platform for detection of Pneumocystis jirovecii. *Future Microbiol,* 12**,** 29-37.

CHOO, J. M., LEONG, L. E. & ROGERS, G. B. 2015. Sample storage conditions significantly influence faecal microbiome profiles. *Sci Rep,* 5**,** 16350.

CHOUKRI, F., MENOTTI, J., SARFATI, C., LUCET, J. C., NEVEZ, G., GARIN, Y. J., DEROUIN, F. & TOTET, A. 2010. Quantification and spread of Pneumocystis jirovecii in the surrounding air of patients with Pneumocystis pneumonia. *Clin Infect Dis,* 51**,** 259-65.

CHOWDHARY, A., AGARWAL, K., RANDHAWA, H. S., KATHURIA, S., GAUR, S. N., NAJAFZADEH, M. J., ROY, P., ARORA, N., KHANNA, G. & MEIS, J. F. 2012. A rare case of allergic bronchopulmonary mycosis caused by Alternaria alternata. *Med Mycol,* 50**,** 890-6.

CHUMPITAZI, B. F., FLORI, P., KERN, J. B., BRENIER-PINCHART, M. P., HINCKY-VITRAT, V., BRION, J. P., THIEBAUT-BERTRAND, A., MINET, C., MAUBON, D. & PELLOUX, H. 2011. Characteristics and clinical relevance of the quantitative touch-down major surface glycoprotein polymerase chain reaction in the diagnosis of Pneumocystis pneumonia. *Med Mycol,* 49**,** 704-13.

CHURCH, D. L., AMBASTA, A., WILMER, A., WILLISCROFT, H., RITCHIE, G., PILLAI, D. R., CHAMPAGNE, S. & GREGSON, D. G. 2015. Development and validation of a Pneumocystis jirovecii real-time polymerase chain reaction assay for diagnosis of Pneumocystis pneumonia. *Can J Infect Dis Med Microbiol,* 26**,** 263-7.

CISSE, O. H., PAGNI, M. & HAUSER, P. M. 2012. De novo assembly of the Pneumocystis jirovecii genome from a single bronchoalveolar lavage fluid specimen from a patient. *MBio,* 4**,** e00428-12.

CISSE, O. H., PAGNI, M. & HAUSER, P. M. 2014. Comparative genomics suggests that the human pathogenic fungus Pneumocystis jirovecii acquired obligate biotrophy through gene loss. *Genome Biol Evol,* 6**,** 1938-48.

CLARKE, P. D., BAIN, B. C., DAVIES, A., LEVIN, G. E. & LAMBERT, H. P. 1981. Aspiration in seriously ill patients: a study of amylase in bronchial secretions. *J Clin Pathol,* 34**,** 803-5.

CLAVEL, A., ARNAL, A. C., SANCHEZ, E. C., CUESTA, J., LETONA, S., AMIGUET, J. A., CASTILLO, F. J., VAREA, M. & GOMEZ-LUS, R. 1996. Respiratory cryptosporidiosis: case series and review of the literature. *Infection,* 24**,** 341-6.

CLERI, D. J., VERNALEO, J. R., LOMBARDI, L. J., RABBAT, M. S., MATHEW, A., MARTON, R. & REYELT, M. C. 1997. Plague pneumonia disease caused by Yersinia pestis. *Semin Respir Infect,* 12**,** 12-23.

CLERINX, J. & SOENTJENS, P. 2017. Epidemiology, pathogenesis, and clinical manifestations of schistosomiasis. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

COHEN, O. J. & STOECKLE, M. Y. 1991. Extrapulmonary Pneumocystis carinii infections in the acquired immunodeficiency syndrome. *Arch Intern Med,* 151**,** 1205-14.

COHEN, Y. Z. & STEAD, W. 2015. Exophiala Pneumonia Presenting with a Cough Productive of Black Sputum. *Case Rep Infect Dis,* 2015**,** 821049.

COLE, E. C. & COOK, C. E. 1998. Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies. *Am J Infect Control,* 26**,** 453-64.

COLTHURST, D. R., SANTOS, M., GRANT, C. M. & TUITE, M. F. 1991. Candida albicans and three other Candida species contain an elongation factor structurally and functionally analogous to elongation factor 3. *FEMS Microbiol Lett,* 64**,** 45-9.

CONCES, D. J., JR., KRAFT, J. L., VIX, V. A. & TARVER, R. D. 1989. Apical Pneumocystis carinii pneumonia after inhaled pentamidine prophylaxis. *AJR Am J Roentgenol,* 152**,** 1193-4.

CONTINI, C., VILLA, M. P., ROMANI, R., MEROLLA, R., DELIA, S. & RONCHETTI, R. 1998. Detection of Pneumocystis carinii among children with chronic respiratory disorders in the absence of HIV infection and immunodeficiency. *J Med Microbiol,* 47**,** 329-33.

COOLEY, L., DENDLE, C., WOLF, J., TEH, B. W., CHEN, S. C., BOUTLIS, C. & THURSKY, K. A. 2014. Consensus guidelines for diagnosis, prophylaxis and management of Pneumocystis jirovecii pneumonia in patients with haematological and solid malignancies, 2014. *Intern Med J,* 44**,** 1350-63.

CORTI, S. & STEPHAN, R. 2002. Detection of Mycobacterium avium subspecies paratuberculosis specific IS900 insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. *BMC Microbiol,* 2**,** 15.

COSTA, C., TERLIZZI, M. E., SOLIDORO, P., LIBERTUCCI, D., BERGALLO, M. & CAVALLO, R. 2009. Detection of parvovirus B19 in the lower respiratory tract. *J Clin Virol,* 46**,** 150-3.

COSTA, S. F. & ALEXANDER, B. D. 2017. Epidemiology, clinical manifestations, and diagnosis of Scedosporium and Lomentospora infections. *In:* THORNER, A. R. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

COX, G. M. 2017. Mucormycosis (zygomycosis). *In:* THORNER, A. R. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

COX, G. M. & PERFECT, J. R. 2017. Cryptococcus neoformans infection outside the central nervous system. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

CREMER, M. A., ROSLONIEC, E. F. & KANG, A. H. 1998. The cartilage collagens: a review of their structure, organization, and role in the pathogenesis of experimental arthritis in animals and in human rheumatic disease. *J Mol Med (Berl),* 76**,** 275-88.

CROOME, R. J. 1953. Acid and alkaline hydrolysis of gelatin. *Journal of Applied Chemistry,* 3**,** 280-286.

CUI, Z., ZHOU, Y., LI, H., ZHANG, Y., ZHANG, S., TANG, S. & GUO, X. 2012. Complex sputum microbial composition in patients with pulmonary tuberculosis. *BMC Microbiol,* 12**,** 276.

CURRIE, B. & ANSTEY, N. M. 2016. Epidemiology, clinical manifestations, and diagnosis of melioidosis. *In:* BLOOM, A. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

CUSHION, M. T. 2004. Pneumocystis: unraveling the cloak of obscurity. *Trends Microbiol,* 12**,** 243-9.

CUSHION, M. T. 2010. Are members of the fungal genus pneumocystis (a) commensals; (b) opportunists; (c) pathogens; or (d) all of the above? *PLoS Pathog,* 6**,** e1001009.

CUSHION, M. T., LINKE, M. J., ASHBAUGH, A., SESTERHENN, T., COLLINS, M. S., LYNCH, K., BRUBAKER, R. & WALZER, P. D. 2010. Echinocandin treatment of pneumocystis pneumonia in rodent models depletes cysts leaving trophic burdens that cannot transmit the infection. *PLoS One,* 5**,** e8524.

DAMIANI, C., LE GAL, S., DA COSTA, C., VIRMAUX, M., NEVEZ, G. & TOTET, A. 2013. Combined quantification of pulmonary Pneumocystis jirovecii DNA and serum (1->3)-beta-D-glucan for differential diagnosis of pneumocystis pneumonia and Pneumocystis colonization. *J Clin Microbiol,* 51**,** 3380-8.

D'AMORE, R., IJAZ, U. Z., SCHIRMER, M., KENNY, J. G., GREGORY, R., DARBY, A. C., SHAKYA, M., PODAR, M., QUINCE, C. & HALL, N. 2016. A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling. *BMC Genomics,* 17**,** 55.

DAVID, G., PERPOINT, T., BOIBIEUX, A., PIALAT, J. B., SALORD, H., DEVOUASSOUX, M., CHIDIAC, C. & PEYRAMOND, D. 2006. Secondary pulmonary syphilis: report of a likely case and literature review. *Clin Infect Dis,* 42**,** e11-5.

DE BOER, M. G., GELINCK, L. B., VAN ZELST, B. D., VAN DE SANDE, W. W., WILLEMS, L. N., VAN DISSEL, J. T., DE JONGE, R. & KROON, F. P. 2011. beta-D-glucan and S-adenosylmethionine serum levels for the diagnosis of Pneumocystis pneumonia in HIV-negative patients: a prospective study. *J Infect,* 62**,** 93-100.

DE BOER, M. G. J., WALZER, P. D. & MORI, S. 2018. Healthcare related transmission of Pneumocystis pneumonia: From key insights toward comprehensive prevention. *Transpl Infect Dis,* e12942.

DE DIOS CABALLERO, J., VIDA, R., COBO, M., MAIZ, L., SUAREZ, L., GALEANO, J., BAQUERO, F., CANTON, R. & DEL CAMPO, R. 2017. Individual Patterns of Complexity in Cystic Fibrosis Lung Microbiota, Including Predator Bacteria, over a 1-Year Period. *MBio,* 8.

DE GOFFAU, M. C., LAGER, S., SALTER, S. J., WAGNER, J., KRONBICHLER, A., CHARNOCK-JONES, D. S., PEACOCK, S. J., SMITH, G. C. S. & PARKHILL, J. 2018. Recognizing the reagent microbiome. *Nat Microbiol,* 3**,** 851-853.

DE LIPTHAY, J. R., ENZINGER, C., JOHNSEN, K., AAMAND, J. & SØRENSEN, S. J. 2004. Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis. *Soil Biology and Biochemistry,* 36**,** 1607-1614.

DE MIGUEL, D., GOMEZ, P., GONZALEZ, R., GARCIA-SUAREZ, J., CUADROS, J. A., BANAS, M. H., ROMANYK, J. & BURGALETA, C. 2005. Nonfatal pulmonary Trichoderma viride infection in an adult patient with acute myeloid leukemia: report of one case and review of the literature. *Diagn Microbiol Infect Dis,* 53**,** 33-7.

DE PAUW, B., WALSH, T. J., DONNELLY, J. P., STEVENS, D. A., EDWARDS, J. E., CALANDRA, T., PAPPAS, P. G., MAERTENS, J., LORTHOLARY, O., KAUFFMAN, C. A., DENNING, D. W., PATTERSON, T. F., MASCHMEYER, G., BILLE, J., DISMUKES, W. E., HERBRECHT, R., HOPE, W. W., KIBBLER, C. C., KULLBERG, B. J., MARR, K. A., MUNOZ, P., ODDS, F. C., PERFECT, J. R., RESTREPO, A., RUHNKE, M., SEGAL, B. H., SOBEL, J. D., SORRELL, T. C., VISCOLI, C., WINGARD, J. R., ZAOUTIS, T., BENNETT, J. E., EUROPEAN ORGANIZATION FOR, R., TREATMENT OF CANCER/INVASIVE FUNGAL INFECTIONS COOPERATIVE, G., NATIONAL INSTITUTE OF, A. & INFECTIOUS DISEASES MYCOSES STUDY GROUP CONSENSUS, G. 2008. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis,* 46**,** 1813-21.

DEESOMCHOK, A. & TANPRAWATE, S. 2006. A 12-case series of Penicillium marneffei pneumonia. *J Med Assoc Thai,* 89**,** 441-7.

DEI-CAS, E. 2000. Pneumocystis infections: the iceberg? *Med Mycol,* 38 Suppl 1**,** 23-32.

DERAGON, J., SINNETT, D., MITCHELL, G., POTIER, M. & LABUDA, D. 1990. Use of γ irradiation to eliminate DNA contamination for PCR. *Nucleic Acids Research,* 18.

DESNEUX, J. & POURCHER, A. M. 2014. Comparison of DNA extraction kits and modification of DNA elution procedure for the quantitation of subdominant bacteria from piggery effluents with real-time PCR. *Microbiologyopen,* 3**,** 437-45.

DEWAVRIN, F., ZERIMECH, F., BOYER, A., MABOUDOU, P., BALDUYCK, M., DUHAMEL, A. & NSEIR, S. 2014. Accuracy of alpha amylase in diagnosing microaspiration in intubated critically-ill patients. *PLoS One,* 9**,** e90851.

DEWHIRST, F. E., CHEN, T., IZARD, J., PASTER, B. J., TANNER, A. C., YU, W. H., LAKSHMANAN, A. & WADE, W. G. 2010. The human oral microbiome. *J Bacteriol,* 192**,** 5002-17.

DHARMADHIKARI, A. S., BASARABA, R. J., VAN DER WALT, M. L., WEYER, K., MPHAHLELE, M., VENTER, K., JENSEN, P. A., FIRST, M. W., PARSONS, S., MCMURRAY, D. N., ORME, I. M. & NARDELL, E. A. 2011. Natural infection of guinea pigs exposed to patients with highly drug-resistant tuberculosis. *Tuberculosis (Edinb),* 91**,** 329-38.

DHARMADHIKARI, A. S., MPHAHLELE, M., STOLTZ, A., VENTER, K., MATHEBULA, R., MASOTLA, T., LUBBE, W., PAGANO, M., FIRST, M., JENSEN, P. A., VAN DER WALT, M. & NARDELL, E. A. 2012. Surgical face masks worn by patients with multidrug-resistant tuberculosis: impact on infectivity of air on a hospital ward. *Am J Respir Crit Care Med,* 185**,** 1104-9.

DHARMASENA, F. M., DAVIES, G. S. & CATOVSKY, D. 1985. Paecilomyces varioti pneumonia complicating hairy cell leukaemia. *Br Med J (Clin Res Ed),* 290**,** 967-8.

DHARMIC, S., NAIR, S. & HARISH, M. 2015. An unusual cause of fungal pneumonia. *Journal of Pharmacy & Bioallied Sciences,* 7**,** S67-S69.

DI PERRI, G., CRUCIANI, M., DANZI, M. C., LUZZATI, R., DE CHECCHI, G., MALENA, M., PIZZIGHELLA, S., MAZZI, R., SOLBIATI, M., CONCIA, E. & ET AL. 1989. Nosocomial epidemic of active tuberculosis among HIV-infected patients. *Lancet,* 2**,** 1502-4.

DICKSON, R. P., ERB-DOWNWARD, J. R., FREEMAN, C. M., MCCLOSKEY, L., FALKOWSKI, N. R., HUFFNAGLE, G. B. & CURTIS, J. L. 2017. Bacterial Topography of the Healthy Human Lower Respiratory Tract. *MBio,* 8.

DICKSON, R. P., ERB-DOWNWARD, J. R. & HUFFNAGLE, G. B. 2013. The role of the bacterial microbiome in lung disease. *Expert Rev Respir Med,* 7**,** 245-57.

DICKSON, R. P., ERB-DOWNWARD, J. R., MARTINEZ, F. J. & HUFFNAGLE, G. B. 2016a. The Microbiome and the Respiratory Tract. *Annu Rev Physiol,* 78**,** 481-504.

DICKSON, R. P. & HUFFNAGLE, G. B. 2015. The Lung Microbiome: New Principles for Respiratory Bacteriology in Health and Disease. *PLoS Pathog,* 11**,** e1004923.

DICKSON, R. P., SINGER, B. H., NEWSTEAD, M. W., FALKOWSKI, N. R., ERB-DOWNWARD, J. R., STANDIFORD, T. J. & HUFFNAGLE, G. B. 2016b. Enrichment of the lung microbiome with gut bacteria in sepsis and the acute respiratory distress syndrome. *Nat Microbiol,* 1**,** 16113.

DINI, L., DU PLESSIS, M., FREAN, J. & FERNANDEZ, V. 2010. High prevalence of dihydropteroate synthase mutations in Pneumocystis jirovecii isolated from patients with Pneumocystis pneumonia in South Africa. *J Clin Microbiol,* 48**,** 2016-21.

DOHN, M. N., WHITE, M. L., VIGDORTH, E. M., RALPH BUNCHER, C., HERTZBERG, V. S., BAUGHMAN, R. P., GEORGE SMULIAN, A. & WALZER, P. D. 2000. Geographic clustering of Pneumocystis carinii pneumonia in patients with HIV infection. *Am J Respir Crit Care Med,* 162**,** 1617-21.

DOIG, C., SEAGAR, A. L., WATT, B. & FORBES, K. J. 2002. The efficacy of the heat killing of Mycobacterium tuberculosis. *J Clin Pathol,* 55**,** 778-9.

DOLLFUSS, R. E., MILIC-EMILI, J. & BATES, D. V. 1967. Regional ventilation of the lung, studied with boluses of 133xenon. *Respiration Physiology,* 2**,** 234-246.

DOLLNER, H., RAMM, C. T., HARSTAD, I., AFSET, J. E. & SAGVIK, E. 2012. Risk of developing tuberculosis after brief exposure in Norwegian children: results of a contact investigation. *BMJ Open,* 2.

DORAK, M. T. 2006. *Real-time PCR,* Oxford, UK, Francis & Taylor Group.

DOYLE, L., VOGEL, S. & PROCOP, G. W. 2017. Pneumocystis PCR: It Is Time to Make PCR the Test of Choice. *Open Forum Infect Dis,* 4**,** ofx193.

DRANCOURT, M., BOLLET, C., CARLIOZ, A., MARTELIN, R., GAYRAL, J. P. & RAOULT, D. 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J Clin Microbiol,* 38**,** 3623-30.

DURAND-JOLY, I., SOULA, F., CHABE, M., DALLE, J. H., LAFITTE, J. J., SENECHAL, M., PINON, A., CAMUS, D. & DEI-CAS, E. 2003. Long-term colonization with Pneumocystis jirovecii in hospital staffs: a challenge to prevent nosocomial pneumocystosis. *J Eukaryot Microbiol,* 50 Suppl**,** 614-5.

DYNER, T. S., LANG, W., BUSCH, D. F. & GORDON, P. R. 1989. Intravascular and pleural involvement by Pneumocystis carinii in a patient with the acquired immunodeficiency syndrome (AIDS). *Ann Intern Med,* 111**,** 94-5.

ECONOMOPOULOU, A., DOMINGUEZ, M., HELYNCK, B., SISSOKO, D., WICHMANN, O., QUENEL, P., GERMONNEAU, P. & QUATRESOUS, I. 2009. Atypical Chikungunya virus infections: clinical manifestations, mortality and risk factors for severe disease during the 2005-2006 outbreak on Reunion. *Epidemiol Infect,* 137**,** 534-41.

EDGAR, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics,* 26**,** 2460-1.

EDGAR, R. C., HAAS, B. J., CLEMENTE, J. C., QUINCE, C. & KNIGHT, R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics,* 27**,** 2194-200.

EDMAN, J. C., KOVACS, J. A., MASUR, H., SANTI, D. V., ELWOOD, H. J. & SOGIN, M. L. 1988. Ribosomal RNA sequence shows Pneumocystis carinii to be a member of the fungi. *Nature,* 334**,** 519-22.

EFFROS, R. M., PETERSON, B., CASABURI, R., SU, J., DUNNING, M., TORDAY, J., BILLER, J. & SHAKER, R. 2005. Epithelial lining fluid solute concentrations in chronic obstructive lung disease patients and normal subjects. *J Appl Physiol (1985),* 99**,** 1286-92.

EL BALI, L., DIMAN, A., BERNARD, A., ROOSENS, N. H. & DE KEERSMAECKER, S. C. 2014. Comparative study of seven commercial kits for human DNA extraction from urine samples suitable for DNA biomarker-based public health studies. *J Biomol Tech,* 25**,** 96-110.

ELLISON III, R. T. & GIEHL, T. G. 1991. Killing of Gram-negative Bacteria by Lactofernn and Lysozyme. *J. Clin. Invest.,* 88**,** 1080-1091.

ENDE, N. 1960. Studies of amylase activity in pleural effusions and ascites. *Cancer,* 13**,** 283-7.

ENDE, N. 1961. Amylase activity in body fluids. *Cancer,* 14**,** 1109-1114.

ENDO, S., HIRONAKA, M., MURAYAMA, F., YAMAGUCHI, T., SOHARA, Y. & SAITO, K. 2002. Scopulariopsis fungus ball. *Ann Thorac Surg,* 74**,** 926-7.

ENINGER, R. M., HOGAN, C. J., BISWAS, P., ADHIKARI, A., REPONEN, T. & GRINSHPUN, S. A. 2009. Electrospray versus Nebulization for Aerosolization and Filter Testing with Bacteriophage Particles. *Aerosol Science and Technology,* 43**,** 298-304.

ERB-DOWNWARD, J. R., THOMPSON, D. L., HAN, M. K., FREEMAN, C. M., MCCLOSKEY, L., SCHMIDT, L. A., YOUNG, V. B., TOEWS, G. B., CURTIS, J. L., SUNDARAM, B., MARTINEZ, F. J. & HUFFNAGLE, G. B. 2011. Analysis of the lung microbiome in the "healthy" smoker and in COPD. *PLoS One,* 6**,** e16384.

ERICSON, P. A., MIRGORODSKAYA, E., HAMMAR, O. S., VIKLUND, E. A., ALMSTRAND, A. R., LARSSON, P. J., RIISE, G. C. & OLIN, A. C. 2016. Low Levels of Exhaled Surfactant Protein A Associated With BOS After Lung Transplantation. *Transplant Direct,* 2**,** e103.

ESAIASSEN, E., HJERDE, E., CAVANAGH, J. P., SIMONSEN, G. S., KLINGENBERG, C. & NORWEGIAN STUDY GROUP ON INVASIVE BIFIDOBACTERIAL, I. 2017. Bifidobacterium Bacteremia: Clinical Characteristics and a Genomic Approach To Assess Pathogenicity. *J Clin Microbiol,* 55**,** 2234-2248.

ESCOMBE, A. R., MOORE, D. A., GILMAN, R. H., NAVINCOPA, M., TICONA, E., MITCHELL, B., NOAKES, C., MARTINEZ, C., SHEEN, P., RAMIREZ, R., QUINO, W., GONZALEZ, A., FRIEDLAND, J. S. & EVANS, C. A. 2009. Upper-room ultraviolet light and negative air ionization to prevent tuberculosis transmission. *PLoS Med,* 6**,** e43.

ESCOMBE, A. R., OESER, C., GILMAN, R. H., NAVINCOPA, M., TICONA, E., MARTINEZ, C., CAVIEDES, L., SHEEN, P., GONZALEZ, A., NOAKES, C., MOORE, D. A., FRIEDLAND, J. S. & EVANS, C. A. 2007. The detection of airborne transmission of tuberculosis from HIV-infected patients, using an in vivo air sampling model. *Clin Infect Dis,* 44**,** 1349-57.

ESOLEN, L. M., FASANO, M. B., FLYNN, J., BURTON, A. & LEDERMAN, H. M. 1992. Pneumocystis carinii osteomyelitis in a patient with common variable immunodeficiency. *N Engl J Med,* 326**,** 999-1001.

FABIAN, P., BRAIN, J., HOUSEMAN, E. A., GERN, J. & MILTON, D. K. 2011. Origin of exhaled breath particles from healthy and human rhinovirus-infected subjects. *J Aerosol Med Pulm Drug Deliv,* 24**,** 137-47.

FABIAN, P., MCDEVITT, J. J., HOUSEMAN, E. A. & MILTON, D. K. 2009. Airborne influenza virus detection with four aerosol samplers using molecular and infectivity assays: considerations for a new infectious virus aerosol sampler. *Indoor Air,* 19**,** 433-41.

FAKHARIAN, A., DORUDINIA, A., ALAVI DARAZAM, I., MANSOURI, D. & MASJEDI, M. R. 2015. Acremonium Pneumonia: Case Report and Literature Review. *Tanaffos,* 14**,** 156-60.

FALKINHAM, J. O., 3RD 2003. Mycobacterial aerosols and respiratory disease. *Emerg Infect Dis,* 9**,** 763-7.

FALKINHAM, J. O., 3RD 2015. Environmental sources of nontuberculous mycobacteria. *Clin Chest Med,* 36**,** 35-41.

FALKINHAM, J. O., GEORGE, K. L., FORD, M. A. & PARKER, B. C. 1990. Collection and characteristics of mycobacteria in aerosols. *In:* MOREY, P. R., FEELEY, J. C. & OTTEN, J. A. (eds.) *Biological contaminants in indoor environments.* Philadelphia: American Society for Testing and Materials.

FANOURGIAKIS, P., GEORGALA, A., VEKEMANS, M., DANEAU, D., HEYMANS, C. & AOUN, M. 2003. Bacteremia due to Stomatococcus mucilaginosus in neutropenic patients in the setting of a cancer institute. *Clinical Microbiology and Infection,* 9**,** 1068-1072.

FASCIGLIONE, G. F., MARINI, S., D'ALESSIO, S., POLITI, V. & COLETTA, M. 2000. pH- and temperature-dependence of functional modulation in metalloproteinases. A comparison between neutrophil collagenase and gelatinases A and B. *Biophys J,* 79**,** 2138-49.

FAUCHIER, T., HASSEINE, L., GARI-TOUSSAINT, M., CASANOVA, V., MARTY, P. M. & POMARES, C. 2016. Detection of Pneumocystis jirovecii by Quantitative PCR To Differentiate Colonization and Pneumonia in Immunocompromised HIV-Positive and HIV-Negative Patients. *J Clin Microbiol,* 54**,** 1487-95.

FENNELLY, K. P. 1998. The role of masks in preventing nosocomial transmission of tuberculosis. *Int J Tuberc Lung Dis,* 2**,** S103-9.

FENNELLY, K. P. 2005. Personal respiratory protection and prevention of occupational tuberculosis. *Int J Tuberc Lung Dis,* 9**,** 476.

FENNELLY, K. P., JONES-LOPEZ, E. C., AYAKAKA, I., KIM, S., MENYHA, H., KIRENGA, B., MUCHWA, C., JOLOBA, M., DRYDEN-PETERSON, S., REILLY, N., OKWERA, A., ELLIOTT, A. M., SMITH, P. G., MUGERWA, R. D., EISENACH, K. D. & ELLNER, J. J. 2012. Variability of infectious aerosols produced during coughing by patients with pulmonary tuberculosis. *Am J Respir Crit Care Med,* 186**,** 450-7.

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FENNELLY, K. P., MARTYNY, J. W., FULTON, K. E., ORME, I. M., CAVE, D. M. & HEIFETS, L. B. 2004. Cough-generated aerosols of Mycobacterium tuberculosis: a new method to study infectiousness. *Am J Respir Crit Care Med,* 169**,** 604-9.

FENNELLY, K. P. & NARDELL, E. A. 1998. The relative efficacy of respirators and room ventilation in preventing occupational tuberculosis. *Infect Control Hosp Epidemiol,* 19**,** 754-9.

FILLAUX, J., MALVY, S., ALVAREZ, M., FABRE, R., CASSAING, S., MARCHOU, B., LINAS, M. D. & BERRY, A. 2008. Accuracy of a routine real-time PCR assay for the diagnosis of Pneumocystis jirovecii pneumonia. *J Microbiol Methods,* 75**,** 258-61.

FILLOUX, B., BEDEL, A., NSEIR, S., MATHIAUX, J., AMADEO, B., CLOUZEAU, B., PILLOT, J., SAGHI, T., VARGAS, F., HILBERT, G., GRUSON, D. & BOYER, A. 2013. Tracheal amylase dosage as a marker for microaspiration: a pilot study. *Minerva Anestesiol,* 79**,** 1003-10.

FISCHER, J., HAMACHER, L., FRIES, J., HALLEK, M., CORNELY, O. A., KOCHANEK, M. & BOELL, B. 2016. Rhodotorula mucilaginosa as a cause of recurrent pulmonary infection and liver infiltration in a patient with CLL. *Ann Hematol,* 95**,** 1569-70.

FISCHER, S., GILL, V. J., KOVACS, J., MIELE, P., KEARY, J., SILCOTT, V., HUANG, S., BORIO, L., STOCK, F., FAHLE, G., BROWN, D., HAHN, B., TOWNLEY, E., LUCEY, D. & MASUR, H. 2001. The use of oral washes to diagnose Pneumocystis carinii pneumonia: a blinded prospective study using a polymerase chain reaction-based detection system. *J Infect Dis,* 184**,** 1485-8.

FISHMAN, J. A. 2017. Pulmonary infections in immunocompromised patients. *In:* BOND, S. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

FISHMAN, J. A., ROTH, R. S., ZANZOT, E., ENOS, E. J. & FERRARO, M. J. 1994. Use of induced sputum specimens for microbiologic diagnosis of infections due to organisms other than Pneumocystis carinii. *J Clin Microbiol,* 32**,** 131-4.

FLETCHER, C. M., ELMES, P. C., FAIRBAIRN, A. S. & WOOD, C. H. 1959. The significance of respiratory symptoms and the diagnosis of chronic bronchitis in a working population. *Br Med J,* 2**,** 257-66.

FLORI, P., BELLETE, B., DURAND, F., RABERIN, H., CAZORLA, C., HAFID, J., LUCHT, F. & SUNG, R. T. 2004. Comparison between real-time PCR, conventional PCR and different staining techniques for diagnosing Pneumocystis jiroveci pneumonia from bronchoalveolar lavage specimens. *J Med Microbiol,* 53**,** 603-7.

FONTANA, G. A., PANTALEO, T., LAVORINI, F., BODDI, V. & PANUCCIO, P. 1997. A noninvasive electromyographic study on threshold and intensity of cough in humans. *European Respiratory Journal,* 10**,** 983-989.

FOROOTAN, A., SJOBACK, R., BJORKMAN, J., SJOGREEN, B., LINZ, L. & KUBISTA, M. 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomol Detect Quantif,* 12**,** 1-6.

FOUHY, F., CLOONEY, A. G., STANTON, C., CLAESSON, M. J. & COTTER, P. D. 2016. 16S rRNA gene sequencing of mock microbial populations- impact of DNA extraction method, primer choice and sequencing platform. *BMC Microbiol,* 16**,** 123.

FOUHY, F., DEANE, J., REA, M. C., O'SULLIVAN, O., ROSS, R. P., O'CALLAGHAN, G., PLANT, B. J. & STANTON, C. 2015. The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations. *PLoS One,* 10**,** e0119355.

FRANGIDES, C. Y. & PNEUMATIKOS, I. 2004. Varicella-zoster virus pneumonia in adults: report of 14 cases and review of the literature. *Eur J Intern Med,* 15**,** 364-370.

FREE, R. C. 2005. Molecular analysis of the lower respiratory tract microbiota.

FUJISAWA, T., SUDA, T., MATSUDA, H., INUI, N., NAKAMURA, Y., SATO, J., TOYOSHIMA, M., NAKANO, Y., YASUDA, K., GEMMA, H., HAYAKAWA, H. & CHIDA, K. 2009. Real-time PCR is more specific than conventional PCR for induced sputum diagnosis of Pneumocystis pneumonia in immunocompromised patients without HIV infection. *Respirology,* 14**,** 203-9.

FURRER, B., CANDRIAN, U., WIELAND, P. & LUTHY, J. 1990. Improving PCR efficiency. *Nature,* 346.

GAGO, S., ESTEBAN, C., VALERO, C., ZARAGOZA, O., PUIG DE LA BELLACASA, J. & BUITRAGO, M. J. 2014. A multiplex real-time PCR assay for identification of Pneumocystis jirovecii, Histoplasma capsulatum, and Cryptococcus neoformans/Cryptococcus gattii in samples from AIDS patients with opportunistic pneumonia. *J Clin Microbiol,* 52**,** 1168-76.

GAJDUSEK, D. C. 1957. Pneumocystis carinii; etiologic agent of interstitial plasma cell pneumonia of premature and young infants. *Pediatrics,* 19**,** 543-65.

GALE, H. 2000. Evaluation of the quantiplex human immunodeficiency virus type 1 RNA 3.0 assay in a tertiary-care center. *Clin Diagn Lab Immunol,* 7**,** 122-4.

448

GANDHI, R. T. 2018. Toxoplasmosis in HIV-infected patients. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

GANZ, T. 2002. Antimicrobial polypeptides in host defense of the respiratory tract. *J Clin Invest,* 109**,** 693-7.

GARCIA-MONTERO, M., RODRIGUEZ-GARCIA, J. L., CALVO, P., GONZALEZ, J. M., FERNANDEZ-GARRIDO, M., LOZA, E. & SERRANO, M. 1995. Pneumonia caused by Listeria monocytogenes. *Respiration,* 62**,** 107-9.

GARTON, N. J., WADDELL, S. J., SHERRATT, A. L., LEE, S. M., SMITH, R. J., SENNER, C., HINDS, J., RAJAKUMAR, K., ADEGBOLA, R. A., BESRA, G. S., BUTCHER, P. D. & BARER, M. R. 2008. Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLoS Med,* 5**,** e75.

GARVY, B. A. & HARMSEN, A. G. 1996. Susceptibility to Pneumocystis carinii infection: host responses of neonatal mice from immune or naive mothers and of immune or naive adults. *Infect Immun,* 64**,** 3987-92.

GARVY, B. A., WILEY, J. A., GIGLIOTTI, F. & HARMSEN, A. G. 1997. Protection against Pneumocystis carinii pneumonia by antibodies generated from either T helper 1 or T helper 2 responses. *Infect Immun,* 65**,** 5052-6.

GASPARETTO, E. L., ESCUISSATO, D. L., INOUE, C., MARCHIORI, E. & MULLER, N. L. 2005. Herpes simplex virus type 2 pneumonia after bone marrow transplantation: high-resolution CT findings in 3 patients. *J Thorac Imaging,* 20**,** 71-3.

GEORGE, L. M., HAIGH, R. D., MISTRY, V., HALDAR, K., BARER, M. R., OGGIONI, M. R. & BRIGHTLING, C. E. 2018. Sputum Moraxella catarrhalis strains exhibit diversity within and between COPD subjects. *Int J Chron Obstruct Pulmon Dis,* 13**,** 3663-3667.

GEORGOPAPADAKOU, N. H. & WALSH, T. J. 1996. Antifungal agents: chemotherapeutic targets and immunologic strategies. *Antimicrob Agents Chemother,* 40**,** 279-91.

GERASIMIDIS, K., BERTZ, M., QUINCE, C., BRUNNER, K., BRUCE, A., COMBET, E., CALUS, S., LOMAN, N. & IJAZ, U. Z. 2016. The effect of DNA extraction methodology on gut microbiota research applications. *BMC Res Notes,* 9**,** 365.

GHAFOURI, B., TAGESSON, C. & LINDAHL, M. 2003. Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. *Proteomics,* 3**,** 1003-15.

GHEBRE, M. A., PANG, P. H., DIVER, S., DESAI, D., BAFADHEL, M., HALDAR, K., KEBADZE, T., COHEN, S., NEWBOLD, P., RAPLEY, L., WOODS, J., RUGMAN, P., PAVORD, I. D., JOHNSTON, S. L., BARER, M., MAY, R. D. & BRIGHTLING, C. E. 2018. Biological exacerbation clusters demonstrate asthma and chronic obstructive pulmonary disease overlap with distinct mediator and microbiome profiles. *J Allergy Clin Immunol,* 141**,** 2027-2036 e12.

GHYSELINCK, J., PFEIFFER, S., HEYLEN, K., SESSITSCH, A. & DE VOS, P. 2013. The effect of primer choice and short read sequences on the outcome of 16S rRNA gene based diversity studies. *PLoS One,* 8**,** e71360.

GIGLIOTTI, F. & HUGHES, W. T. 1988. Passive immunoprophylaxis with specific monoclonal antibody confers partial protection against Pneumocystis carinii pneumonitis in animal models. *J Clin Invest,* 81**,** 1666-8.

GIGLIOTTI, F., LIMPER, A. H. & WRIGHT, T. 2014. Pneumocystis. *Cold Spring Harb Perspect Med,* 4.

GIGLIOTTI, F. & WRIGHT, T. W. 2005. Immunopathogenesis of Pneumocystis carinii pneumonia. *Expert Rev Mol Med,* 7**,** 1-16.

GILL, P. K. & GILL, J. S. 2011. Blastoschizomyces capitatus pneumonia in an immuno-competent female. *Indian J Tuberc,* 58**,** 88-9.

GLASEL, J. A. 1995. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. *Biotechniques,* 18**,** 62-3.

GLEESON, K., EGGLI, D. F. & MAXWELL, S. L. 1997. Quantitative aspiration during sleep in normal subjects. *Chest,* 111**,** 1266-72.

GLENDINNING, L., COLLIE, D., WRIGHT, S., RUTHERFORD, K. M. D. & MCLACHLAN, G. 2017b. Comparing microbiotas in the upper aerodigestive and lower respiratory tracts of lambs. *Microbiome,* 5**,** 145.

GLENDINNING, L., WRIGHT, S., TENNANT, P., GILL, A. C., COLLIE, D. & MCLACHLAN, G. 2017a. Microbiota in Exhaled Breath Condensate and the Lung. *Appl Environ Microbiol,* 83.

GLENNY, R. W. & PIERSON, D. J. 1992. Cost reduction in diagnosing Pneumocystis carinii pneumonia. Sputum induction versus bronchoalveolar lavage as the initial diagnostic procedure. *Am Rev Respir Dis,* 145**,** 1425-8.

GLOBAL BURDEN OF DISEASE COLLABORATIVE NETWORK. 2017. Global Burden of Disease Study 2016 (GBD 2016) Data Resources. Seattle, United States: Institute for Health Metrics and Evaluation (IHME).

GOESCH, T. R., GOTZ, G., STELLBRINCK, K. H., ALBRECHT, H., WEH, H. J. & HOSSFELD, D. K. 1990. Possible transfer of Pneumocystis carinii between immunodeficient patients. *Lancet,* 336**,** 627.

GOH, L. Y., CARD, T., FOGARTY, A. W. & MCKEEVER, T. M. 2014. The association of exposure to hepatitis B and C viruses with lung function and respiratory disease: a population based study from the NHANES III database. *Respir Med,* 108**,** 1733-40.

GOHL, D. M., VANGAY, P., GARBE, J., MACLEAN, A., HAUGE, A., BECKER, A., GOULD, T. J., CLAYTON, J. B., JOHNSON, T. J., HUNTER, R., KNIGHTS, D. & BECKMAN, K. B. 2016. Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. *Nat Biotechnol,* 34**,** 942-9.

GOLDBERG, M. B. 2018. Capnocytophaga. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

GÓMEZ-GUILLÉN, M. C., TURNAY, J., FERNÁNDEZ-DíAZ, M. D., ULMO, N., LIZARBE, M. A. & MONTERO, P. 2002. Structural and physical properties of gelatin extracted from different marine species: a comparative study. *Food Hydrocolloids,* 16**,** 25-34.

GOTELLI, N. J. & COLWELL, R. K. 2011. Estimating species richness. *In:* MAGURRAN, A. E. & MCGILL, B. J. (eds.) *Biological diversity: Frontiers in measuringbiodiversity.* Oxford, United Kingdom: Oxford University Press.

GRAVA, S., LOPES, F. A. D., CAVALLAZZI, R. S., GRASSI, M. & SVIDZINSKI, T. I. E. 2016. A rare case of hemorrhagic pneumonia due to Cladosporium cladosporioides. *J Bras Pneumol,* 42**,** 392-4.

GRIESE, M., NOSS, J. & VON BREDOW, C. 2002. Protein pattern of exhaled breath condensate and saliva. *Proteomics, 2***,** 690-6.

GRIFFITH, D. E. 2017. Overview of nontuberculous mycobacterial infections in HIV-negative patients. *In:* BLOOM, A. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

GRUPPER, M., KRAVTSOV, A. & POTASMAN, I. 2009. Enterococcal-associated lower respiratory tract infections: a case report and literature review. *Infection,* 37**,** 60-4.

GRUSON, D., HILBERT, G., PIGNEUX, A., VARGAS, F., GUISSET, O., TEXIER, J., BOIRON, J. M., REIFFERS, J., GBIKPI-BENISSAN, G. & CARDINAUD, J. P. 1998. Severe infection caused by Stomatococcus mucilaginosus in a neutropenic patient: case report and review of the literature. *Hematol Cell Ther,* 40**,** 167-9.

GUILLAUD-SAUMUR, T., NEVEZ, G., BAZIRE, A., VIRMAUX, M., PAPON, N. & LE GAL, S. 2017. Comparison of a commercial real-time PCR assay, RealCycler(R) PJIR kit, progenie molecular, to an in-house real-time PCR assay for the diagnosis of Pneumocystis jirovecii infections. *Diagn Microbiol Infect Dis,* 87**,** 335-337.

GUO, F. & ZHANG, T. 2013. Biases during DNA extraction of activated sludge samples revealed by high throughput sequencing. *Appl Microbiol Biotechnol,* 97**,** 4607-16.

GUO, X., XIA, X., TANG, R., ZHOU, J., ZHAO, H. & WANG, K. 2008. Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett Appl Microbiol,* 47**,** 367-73.

GUPTA, R., IYER, V. K., MIRDHA, B. R., GULERIA, R., KUMAR, L. & AGARWAL, S. K. 2010. Role of cytology and polymerase chain reaction based detection of Pneumocystis jirovecii infection in bronchoalveolar lavage fluid. *Acta Cytol,* 54**,** 296-302.

GURCAN, S., TUGRUL, H. M., YORUK, Y., OZER, B., TATMAN-OTKUN, M. & OTKUN, M. 2006. First case report of empyema caused by Beauveria bassiana. *Mycoses,* 49**,** 246-8.

GUTTLER, R., SINGER, P. A., AXLINE, S. G., GREAVES, T. S. & MCGILL, J. J. 1993. Pneumocystis carinii thyroiditis. Report of three cases and review of the literature. *Arch Intern Med,* 153**,** 393-6.

HAHN, A., BENDALL, M. L., GIBSON, K. M., CHANEY, H., SAMI, I., PEREZ, G. F., KOUMBOURLIS, A. C., MCCAFFREY, T. A., FREISHTAT, R. J. & CRANDALL, K. A. 2018. Benchmark Evaluation of True Single Molecular Sequencing to Determine Cystic Fibrosis Airway Microbiome Diversity. *Front Microbiol,* 9**,** 1069.

HALDAR, K., BAFADHEL, M., LAU, K., BERG, A., KWAMBANA, B., KEBADZE, T., RAMSHEH, M. Y., BARKER, B., HALDAR, P., JOHNSTON, S., KETLEY, J. M., BRIGHTLING, C. E. & BARER, M. R. 2017. Microbiome balance in sputum determined by PCR stratifies COPD exacerbations and shows potential for selective use of antibiotics. *PLoS One,* 12**,** e0182833.

HALDAR, K. S. 2014. *Profiling of Bacterial Communities in Chronic Obstructive Pulmonary Disease.* Doctor of Philosophy, University of Leicester.

HARADA, K. & KITAMURA, M. 1971. [Amylase isoenzyme in a patient with primary lung cancer]. *Igaku To Seibutsugaku,* 82**,** 155-8.

HARDAK, E., BROOK, O. & YIGLA, M. 2010. Radiological features of Pneumocystis jirovecii Pneumonia in immunocompromised patients with and without AIDS. *Lung,* 188**,** 159-63.

HARMSEN, A. G., CHEN, W. & GIGLIOTTI, F. 1995. Active immunity to Pneumocystis carinii reinfection in T-cell-depleted mice. *Infect Immun,* 63**,** 2391-5.

HARO, A., TAMIYA, S. & NAGASHIMA, A. 2016. A rare case of human pulmonary dirofilariasis with a growing pulmonary nodule after migrating infiltration shadows, mimicking primary lung carcinoma. *Int J Surg Case Rep,* 22**,** 8-11.

450

HARON, E., BODEY, G. P., LUNA, M. A., DEKMEZIAN, R. & ELTING, L. 1988. Has the incidence of Pneumocystis carinii pneumonia in cancer patients increased with the AIDS epidemic? *Lancet,* 2**,** 904-5.

HARRIES, A. D., MAHER, D. & NUNN, P. 1997. Practical and affordable measures for the protection of health care workers from tuberculosis in low-income countries. *Bull World Health Organ,* 75**,** 477-89.

HARRIS, K., MAROUN, R., CHALHOUB, M. & ELSAYEGH, D. 2012. Unusual presentation of pneumocystis pneumonia in an immunocompetent patient diagnosed by open lung biopsy. *Heart Lung Circ,* 21**,** 221-4.

HART, M. L., MEYER, A., JOHNSON, P. J. & ERICSSON, A. C. 2015. Comparative Evaluation of DNA Extraction Methods from Feces of Multiple Host Species for Downstream Next-Generation Sequencing. *PLoS One,* 10**,** e0143334.

HARTMAN, T. E., PRIMACK, S. L., MULLER, N. L. & STAPLES, C. A. 1994. Diagnosis of thoracic complications in AIDS: accuracy of CT. *AJR Am J Roentgenol,* 162**,** 547-53.

HATAGISHI, E., OKAMOTO, M., OHMIYA, S., YANO, H., HORI, T., SAITO, W., MIKI, H., SUZUKI, Y., SAITO, R., YAMAMOTO, T., SHOJI, M., MORISAKI, Y., SAKATA, S. & NISHIMURA, H. 2014. Establishment and clinical applications of a portable system for capturing influenza viruses released through coughing. *PLoS One,* 9**,** e103560.

HAUSER, P. M. 2014. Genomic insights into the fungal pathogens of the genus pneumocystis: obligate biotrophs of humans and other mammals. *PLoS Pathog,* 10**,** e1004425.

HAUSER, P. M., BILLE, J., LASS-FLORL, C., GELTNER, C., FELDMESSER, M., LEVI, M., PATEL, H., MUGGIA, V., ALEXANDER, B., HUGHES, M., FOLLETT, S. A., CUI, X., LEUNG, F., MORGAN, G., MOODY, A., PERLIN, D. S. & DENNING, D. W. 2011. Multicenter, prospective clinical evaluation of respiratory samples from subjects at risk for Pneumocystis jirovecii infection by use of a commercial real-time PCR assay. *J Clin Microbiol,* 49**,** 1872-8.

HAYASHI, A. & OH, S. C. 1983. Gelation of gelatin solution. *Agric. Biol. Chem.,* 47**,** 1711-1716.

HAYASHI, Y., FUKAYAMA, M., KOIKE, M. & NAKAYAMA, T. 1986. Amylase in human lungs and the female genital tract. Histochemical and immunohistochemical localization. *Histochemistry,* 85**,** 491-6.

HE, Y., CAPORASO, J. G., JIANG, X. T., SHENG, H. F., HUSE, S. M., RIDEOUT, J. R., EDGAR, R. C., KOPYLOVA, E., WALTERS, W. A., KNIGHT, R. & ZHOU, H. W. 2015. Stability of operational taxonomic units: an important but neglected property for analyzing microbial diversity. *Microbiome,* 3**,** 20.

HEIMBUCH, B. K., KINNEY, K., LUMLEY, A. E., HARNISH, D. A., BERGMAN, M. & WANDER, J. D. 2014. Cleaning of filtering facepiece respirators contaminated with mucin and Staphylococcus aureus. *Am J Infect Control,* 42**,** 265-70.

HELWEG-LARSEN, J., BENFIELD, T. L., EUGEN-OLSEN, J., LUNDGREN, J. D. & LUNDGREN, B. 1999. Effects of mutations in Pneumocystis carinii dihydropteroate synthase gene on outcome of AIDS-associated P. carinii pneumonia. *Lancet,* 354**,** 1347-51.

HELWEG-LARSEN, J., JENSEN, J. S., BENFIELD, T., SVENDSEN, U. G., LUNDGREN, J. D. & LUNDGREN, B. 1998b. Diagnostic use of PCR for detection of Pneumocystis carinii in oral wash samples. *J Clin Microbiol,* 36**,** 2068-72.

HELWEG-LARSEN, J., LEE, C. H., JIN, S., HSUEH, J. Y., BENFIELD, T. L., HANSEN, J., LUNDGREN, J. D. & LUNDGREN, B. 2001. Clinical correlation of variations in the internal transcribed spacer regions of rRNA genes in Pneumocystis carinii f.sp. hominis. *AIDS,* 15**,** 451-9.

HELWEG-LARSEN, J., TSOLAKI, A. G., MILLER, R. F., LUNDGREN, B. & WAKEFIELD, A. E. 1998a. Clusters of Pneumocystis carinii pneumonia: analysis of person-to-person transmission by genotyping. *QJM,* 91**,** 813-20.

HENDERSON, G., COX, F., KITTELMANN, S., MIRI, V. H., ZETHOF, M., NOEL, S. J., WAGHORN, G. C. & JANSSEN, P. H. 2013. Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One,* 8**,** e74787.

HENRIQUES, A., CEREIJA, T., MACHADO, A. & CERCA, N. 2012. In silico vs in vitro analysis of primer specificity for the detection of Gardnerella vaginalis, Atopobium vaginae and Lactobacillus spp. *BMC Res Notes,* 5**,** 637.

HERNANDEZ-GARDUNO, E., COOK, V., KUNIMOTO, D., ELWOOD, R. K., BLACK, W. A. & FITZGERALD, J. M. 2004. Transmission of tuberculosis from smear negative patients: a molecular epidemiology study. *Thorax,* 59**,** 286-90.

HIGUCHI, R., FOCKLER, C., DOLLINGER, G. & WATSON, R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y),* 11**,** 1026-30.

HILTY, M., BURKE, C., PEDRO, H., CARDENAS, P., BUSH, A., BOSSLEY, C., DAVIES, J., ERVINE, A., POULTER, L., PACHTER, L., MOFFATT, M. F. & COOKSON, W. O. 2010. Disordered microbial communities in asthmatic airways. *PLoS One,* 5**,** e8578.

HORVATH, I., HUNT, J., BARNES, P. J., ALVING, K., ANTCZAK, A., BARALDI, E., BECHER, G., VAN BEURDEN, W. J., CORRADI, M., DEKHUIJZEN, R., DWEIK, R. A., DWYER, T., EFFROS, R., ERZURUM, S., GASTON, B., GESSNER, C., GREENING, A., HO, L. P., HOHLFELD, J., JOBSIS, Q., LASKOWSKI, D., LOUKIDES, S., MARLIN, D., MONTUSCHI, P., OLIN, A. C., REDINGTON, A. E., REINHOLD, P., VAN RENSEN, E. L., RUBINSTEIN, I., SILKOFF, P., TOREN, K., VASS, G.,

451

VOGELBERG, C., WIRTZ, H. & CONDENSATE, A. E. T. F. O. E. B. 2005. Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J,* 26, 523-48.

HUANG, Y. J. & LIPUMA, J. J. 2016. The Microbiome in Cystic Fibrosis. *Clin Chest Med,* 37, 59-67.

HUFFNAGLE, G. B., DICKSON, R. P. & LUKACS, N. W. 2017. The respiratory tract microbiome and lung inflammation: a two-way street. *Mucosal Immunol,* 10, 299-306.

HUFFNAGLE, G. B. & NOVERR, M. C. 2013. The emerging world of the fungal microbiome. *Trends Microbiol,* 21, 334-41.

HUGGETT, J. F., TAYLOR, M. S., KOCJAN, G., EVANS, H. E., MORRIS-JONES, S., GANT, V., NOVAK, T., COSTELLO, A. M., ZUMLA, A. & MILLER, R. F. 2008. Development and evaluation of a real-time PCR assay for detection of Pneumocystis jirovecii DNA in bronchoalveolar lavage fluid of HIV-infected patients. *Thorax,* 63, 154-9.

HUGHES, W. T. 1977. Pneumocystis carinii pneumonia. *N Engl J Med,* 297, 1381-3.

HUGHES, W. T. 1987. Pneumocystis carinii pneumonitis. *N Engl J Med,* 317, 1021-3.

HUXLEY, E. J., VIROSLAV, J., GRAY, W. R. & PIERCE, A. K. 1978. Pharyngeal aspiration in normal adults and patients with depressed consciousness. *Am J Med,* 64, 564-8.

HUYNH, K. N., OLIVER, B. G., STELZER, S., RAWLINSON, W. D. & TOVEY, E. R. 2008. A new method for sampling and detection of exhaled respiratory virus aerosols. *Clin Infect Dis,* 46, 93-5.

IBRAHIM, H. R., THOMAS, U. & PELLEGRINI, A. 2001. A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *J Biol Chem,* 276, 43767-74.

ITO, K., LI, L. F., NISHIWAKI, M., OKADA, Y. & MINAMIURA, N. 1992. Evidence for conversion of human salivary alpha-amylase family A to family B by an enzyme action. *J Biochem,* 112, 88-92.

JACCARD, P. 1912. THE DISTRIBUTION OF THE FLORA IN THE ALPINE ZONE.1. *New Phytologist,* 11, 37-50.

JACOBS, J. L., LIBBY, D. M., WINTERS, R. A., GELMONT, D. M., FRIED, E. D., HARTMAN, B. J. & LAURENCE, J. 1991. A cluster of Pneumocystis carinii pneumonia in adults without predisposing illnesses. *N Engl J Med,* 324, 246-50.

JAIN, R., SCHRIEVER, C. A., DANZIGER, L. H., CHO, S. H. & RUBINSTEIN, I. 2007. The IS6110 repetitive DNA element of Mycobacterium tuberculosis is not detected in exhaled breath condensate of patients with active pulmonary tuberculosis. *Respiration,* 74, 329-33.

JANKUTE, M., COX, J. A., HARRISON, J. & BESRA, G. S. 2015. Assembly of the Mycobacterial Cell Wall. *Annu Rev Microbiol,* 69, 405-23.

JANKUTE, M., NATARAJ, V., LEE, O. Y., WU, H. H. T., RIDELL, M., GARTON, N. J., BARER, M. R., MINNIKIN, D. E., BHATT, A. & BESRA, G. S. 2017. The role of hydrophobicity in tuberculosis evolution and pathogenicity. *Sci Rep,* 7, 1315.

JANSSEN, P. H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol,* 72, 1719-28.

JAOUDE, P. A., KNIGHT, P. R., OHTAKE, P. & EL-SOLH, A. A. 2010. Biomarkers in the diagnosis of aspiration syndromes. *Expert Rev Mol Diagn,* 10, 309-19.

JASCHHOF, H. 1992. Sampling virus aerosols using the gelatin membrane filter—collection using a membrane filter at a high sampling rate. *Bio Tec,* 6.

JENNINGS, C., HARTY, B., GRANGER, S., WAGER, C., CRUMP, J. A., FISCUS, S. A. & BREMER, J. W. 2012. Cross-platform analysis of HIV-1 RNA data generated by a multicenter assay validation study with wide geographic representation. *J Clin Microbiol,* 50, 2737-47.

JENSEN, B. N., LISSE, I. M., GERSTOFT, J., BORGESKOV, S. & SKINHOJ, P. 1991. Cellular profiles in bronchoalveolar lavage fluid of HIV-infected patients with pulmonary symptoms: relation to diagnosis and prognosis. *AIDS,* 5, 527-33.

JERVIS-BARDY, J., LEONG, L. E., MARRI, S., SMITH, R. J., CHOO, J. M., SMITH-VAUGHAN, H. C., NOSWORTHY, E., MORRIS, P. S., O'LEARY, S., ROGERS, G. B. & MARSH, R. L. 2015. Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. *Microbiome,* 3, 19.

JESSIE, K., PANG, W. W., HAJI, Z., RAHIM, A. & HASHIM, O. H. 2010. Proteomic analysis of whole human saliva detects enhanced expression of interleukin-1 receptor antagonist, thioredoxin and lipocalin-1 in cigarette smokers compared to non-smokers. *Int J Mol Sci,* 11, 4488-505.

JIANCHENG, W., MINJUN, H., YI-JUN, A., LAN, S., ZENGZHU, G., JIANRONG, S. & XIXIONG, K. 2009. Screening Pneumocystis carinii pneumonia in non-HIV-infected immunocompromised patients using polymerase chain reaction. *Diagn Microbiol Infect Dis,* 64, 396-401.

JOHNSON, G. R. & MORAWSKA, L. 2009. The mechanism of breath aerosol formation. *J Aerosol Med Pulm Drug Deliv,* 22, 229-37.

JOHNSON, G. R., MORAWSKA, L., RISTOVSKI, Z. D., HARGREAVES, M., MENGERSEN, K., CHAO, C. Y. H., WAN, M. P., LI, Y., XIE, X., KATOSHEVSKI, D. & CORBETT, S. 2011. Modality of human expired aerosol size distributions. *Journal of Aerosol Science,* 42**,** 839-851.

JONES, R. M. & BROSSEAU, L. M. 2015. Aerosol transmission of infectious disease. *J Occup Environ Med,* 57**,** 501-8.

JONES-LOPEZ, E. C., NAMUGGA, O., MUMBOWA, F., SSEBIDANDI, M., MBABAZI, O., MOINE, S., MBOOWA, G., FOX, M. P., REILLY, N., AYAKAKA, I., KIM, S., OKWERA, A., JOLOBA, M. & FENNELLY, K. P. 2013. Cough aerosols of Mycobacterium tuberculosis predict new infection: a household contact study. *Am J Respir Crit Care Med,* 187**,** 1007-15.

JONSSON, B., RIDELL, M. & WOLD, A. E. 2013. Phagocytosis and cytokine response to rough and smooth colony variants of Mycobacterium abscessus by human peripheral blood mononuclear cells. *APMIS,* 121**,** 45-55.

JONSSON, B. E., GILLJAM, M., LINDBLAD, A., RIDELL, M., WOLD, A. E. & WELINDER-OLSSON, C. 2007. Molecular epidemiology of Mycobacterium abscessus, with focus on cystic fibrosis. *J Clin Microbiol,* 45**,** 1497-504.

JULES-ELYSEE, K. M., STOVER, D. E., ZAMAN, M. B., BERNARD, E. M. & WHITE, D. A. 1990. Aerosolized pentamidine: effect on diagnosis and presentation of Pneumocystis carinii pneumonia. *Ann Intern Med,* 112**,** 750-7.

KAEVSKA, M. & SLANA, I. 2015. Comparison of filtering methods, filter processing and DNA extraction kits for detection of mycobacteria in water. *Ann Agric Environ Med,* 22**,** 429-32.

KALLE, E., KUBISTA, M. & RENSING, C. 2014. Multi-template polymerase chain reaction. *Biomol Detect Quantif,* 2**,** 11-29.

KAMIO, T., SAMESHIMA, K., IRIE, J., SHIGEMATSU, K., KAWAI, K., TSUCHIYAMA, H. & NAKANO, M. 1989. [Two cases of amylase-producing lung cancer]. *Gan No Rinsho,* 35**,** 735-40.

KANAFANI, Z. A. & KANJ, S. S. 2018. Acinetobacter infection: Epidemiology, microbiology, pathogenesis, clinical features, and diagnosis. *In:* BLOOM, A. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

KANAGAWA, T. 2003. Bias and artifacts in multitemplate polymerase chain reactions (PCR). *J Biosci Bioeng,* 96**,** 317-23.

KANE, S. L., DASTA, J. F. & COOK, C. H. 2002. Amphotericin B lipid complex for Hansenula anomala pneumonia. *Ann Pharmacother,* 36**,** 59-62.

KAPLANSKI, G., GRANEL, B., DI STEFANO, D., DURAND, J. M. & SOUBEYRAND, J. 1996. Pleural pseudotumoral mass revealing an extrapulmonary Pneumocystis carinii infection. *Clin Infect Dis,* 23**,** 199-200.

KASER, M., RUF, M. T., HAUSER, J., MARSOLLIER, L. & PLUSCHKE, G. 2009. Optimized method for preparation of DNA from pathogenic and environmental mycobacteria. *Appl Environ Microbiol,* 75**,** 414-8.

KASPER, L. H. & BUZONI-GATEL, D. 1998. Some Opportunistic Parasitic Infections in AIDS: Candidiasis, Pneumocystosis, Cryptosporidiosis, Toxoplasmosis. *Parasitology Today,* 14**,** 150-156.

KATRAGKOU, A., PANA, Z. D., PERLIN, D. S., KONTOYIANNIS, D. P., WALSH, T. J. & ROILIDES, E. 2014. Exserohilum infections: review of 48 cases before the 2012 United States outbreak. *Med Mycol,* 52**,** 376-86.

KAUFFMAN, C. A. 2016. Diagnosis and treatment of pulmonary histoplasmosis. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

KAUFFMAN, C. A. 2017. Primary coccidioidal infection. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

KAUFFMAN, C. A. 2018. Clinical features and diagnosis of sporotrichosis. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

KAWADA, H., YAMAZATO, M., SHINOZAWA, Y., SUZUKI, K., OTANI, S., NEMOTO, I. & MIYAIRI, M. 2008. [Exhaled breath-generated aerosols of mycobacterium tuberculosis in patients with active pulmonary tuberculosis during normal breathing]. *Kekkaku,* 83**,** 387-91.

KEHRMANN, J., VECKOLLARI, B., SCHMIDT, D., SCHILDGEN, O., SCHILDGEN, V., WAGNER, N., ZESCHNIGK, M., KLEIN-HITPASS, L., WITZKE, O., BUER, J. & STEINMANN, J. 2017. The lung microbiome in patients with pneumocystosis. *BMC Pulm Med,* 17**,** 170.

KELLY, M. N. & SHELLITO, J. E. 2010. Current understanding of Pneumocystis immunology. *Future Microbiol,* 5**,** 43-65.

KENNEDY, M., RAMSHEH, M. Y., WILLIAMS, C. M. L., AUTY, J., HALDAR, K., ABDULWHHAB, M., BRIGHTLING, C. E. & BARER, M. R. 2018. Face mask sampling reveals antimicrobial resistance genes in exhaled aerosols from patients with chronic obstructive pulmonary disease and healthy volunteers. *BMJ Open Respir Res,* 5**,** e000321.

KENNEDY, N. A., WALKER, A. W., BERRY, S. H., DUNCAN, S. H., FARQUARSON, F. M., LOUIS, P., THOMSON, J. M., SATSANGI, J., FLINT, H. J., PARKHILL, J., LEES, C. W. & HOLD, G. L. 2014. The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PLoS One,* 9**,** e88982.

KENNEY, R. T., KWON-CHUNG, K. J., WAYTES, A. T., MELNICK, D. A., PASS, H. I., MERINO, M. J. & GALLIN, J. I. 1992. Successful treatment of systemic Exophiala dermatitidis infection in a patient with chronic granulomatous disease. *Clin Infect Dis,* 14**,** 235-42.

KENNEY, R. T., KWON-CHUNG, K. J., WITEBSKY, F. G., MELNICK, D. A., MALECH, H. L. & GALLIN, J. I. 1990. Invasive infection with Sarcinosporon inkin in a patient with chronic granulomatous disease. *Am J Clin Pathol,* 94**,** 344-50.

KENWARD, M. G. & ROGER, J. H. 1997. Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics,* 53**,** 983-97.

KIM, T., LEE, S. O., HONG, H. L., LEE, J. Y., KIM, S. H., CHOI, S. H., KIM, M. N., KIM, Y. S., WOO, J. H. & SUNG, H. 2015. Clinical characteristics of hospital-onset Pneumocystis pneumonia and genotypes of Pneumocystis jirovecii in a single tertiary centre in Korea. *BMC Infect Dis,* 15**,** 102.

KIM, T., SUNG, H., LEE, Y. M., HONG, H. L., KIM, S. H., CHOI, S. H., WOO, J. H., KIM, Y. S. & LEE, S. O. 2012. No recurrence of Pneumocystis jirovecii Pneumonia after solid organ transplantation regardless of secondary prophylaxis. *Antimicrob Agents Chemother,* 56**,** 6041-3.

KIRK, R. E. 1996. Practical Significance: A Concept Whose Time Has Come. *Educational and Psychological Measurement,* 56**,** 746-759.

KITAZAWA, M., NAKAGAWA, M., BABA, O., SUMIYOSHI, K., SAITO, Y., NISHIMURA, T., BAMBA, T. & HOSODA, S. 1993. [A case of amylase producing lung cancer]. *Kokyu To Junkan,* 41**,** 393-6.

KLEIN, N. C. & CUNHA, B. A. 1997. Pasteurella multocida pneumonia. *Semin Respir Infect,* 12**,** 54-6.

KLOMPAS, M. 2018. Epidemiology, pathogenesis, microbiology, and diagnosis of hospital-acquired and ventilator-associated pneumonia in adults. *In:* BOND, S. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

KO, H. W., TSAI, Y. H., YU, C. T., HUANG, C. Y. & CHEN, C. H. 2008. Good response to gefitinib for lung adenocarcinoma with hyperamylasemia: a case report. *Chang Gung Med J,* 31**,** 606-11.

KOFTERIDIS, D. P., CHRISTOFAKI, M., MANTADAKIS, E., MARAKI, S., DRYGIANNAKIS, I., PAPADAKIS, J. A. & SAMONIS, G. 2009. Bacteremic community-acquired pneumonia due to Pasteurella multocida. *International Journal of Infectious Diseases,* 13**,** e81-e83.

KONSTANTINIDI, E. M., LAPPAS, A. S., TZORTZI, A. S. & BEHRAKIS, P. K. 2015. Exhaled Breath Condensate: Technical and Diagnostic Aspects. *ScientificWorldJournal,* 2015**,** 435160.

KORSHOLM, T. L., HAAHR, V. & PRAG, J. 2007. Eight cases of lower respiratory tract infection caused by Stomatococcus mucilaginosus. *Scandinavian Journal of Infectious Diseases,* 39**,** 913-917.

KOSHY, G., KOSHY, J., JOHN, M. & DEODHAR, D. 2015. Pneumocystis jirovecii pneumonia in an immunocompetent host. *Annals of Tropical Medicine and Public Health,* 8**,** 122-124.

KOSKINEN, K., PAUSAN, M. R., PERRAS, A. K., BECK, M., BANG, C., MORA, M., SCHILHABEL, A., SCHMITZ, R. & MOISSL-EICHINGER, C. 2017. First Insights into the Diverse Human Archaeome: Specific Detection of Archaea in the Gastrointestinal Tract, Lung, and Nose and on Skin. *MBio,* 8.

KOSKINEN, K., REICHERT, J. L., HOIER, S., SCHACHENREITER, J., DULLER, S., MOISSL-EICHINGER, C. & SCHÖPF, V. 2018. The nasal microbiome mirrors and potentially shapes olfactory function. *Scientific Reports,* 8**,** 1296.

KOTTOM, T. J., BURGESS, J. W. & LIMPER, A. H. 2011. Pneumocystis carinii interactions with lung epithelial cells and matrix proteins induce expression and activity of the PcSte20 kinase with subsequent phosphorylation of the downstream cell wall biosynthesis kinase PcCbk1. *Infect Immun,* 79**,** 4157-64.

KOTTOM, T. J., KOHLER, J. R., THOMAS, C. F., JR., FINK, G. R. & LIMPER, A. H. 2003. Lung epithelial cells and extracellular matrix components induce expression of Pneumocystis carinii STE20, a gene complementing the mating and pseudohyphal growth defects of STE20 mutant yeast. *Infect Immun,* 71**,** 6463-71.

KOTTOM, T. J. & LIMPER, A. H. 2013. The Pneumocystis Ace2 transcription factor regulates cell wall-remodeling genes and organism virulence. *J Biol Chem,* 288**,** 23893-902.

KOUFAKIS, T., CHATZOPOULOU, M., MARGARITIS, A., TSIAKALOU, M. & GABRANIS, I. 2015. Pneumonia by Listeria monocytogenes: A Common Infection by an Uncommon Pathogen. *Case Rep Infect Dis,* 2015**,** 627073.

KRALIK, P. & RICCHI, M. 2017. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. *Front Microbiol,* 8**,** 108.

KUTYAVIN, I. V., AFONINA, I. A., MILLS, A., GORN, V. V., LUKHTANOV, E. A., BELOUSOV, E. S., SINGER, M. J., WALBURGER, D. K., LOKHOV, S. G., GALL, A. A., DEMPCY, R., REED, M. W., MEYER, R. B. & HEDGPETH, J. 2000. 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res,* 28**,** 655-61.

KWA, A. L., TAM, V. H. & RYBAK, M. J. 2001. Rhodococcus equi pneumonia in a patient with human immunodeficiency virus: case report and review. *Pharmacotherapy,* 21**,** 998-1002.

LAAKKONEN, J. 1998. Pneumocystis carinii in wildlife. *International Journal for Parasitology,* 28**,** 241-252.

LADHANI, L., PARDON, G., MEEUWS, H., VAN WESENBEECK, L., SCHMIDT, K., STUYVER, L. & VAN DER WIJNGAART, W. 2017. Sampling and detection of airborne influenza virus towards point-of-care applications. *PLoS One,* 12**,** e0174314.

LAGIER, J. C., PAPAZIAN, L., FENOLLAR, F., EDOUARD, S., MELENOTTE, C., LAROUMAGNE, S., MICHEL, G., MARTIN, C., GAINNIER, M., LIONS, C., CARRIERI, P., STEIN, A., BROUQUI, P. & RAOULT, D. 2016. Tropheryma whipplei DNA in bronchoalveolar lavage samples: a case control study. *Clin Microbiol Infect,* 22**,** 875-879.

LAI, C. C., TENG, L. J., HSUEH, P. R., YUAN, A., TSAI, K. C., TANG, J. L. & TIEN, H. F. 2004. Clinical and microbiological characteristics of Rhizobium radiobacter infections. *Clin Infect Dis,* 38**,** 149-53.

LAMBOTTE, O., DEBORD, T., SOLER, C. & ROUÉ, R. 1999. Pneumonia due to Stomatococcus mucilaginosus in an AIDS patient: case report and literature review. *Clinical Microbiology and Infection,* 5**,** 112-114.

LANDMAN, W. J., CORBANIE, E. A., FEBERWEE, A. & VAN ECK, J. H. 2004. Aerosolization of Mycoplasma synoviae compared with Mycoplasma gallisepticum and Enterococcus faecalis. *Avian Pathol,* 33**,** 210-5.

LANKEN, P. N., MINDA, M., PIETRA, G. G. & FISHMAN, A. P. 1980. Alveolar response to experimental Pneumocystis carinii pneumonia in the rat. *Am J Pathol,* 99**,** 561-88.

LARSEN 2000. Some Common Methods in Mycobacterial Genetics. *In:* HATFULL, G. F. & WILLIAM R. JACOBS, J. (eds.) *Molecular genetics of mycobacteria.* Washington, D.C.: ASM Press.

LARSEN, H. H., HUANG, L., KOVACS, J. A., CROTHERS, K., SILCOTT, V. A., MORRIS, A., TURNER, J. R., BEARD, C. B., MASUR, H. & FISCHER, S. H. 2004. A prospective, blinded study of quantitative touch-down polymerase chain reaction using oral-wash samples for diagnosis of Pneumocystis pneumonia in HIV-infected patients. *J Infect Dis,* 189**,** 1679-83.

LARSEN, H. H., MASUR, H., KOVACS, J. A., GILL, V. J., SILCOTT, V. A., KOGULAN, P., MAENZA, J., SMITH, M., LUCEY, D. R. & FISCHER, S. H. 2002. Development and evaluation of a quantitative, touch-down, real-time PCR assay for diagnosing Pneumocystis carinii pneumonia. *J Clin Microbiol,* 40**,** 490-4.

LARSSON, P., BAKE, B., WALLIN, A., HAMMAR, O., ALMSTRAND, A. C., LARSTAD, M., LJUNGSTROM, E., MIRGORODSKAYA, E. & OLIN, A. C. 2017. The effect of exhalation flow on endogenous particle emission and phospholipid composition. *Respir Physiol Neurobiol,* 243**,** 39-46.

LARSSON, P., LARSTAD, M., BAKE, B., HAMMAR, O., BREDBERG, A., ALMSTRAND, A. C., MIRGORODSKAYA, E. & OLIN, A. C. 2015. Exhaled particles as markers of small airway inflammation in subjects with asthma. *Clin Physiol Funct Imaging,* 37**,** 489-497.

LARSSON, P., MIRGORODSKAYA, E., SAMUELSSON, L., BAKE, B., ALMSTRAND, A. C., BREDBERG, A. & OLIN, A. C. 2012. Surfactant protein A and albumin in particles in exhaled air. *Respir Med,* 106**,** 197-204.

LARSTAD, M., ALMSTRAND, A. C., LARSSON, P., BAKE, B., LARSSON, S., LJUNGSTROM, E., MIRGORODSKAYA, E. & OLIN, A. C. 2015. Surfactant Protein A in Exhaled Endogenous Particles Is Decreased in Chronic Obstructive Pulmonary Disease (COPD) Patients: A Pilot Study. *PLoS One,* 10**,** e0144463.

LAU, S. K. P., CHAN, J. F. W., TSANG, C.-C., CHAN, S.-M., HO, M.-L., QUE, T.-L., LAU, Y.-L. & WOO, P. C. Y. 2016. Human oropharynx as natural reservoir of Streptobacillus hongkongensis. *Scientific Reports,* 6**,** 24419.

LAUBER, C. L., ZHOU, N., GORDON, J. I., KNIGHT, R. & FIERER, N. 2010. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. *FEMS Microbiol Lett,* 307**,** 80-6.

LAWN, S. D. & NICOL, M. P. 2011. Xpert(R) MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. *Future Microbiol,* 6**,** 1067-82.

LE GAL, S., DAMIANI, C., ROUILLE, A., GRALL, A., TREGUER, L., VIRMAUX, M., MOALIC, E., QUINIO, D., MOAL, M. C., BERTHOU, C., SALIOU, P., LE MEUR, Y., TOTET, A. & NEVEZ, G. 2012. A cluster of Pneumocystis infections among renal transplant recipients: molecular evidence of colonized patients as potential infectious sources of Pneumocystis jirovecii. *Clin Infect Dis,* 54**,** e62-71.

LE GAL, S., POUGNET, L., DAMIANI, C., FREALLE, E., GUEGUEN, P., VIRMAUX, M., ANSART, S., JAFFUEL, S., COUTURAUD, F., DELLUC, A., TONNELIER, J. M., CASTELLANT, P., LE MEUR, Y., LE FLOCH, G., TOTET, A., MENOTTI, J. & NEVEZ, G. 2015. Pneumocystis jirovecii in the air surrounding patients with Pneumocystis pulmonary colonization. *Diagn Microbiol Infect Dis,* 82**,** 137-42.

LE GAL, S., ROBERT-GANGNEUX, F., PEPINO, Y., BELAZ, S., DAMIANI, C., GUEGUEN, P., PITOUS, M., VIRMAUX, M., LISSILLOUR, E., POUGNET, L., GUILLAUD-SAUMUR, T., TOUBAS, D., VALOT, S., HENNEQUIN, C., MORIO, F., HASSEINE, L., BOUCHARA, J. P., TOTET, A. & NEVEZ, G. 2017. A misleading false-negative result of

Pneumocystis real-time PCR assay due to a rare punctual mutation: A French multicenter study. *Med Mycol,* 55**,** 180-184.

LEDER, K. & WELLER, P. F. 2017. Extraintestinal Entamoeba histolytica amebiasis. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

LEDER, K. & WELLER, P. F. 2018a. Microsporidiosis. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

LEDER, K. & WELLER, P. F. 2018b. Ascariasis. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

LEDER, K. & WELLER, P. F. 2018c. Miscellaneous nematodes. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

LEDER, K. & WELLER, P. F. 2018d. Paragonimiasis. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

LEDER, K. & WELLER, P. F. 2018e. Strongyloidiasis. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

LEE, H. Y., KANG, H. S., LEE, H. Y., RHEE, C. K., LEE, S. Y., KIM, S. C., KIM, S. J., PARK, Y. J., KIM, Y. K. & KANG, J. Y. 2017b. Clinical significance of positive Pneumocystis jirovecii polymerase chain reaction in non-human immunodeficiency virus immunocompromised patients in a real practice. *Korean J Intern Med,* 32**,** 478-485.

LEE, J. Y. 2016. Tuberculosis Infection Control in Health-Care Facilities: Environmental Control and Personal Protection. *Tuberc Respir Dis (Seoul),* 79**,** 234-240.

LEE, J. Y., PARK, H. J., KIM, Y. K., YU, S., CHONG, Y. P., KIM, S. H., SUNG, H., LEE, S. O., KIM, M. N., LIM, C. M., KIM, Y. S., KOH, Y., WOO, J. H. & CHOI, S. H. 2015b. Cellular Profiles of Bronchoalveolar Lavage Fluid and Their Prognostic Significance for Non-HIV-Infected Patients with Pneumocystis jirovecii Pneumonia. *J Clin Microbiol,* 53**,** 1310-6.

LEE, K. C. Y., DUNFIELD, P. F. & STOTT, M. B. 2014. The Phylum Armatimonadetes. *In:* ROSENBERG, E., DELONG, E. F., LORY, S., STACKEBRANDT, E. & THOMPSON, F. (eds.) *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea.* Berlin, Heidelberg: Springer Berlin Heidelberg.

LEE, K. K., MATOS, S., WARD, K., RAFFERTY, G. F., MOXHAM, J., EVANS, D. H. & BIRRING, S. S. 2017a. Sound: a non-invasive measure of cough intensity. *BMJ Open Respir Res,* 4**,** e000178.

LEE, K. K., SAVANI, A., MATOS, S., EVANS, D. H., PAVORD, I. D. & BIRRING, S. S. 2012. Four-hour cough frequency monitoring in chronic cough. *Chest,* 142**,** 1237-1243.

LEE, K. K., WARD, K., RAFFERTY, G. F., MOXHAM, J. & BIRRING, S. S. 2015a. The Intensity of Voluntary, Induced, and Spontaneous Cough. *Chest,* 148**,** 1259-1267.

LENLER-PETERSEN, P., GROVE, A., BROCK, A. & JELNES, R. 1994. alpha-Amylase in resectable lung cancer. *Eur Respir J,* 7**,** 941-5.

LEWIS, K. 2010. Persister cells. *Annu Rev Microbiol,* 64**,** 357-72.

LI, R., TUN, H. M., JAHAN, M., ZHANG, Z., KUMAR, A., FERNANDO, D., FARENHORST, A. & KHAFIPOUR, E. 2017. Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. *Sci Rep,* 7**,** 5752.

LIMPER, A. H. 1991a. Parasitic adherence and host responses in the development of Pneumocystis carinii pneumonia. *Semin Respir Infect,* 6**,** 19-26.

LIMPER, A. H. & MARTIN, W. J., II 1989a. Pathogenesis of Pneumocystis carinii pneumonia. *CHEST,* 95**,** 180S-181S.

LIMPER, A. H. & MARTIN, W. J., 2ND 1990. Pneumocystis carinii: inhibition of lung cell growth mediated by parasite attachment. *J Clin Invest,* 85**,** 391-6.

LIMPER, A. H., STANDING, J. E., HOFFMAN, O. A., CASTRO, M. & NEESE, L. W. 1993. Vitronectin binds to Pneumocystis carinii and mediates organism attachment to cultured lung epithelial cells. *Infect Immun,* 61**,** 4302-9.

LINSSEN, C. F., JACOBS, J. A., BECKERS, P., TEMPLETON, K. E., BAKKERS, J., KUIJPER, E. J., MELCHERS, W. J., DRENT, M. & VINK, C. 2006. Inter-laboratory comparison of three different real-time PCR assays for the detection of Pneumocystis jiroveci in bronchoalveolar lavage fluid samples. *J Med Microbiol,* 55**,** 1229-35.

LIPSCHIK, G. Y., GILL, V. J., LUNDGREN, J. D., ANDRAWIS, V. A., NELSON, N. A., NIELSEN, J. O., OGNIBENE, F. P. & KOVACS, J. A. 1992. Improved diagnosis of Pneumocystis carinii infection by polymerase chain reaction on induced sputum and blood. *Lancet,* 340**,** 203-6.

LIPSCHIK, G. Y., TREML, J. F., MOORE, S. D. & BEERS, M. F. 1998. Pneumocystis carinii glycoprotein A inhibits surfactant phospholipid secretion by rat alveolar type II cells. *J Infect Dis,* 177**,** 182-7.

LIU, H., ZHU, J., HU, Q. & RAO, X. 2016. Morganella morganii, a non-negligent opportunistic pathogen. *International Journal of Infectious Diseases,* 50**,** 10-17.

LIU, S. & NOVOSELAC, A. 2014. *Transport of Airborne Particles from an Unobstructed Cough Jet.*

LJUNGKVIST, G., ULLAH, S., TINGLEV, A., STEIN, K., BAKE, B., LARSSON, P., ALMSTRAND, A. C., VIKLUND, E., HAMMAR, O., SANDQVIST, S., BECK, O. & OLIN, A. C. 2017. Two techniques to sample non-volatiles in breath-exemplified by methadone. *J Breath Res,* 12**,** 016011.

LOLIS, N., VELDEKIS, D., MORAITOU, H., KANAVAKI, S., VELEGRAKI, A., TRIANDAFYLLIDIS, C., TASIOUDIS, C., PEFANIS, A. & PNEUMATIKOS, I. 2008. Saccharomyces boulardii fungaemia in an intensive care unit patient treated with caspofungin. *Crit Care,* 12**,** 414.

LONG, E. G., SMITH, J. S. & MEIER, J. L. 1986. Attachment of Pneumocystis carinii to rat pneumocytes. *Lab Invest,* 54**,** 609-15.

LORBER, B. & SWENSON, R. M. 1974. Bacteriology of aspiration pneumonia. A prospective study of community- and hospital-acquired cases. *Ann Intern Med,* 81**,** 329-31.

LOUDON, R. G., BUMGARNER, L. R., LACY, J. & COFFMAN, G. K. 1969. Aerial transmission of mycobacteria. *Am Rev Respir Dis,* 100**,** 165-71.

LOUDON, R. G. & ROBERTS, R. M. 1967. Droplet expulsion from the respiratory tract. *Am Rev Respir Dis,* 95**,** 435-42.

LOUDON, R. G. & ROBERTS, R. M. 1968. Singing and the dissemination of tuberculosis. *Am Rev Respir Dis,* 98**,** 297-300.

LOUDON, R. G. & SPOHN, S. K. 1969. Cough frequency and infectivity in patients with pulmonary tuberculosis. *Am Rev Respir Dis,* 99**,** 109-11.

LOUIS, M., GUITARD, J., JODAR, M., ANCELLE, T., MAGNE, D., LASCOLS, O. & HENNEQUIN, C. 2015. Impact of HIV Infection Status on Interpretation of Quantitative PCR for Detection of Pneumocystis jirovecii. *J Clin Microbiol,* 53**,** 3870-5.

LOWE, J. S., ANDERSON, P. G., ANDERSON, S. I. & STEVENS, A. 2019. *Stevens & Lowe's Human Histology,* Philadelphia, Elsevier.

LOZUPONE, C., LLADSER, M. E., KNIGHTS, D., STOMBAUGH, J. & KNIGHT, R. 2011. UniFrac: an effective distance metric for microbial community comparison. *ISME J,* 5**,** 169-72.

LUGINBUEHL, M., IMHOF, A. & KLARER, A. 2017. Herpes simplex type 1 pneumonitis and acute respiratory distress syndrome in a patient with chronic lymphatic leukemia: a case report. *Journal of Medical Case Reports,* 11**,** 329.

LUHR, K. 1951. [Increase of blood diastase as early symptom of bronchogenic carcinoma]. *Munch Med Wochenschr,* 93**,** 2407.

LURIE, M. B. & WELLS, W. F. 1941. EXPERIMENTAL AIRBORNE DISEASE: QUANTITATIVE NATURAL RESPIRATORY CONTAGION OF TUBERCULOSIS1. *American Journal of Epidemiology,* 34-SectionB**,** 21-40.

LYNCH, S. V. & BRUCE, K. D. 2013. The cystic fibrosis airway microbiome. *Cold Spring Harb Perspect Med,* 3**,** a009738.

MA, L., CISSE, O. H. & KOVACS, J. A. 2018. A Molecular Window into the Biology and Epidemiology of Pneumocystis spp. *Clin Microbiol Rev,* 31.

MABEZA, G. F. & MACFARLANE, J. 2003. Pulmonary actinomycosis. *Eur Respir J,* 21**,** 545-51.

MACOVEI, L., MCCAFFERTY, J., CHEN, T., TELES, F., HASTURK, H., PASTER, B. J. & CAMPOS-NETO, A. 2015. The hidden 'mycobacteriome' of the human healthy oral cavity and upper respiratory tract. *J Oral Microbiol,* 7**,** 26094.

MAEDA, M., OTSUKI, M., YUU, H., SAEKI, S., YAMASAKI, T. & BABA, S. 1982. Salivary-type hyperamylasemia in primary lung cancer: observation of a possible precursor of the salivary-type isoamylase. *Eur J Cancer Clin Oncol,* 18**,** 123-8.

MAILLET, M., MAUBON, D., BRION, J. P., FRANCOIS, P., MOLINA, L., STAHL, J. P., EPAULARD, O., BOSSERAY, A. & PAVESE, P. 2014. Pneumocystis jirovecii (Pj) quantitative PCR to differentiate Pj pneumonia from Pj colonization in immunocompromised patients. *Eur J Clin Microbiol Infect Dis,* 33**,** 331-6.

MAINI, R., HENDERSON, K. L., SHERIDAN, E. A., LAMAGNI, T., NICHOLS, G., DELPECH, V. & PHIN, N. 2013. Increasing Pneumocystis pneumonia, England, UK, 2000-2010. *Emerg Infect Dis,* 19**,** 386-92.

MAN, W. H., DE STEENHUIJSEN PITERS, W. A. & BOGAERT, D. 2017. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol,* 15**,** 259-270.

MANCINI, F., CARNIATO, A. & CIERVO, A. 2009. Pneumonia caused by Shigella sonnei in man returned from India. *Emerg Infect Dis,* 15**,** 1874-6.

MANOLOFF, E. S., FRANCIOLI, P., TAFFE, P., VAN MELLE, G., BILLE, J. & HAUSER, P. M. 2003. Risk for Pneumocystis carinii transmission among patients with pneumonia: a molecular epidemiology study. *Emerg Infect Dis,* 9**,** 132-4.

MANSOUR, A. & JORDAN, K. 2014. Disseminated Cladophialophora bantiana disease in a patient with prediabetes. *BMJ Case Rep,* 2014.

MARCOTTE, H., LEVESQUE, D., DELANAY, K., BOURGEAULT, A., DE LA DURANTAYE, R., BROCHU, S. & LAVOIE, M. C. 1996. Pneumocystis carinii infection in transgenic B cell-deficient mice. *J Infect Dis,* 173**,** 1034-7.

MARIK, P. E. & CAREAU, P. 1999. The role of anaerobes in patients with ventilator-associated pneumonia and aspiration pneumonia: a prospective study. *Chest,* 115**,** 178-83.

MARIUZ, P., RAVIGLIONE, M. C., GOULD, I. A. & MULLEN, M. P. 1991. Pleural Pneumocystis carinii infection. *Chest,* 99**,** 774-6.

MARPLE, V. A., RUBOW, K. L. & BEHM, S. M. 1991. A Microorifice Uniform Deposit Impactor (MOUDI): Description, Calibration, and Use. *Aerosol Science and Technology,* 14**,** 434-446.

MARRIE, T. J. & FILE, T. M. 2018. Epidemiology, pathogenesis, and microbiology of community-acquired pneumonia in adults. *In:* BOND, S. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

MARSLAND, B. J., TROMPETTE, A. & GOLLWITZER, E. S. 2015. The Gut-Lung Axis in Respiratory Disease. *Ann Am Thorac Soc,* 12 Suppl 2**,** S150-6.

MARTIN, P. C. & SARMA, D. P. 1982. Amylase-producing lung cancer. *J Surg Oncol,* 21**,** 30-2.

MARTINEZ, A., HALLIEZ, M. C., ALIOUAT EL, M., CHABE, M., STANDAERT-VITSE, A., FREALLE, E., GANTOIS, N., POTTIER, M., PINON, A., DEI-CAS, E. & ALIOUAT-DENIS, C. M. 2013. Growth and airborne transmission of cell-sorted life cycle stages of Pneumocystis carinii. *PLoS One,* 8**,** e79958.

MARTY, F. M. & KOO, S. 2009. Role of (1-->3)-beta-D-glucan in the diagnosis of invasive aspergillosis. *Med Mycol,* 47 Suppl 1**,** S233-40.

MASTORIDES, S. M., OEHLER, R. L., GREENE, J. N., SINNOTT, J. T. T., KRANIK, M. & SANDIN, R. L. 1999. The detection of airborne Mycobacterium tuberculosis using micropore membrane air sampling and polymerase chain reaction. *Chest,* 115**,** 19-25.

MASUR, H. 1992. Prevention and treatment of pneumocystis pneumonia. *N Engl J Med,* 327**,** 1853-60.

MATOS, O., COSTA, M. C., LUNDGREN, B., CALDEIRA, L., AGUIAR, P. & ANTUNES, F. 2001. Effect of oral washes on the diagnosis of Pneumocystis carinii pneumonia with a low parasite burden and on detection of organisms in subclinical infections. *Eur J Clin Microbiol Infect Dis,* 20**,** 573-5.

MATSUMURA, Y., ITO, Y., IINUMA, Y., YASUMA, K., YAMAMOTO, M., MATSUSHIMA, A., NAGAO, M., TAKAKURA, S. & ICHIYAMA, S. 2012. Quantitative real-time PCR and the (1-->3)-beta-D-glucan assay for differentiation between Pneumocystis jirovecii pneumonia and colonization. *Clin Microbiol Infect,* 18**,** 591-7.

MAY, A. K., BRADY, J. S., ROMANO-KEELER, J., DRAKE, W. P., NORRIS, P. R., JENKINS, J. M., ISAACS, R. J. & BOCZKO, E. M. 2015. A Pilot Study of the Noninvasive Assessment of the Lung Microbiota as a Potential Tool for the Early Diagnosis of Ventilator-Associated Pneumonia. *CHEST,* 147**,** 1494-1502.

MCCROSKERY, P. A., WOOD, S. & HARRIS, E. D. 1973. Gelatin: A Poor Substrate for a Mammalian Collagenase. *Science,* 182**,** 70-71.

MCKAIN, N., GENC, B., SNELLING, T. J. & WALLACE, R. J. 2013. Differential recovery of bacterial and archaeal 16S rRNA genes from ruminal digesta in response to glycerol as cryoprotectant. *J Microbiol Methods,* 95**,** 381-3.

MCTAGGART, L. R., WENGENACK, N. L. & RICHARDSON, S. E. 2012. Validation of the MycAssay Pneumocystis kit for detection of Pneumocystis jirovecii in bronchoalveolar lavage specimens by comparison to a laboratory standard of direct immunofluorescence microscopy, real-time PCR, or conventional PCR. *J Clin Microbiol,* 50**,** 1856-9.

MEADOW, J. F., ALTRICHTER, A. E., BATEMAN, A. C., STENSON, J., BROWN, G. Z., GREEN, J. L. & BOHANNAN, B. J. 2015. Humans differ in their personal microbial cloud. *PeerJ,* 3**,** e1258.

MEDRANO, F. J., MONTES-CANO, M., CONDE, M., DE LA HORRA, C., RESPALDIZA, N., GASCH, A., PEREZ-LOZANO, M. J., VARELA, J. M. & CALDERON, E. J. 2005. Pneumocystis jirovecii in general population. *Emerg Infect Dis,* 11**,** 245-50.

MEDRANO, F. J., RESPALDIZA, N., MEDRANO, A., VARELA, J. M., MONTES-CANO, M., DE LA HORRA, C., WICHMANN, I., FERRER, S. & CALDERON, E. 2003. Seroprevalence of Pneumocystis human infection in southern Spain. *J Eukaryot Microbiol,* 50 Suppl**,** 649-50.

MELIANI, L., DEVELOUX, M., MARTEAU-MILTGEN, M., MAGNE, D., BARBU, V., POIROT, J. L. & ROUX, P. 2003. Real time quantitative PCR assay for Pneumocystis jirovecii detection. *J Eukaryot Microbiol,* 50 Suppl**,** 651.

MENNINK-KERSTEN, M. A., RUEGEBRINK, D. & VERWEIJ, P. E. 2008. Pseudomonas aeruginosa as a cause of 1,3-beta-D-glucan assay reactivity. *Clin Infect Dis,* 46**,** 1930-1.

MENNINK-KERSTEN, M. A. & VERWEIJ, P. E. 2006. Non-culture-based diagnostics for opportunistic fungi. *Infect Dis Clin North Am,* 20**,** 711-27, viii.

MERCALDO, N. D., LAU, K. F. & ZHOU, X. H. 2007. Confidence intervals for predictive values with an emphasis to case-control studies. *Stat Med,* 26**,** 2170-83.

MERIDEN, Z., MARR, K. A., LEDERMAN, H. M., ILLEI, P. B., VILLA, K., RIEDEL, S., CARROLL, K. C. & ZHANG, S. X. 2012. Ochroconis gallopava infection in a patient with chronic granulomatous disease: case report and review of the literature. *Med Mycol,* 50**,** 883-9.

METERSKY, M. L., ASLENZADEH, J. & STELMACH, P. 1998. A comparison of induced and expectorated sputum for the diagnosis of Pneumocystis carinii pneumonia. *Chest,* 113**,** 1555-9.

METERSKY, M. L. & CATANZARO, A. 1991. Diagnostic approach to Pneumocystis carinii pneumonia in the setting of prophylactic aerosolized pentamidine. *Chest,* 100**,** 1345-9.

METHENY, N. A., CLOUSE, R. E., CHANG, Y. H., STEWART, B. J., OLIVER, D. A. & KOLLEF, M. H. 2006. Tracheobronchial aspiration of gastric contents in critically ill tube-fed patients: frequency, outcomes, and risk factors. *Crit Care Med,* 34**,** 1007-15.

MEUWISSEN, J. H., TAUBER, I., LEEUWENBERG, A. D., BECKERS, P. J. & SIEBEN, M. 1977. Parasitologic and serologic observations of infection with Pneumocystis in humans. *J Infect Dis,* 136**,** 43-9.

MEYNARD, J. L., MEYOHAS, M. C., BINET, D., CHOUAID, C. & FROTTIER, J. 1996. Pulmonary cryptosporidiosis in the acquired immunodeficiency syndrome. *Infection,* 24**,** 328-31.

MILLARD, P. R., WAKEFIELD, A. E. & HOPKIN, J. M. 1990. A sequential ultrastructural study of rat lungs infected with Pneumocystis carinii to investigate the appearances of the organism, its relationships and its effects on pneumocytes. *Int J Exp Pathol,* 71**,** 895-904.

MILLER, R. F., AMBROSE, H. E., NOVELLI, V. & WAKEFIELD, A. E. 2002. Probable mother-to-infant transmission of Pneumocystis carinii f. sp. hominis infection. *J Clin Microbiol,* 40**,** 1555-7.

MILLER, R. F., AMBROSE, H. E. & WAKEFIELD, A. E. 2001. Pneumocystis carinii f. sp. hominis DNA in immunocompetent health care workers in contact with patients with P. carinii pneumonia. *J Clin Microbiol,* 39**,** 3877-82.

MILLS, B., RATRA, A., EL-BAKUSH, A., KAMBALI, S. & NUGENT, K. 2014. Herpes Simplex Pneumonia in an Immunocompetent Patient With Progression to Organizing Pneumonia. *J Investig Med High Impact Case Rep,* 2**,** 2324709614530560.

MILLS, J. 1986. Pneumocystis carinii and Toxoplasma gondii infections in patients with AIDS. *Rev Infect Dis,* 8**,** 1001-11.

MINAMI, S., JOKOJI, R., YAMAMOTO, S., OGATA, Y., KOBA, T., FUTAMI, S., NISHIJIMA, Y., YAGA, M., MASUHIRO, K., TSUJIMOTO, M. & KOMUTA, K. 2014. Amylase-Producing Lung Cancer with a Positive Epidermal Growth Factor Receptor Mutation Treated With Gefitinib: A Case Report. *World J Oncol,* 5**,** 41-46.

MIRDHA, B. R. & GULERIA, R. 2000. Comparative yield of different respiratory samples for diagnosis of Pneumocystis carinii infections in HIV-seropositive and seronegative individuals in India. *Southeast Asian J Trop Med Public Health,* 31**,** 473-7.

MITA, S., SUGIMOTO, M., NAKAMURA, M., MURAKAMI, T., TOKUNAGA, M., UYAMA, E. & ARAKI, S. 1993. Increased human T lymphotropic virus type-1 (HTLV-1) proviral DNA in peripheral blood mononuclear cells and bronchoalveolar lavage cells from Japanese patients with HTLV-1-associated myelopathy. *Am J Trop Med Hyg,* 48**,** 170-7.

MITZ, H. S. & FARBER, S. S. 1993. Demonstration of Helicobacter pylori in tracheal secretions. *J Am Osteopath Assoc,* 93**,** 87-91.

MOFFATT, M. F. & COOKSON, W. O. 2017. The lung microbiome in health and disease. *Clin Med (Lond),* 17**,** 525-529.

MOGRIDGE, R., STACEY, P. & FORDER, J. 2016. A New Miniature Respirable Sampler for In-mask Sampling: Part 2—Tests Performed Inside the Mask. *Annals of Work Exposures and Health,* 60**,** 1084-1091.

MOHAN, S., HAMID, N. S. & CUNHA, B. A. 2006. A cluster of nosocomial herpes simplex virus type 1 pneumonia in a medical intensive care unit. *Infect Control Hosp Epidemiol,* 27**,** 1255-7.

MONTES-CANO, M. A., CHABE, M., FONTILLON-ALBERDI, M., DE-LAHORRA, C., RESPALDIZA, N., MEDRANO, F. J., VARELA, J. M., DEI-CAS, E. & CALDERON, E. J. 2009. Vertical transmission of Pneumocystis jirovecii in humans. *Emerg Infect Dis,* 15**,** 125-7.

MONTESINOS, I., BRANCART, F., SCHEPERS, K., JACOBS, F., DENIS, O. & DELFORGE, M. L. 2015. Comparison of 2 real-time PCR assays for diagnosis of Pneumocystis jirovecii pneumonia in human immunodeficiency virus (HIV) and non-HIV immunocompromised patients. *Diagn Microbiol Infect Dis,* 82**,** 143-7.

MONTESINOS, I., DELFORGE, M. L., AJJAHAM, F., BRANCART, F., HITES, M., JACOBS, F. & DENIS, O. 2017. Evaluation of a new commercial real-time PCR assay for diagnosis of Pneumocystis jirovecii pneumonia and identification of dihydropteroate synthase (DHPS) mutations. *Diagn Microbiol Infect Dis,* 87**,** 32-36.

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MONTI, M., DIANO, D., ALLEGRINI, F., DELMONTE, A., FAUSTI, V., CRAVERO, P., MARCANTOGNINI, G. & FRASSINETI, G. L. 2017. Bordetella bronchiseptica pneumonia in a patient with lung cancer; a case report of a rare infection. *BMC Infect Dis,* 17**,** 644.

MOODLEY, B., TEMPIA, S. & FREAN, J. A. 2017. Comparison of quantitative real-time PCR and direct immunofluorescence for the detection of Pneumocystis jirovecii. *PLoS One,* 12**,** e0180589.

MORI, H., OHNO, Y., ITO, F., ENDO, J., YANASE, K., FUNAGUCHI, N., BAI LA, B. L. & MINATOGUCHI, S. 2010b. Polymerase chain reaction positivity of Pneumocystis jirovecii during primary lung cancer treatment. *Jpn J Clin Oncol,* 40**,** 658-62.

MORI, S., CHO, I. & SUGIMOTO, M. 2010a. A cluster of Pneumocystis jirovecii infection among outpatients with rheumatoid arthritis. *J Rheumatol,* 37**,** 1547-8.

MOROHOSHI, T., NAKAMURA, N., HAYASHI, K. & KANDA, M. 1980. Amylase producing lung cancer. *Virchows Archiv A,* 387**,** 125-132.

MORRIS, A., BECK, J. M., SCHLOSS, P. D., CAMPBELL, T. B., CROTHERS, K., CURTIS, J. L., FLORES, S. C., FONTENOT, A. P., GHEDIN, E., HUANG, L., JABLONSKI, K., KLEERUP, E., LYNCH, S. V., SODERGREN, E., TWIGG, H., YOUNG, V. B., BASSIS, C. M., VENKATARAMAN, A., SCHMIDT, T. M., WEINSTOCK, G. M. & LUNG, H. I. V. M. P. 2013. Comparison of the respiratory microbiome in healthy nonsmokers and smokers. *Am J Respir Crit Care Med,* 187**,** 1067-75.

MORRIS, A. & NORRIS, K. A. 2012. Colonization by Pneumocystis jirovecii and its role in disease. *Clin Microbiol Rev,* 25**,** 297-317.

MORRIS, A., PAULSON, J. N., TALUKDER, H., TIPTON, L., KLING, H., CUI, L., FITCH, A., POP, M., NORRIS, K. A. & GHEDIN, E. 2016. Longitudinal analysis of the lung microbiota of cynomolgous macaques during long-term SHIV infection. *Microbiome,* 4**,** 38.

MORRIS, A. M., SWANSON, M., HA, H. & HUANG, L. 2000. Geographic distribution of human immunodeficiency virus-associated Pneumocystis carinii pneumonia in San Francisco. *Am J Respir Crit Care Med,* 162**,** 1622-6.

MORSKY, P. 1983. Turbidimetric determination of lysozyme with Micrococcus lysodeikticus cells: reexamination of reaction conditions. *Anal Biochem,* 128**,** 77-85.

MORTIER, E., POUCHOT, J., BOSSI, P. & MOLINIE, V. 1995. Maternal-fetal transmission of Pneumocystis carinii in human immunodeficiency virus infection. *N Engl J Med,* 332**,** 825.

MU, X. D., JIA, P., GAO, L., SU, L., ZHANG, C., WANG, R. G. & WANG, G. F. 2016. Relationship between Radiological Stages and Prognoses of Pneumocystis Pneumonia in Non-AIDS Immunocompromised Patients. *Chin Med J (Engl),* 129**,** 2020-5.

MU, X. D., WANG, G. F. & SU, L. 2011. A clinical comparative study of polymerase chain reaction assay for diagnosis of pneumocystis pneumonia in non-AIDS patients. *Chin Med J (Engl),* 124**,** 2683-6.

MUHLETHALER, K., BOGLI-STUBER, K., WASMER, S., VON GARNIER, C., DUMONT, P., RAUCH, A., MUHLEMANN, K. & GARZONI, C. 2012. Quantitative PCR to diagnose Pneumocystis pneumonia in immunocompromised non-HIV patients. *Eur Respir J,* 39**,** 971-8.

MUHLING, M., WOOLVEN-ALLEN, J., MURRELL, J. C. & JOINT, I. 2008. Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *ISME J,* 2**,** 379-92.

MUKAMOLOVA, G. V., TURAPOV, O., MALKIN, J., WOLTMANN, G. & BARER, M. R. 2010. Resuscitation-promoting factors reveal an occult population of tubercle Bacilli in Sputum. *Am J Respir Crit Care Med,* 181**,** 174-80.

MURPHY, M. J., PIFER, L. L. & HUGHES, W. T. 1977. Pneumocystis carinii in vitro: A study by scanning electron microscopy. *Am J Pathol,* 86**,** 387-401.

NAGDEV, K. J., KASHYAP, R. S., DESHPANDE, P. S., PUROHIT, H. J., TAORI, G. M. & DAGINAWALA, H. F. 2010. Determination of polymerase chain reaction efficiency for diagnosis of tuberculous meningitis in Chelex-100 extracted DNA samples. *Int J Tuberc Lung Dis,* 14**,** 1032-8.

NAGENDRA, H. 2002. Opposite trends in response for the Shannon and Simpson indices of landscape diversity. *Applied Geography,* 22**,** 175-186.

NAHID, H., STAUB, D., HACHLER, H. & ALBRICH, W. C. 2017. Detection of Shigella sonnei in a respiratory specimen in a patient with subacute atypical pneumonia. *Infection,* 45**,** 241-244.

NAKAMURA, Y., TOMITA, N., NISHIDE, T., EMI, M., HORII, A., OGAWA, M., MORI, T., KOSAKI, G., OKABE, T., FUJISAWA, M. & ET AL. 1989. Production of salivary type alpha-amylase in human lung cancer. *Gene,* 77**,** 107-12.

NAKAO, M., KODAMA, T., SASAKI, M. & KURODA, K. 1996. [A case of ectopic amylase-producing lung cancer]. *Kyobu Geka,* 49**,** 1069-73.

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NAKATANI, S. M., BURGER, M., ASSEF, M. C., BROCKELT, S. R., COGO, L. L. & MESSIAS-REASON, I. J. 2004. Efficient method for mycobacterial DNA extraction in blood cultures aids rapid PCR identification of Mycobacterium tuberculosis and Mycobacterium avium. *Eur J Clin Microbiol Infect Dis,* 23**,** 851-4.

NAKATSUJI, T. 2008. Salivary-type amylase producing lung cancers examined clinically and pathologically in 260 Japanese patients of lung lobectomy or segmentectomy. *Comparative Clinical Pathology,* 17**,** 43.

NANDAPALAN, V., MCILWAIN, J. C. & ENGLAND, J. 1995. Amylase activity in tracheobronchial secretions of laryngectomized patients. *J Laryngol Otol,* 109**,** 637-9.

NARDELL, E. & DHARMADHIKARI, A. 2010. Turning off the spigot: reducing drug-resistant tuberculosis transmission in resource-limited settings. *Int J Tuberc Lung Dis,* 14**,** 1233-43.

NARDELL, E. A. 1991. Nosocomial tuberculosis in the AIDS era: strategies for interrupting transmission in developed countries. *Bull Int Union Tuberc Lung Dis,* 66**,** 107-11.

NARDELL, E. A. 1999. Air sampling for tuberculosis- homage to the lowly guinea pig. *Chest,* 116**,** 1143-5.

NARDELL, E. A., WILLIAMS, C. M., BELL, A. J., MALINGA, L., RAMSHEH, M. Y., BAKIR, A., GARTON, N. J., STOLTZ, A., DEKOCK, E., WADDELL, S., HINTON, J., FENNELLY, K. & BARER, M. 2016. TB Airborne Transmission: First Gene Expression Signatures of Captured, Uncultured M. tuberculosis from Human Source Aerosol [abstract]. *Am J Respir Crit Care Med,* 193**,** A7934.

NEHME, B., GILBERT, Y., LETOURNEAU, V., FORSTER, R. J., VEILLETTE, M., VILLEMUR, R. & DUCHAINE, C. 2009. Culture-independent characterization of archaeal biodiversity in swine confinement building bioaerosols. *Appl Environ Microbiol,* 75**,** 5445-50.

NEVE, H., LABORIUS, A. & HELLER, K. J. 2003. Testing of the applicability of battery-powered portable microbial air samplers for detection and enumeration of air-borne dairy
Lactococcus lactis bacteriophages. *Kieler Milchwirtschaftliche Forschungsberichte,* 55**,** 301-315.

NEVEZ, G., TOTET, A., JOUNIEAUX, V., SCHMIT, J.-L., DEI-CAS, E. & RACCURT, C. 2003. Pneumocystis jiroveci Internal Transcribed Spacer Types in Patients Colonized by the Fungus and in Patients with Pneumocystosis from the Same French Geographic Region. *Journal of Clinical Microbiology,* 41**,** 181-186.

NEZAFAT, N., NEGAHDARIPOUR, M., GHOLAMI, A. & GHASEMI, Y. 2015. *Computational analysis of collagenase from different Vibrio, Clostridium and Bacillus strains to find new enzyme sources*.

NG, V. L., YAJKO, D. M. & HADLEY, W. K. 1997. Extrapulmonary pneumocystosis. *Clin Microbiol Rev,* 10**,** 401-18.

NGUYEN, L. D., VISCOGLIOSI, E. & DELHAES, L. 2015. The lung mycobiome: an emerging field of the human respiratory microbiome. *Front Microbiol,* 6**,** 89.

NICAS, M. & SUN, G. 2006. An integrated model of infection risk in a health-care environment. *Risk Anal,* 26**,** 1085-96.

NIEDERMAIER, G., MERLO, C. & KRAUSE, M. 1997. [Pneumocystis carinii pneumonia in a, until now, healthy 46-year-old HIV-negative man]. *Schweiz Med Wochenschr,* 127**,** 631-5.

NIKNAM, N., MANKAME, S., HA, L. & GAUTAM-GOYAL, P. 2017. Acremonium pneumonia in an AIDS patient. *IDCases,* 8**,** 75-76.

NILAKANTA, H., DREWS, K. L., FIRRELL, S., FOULKES, M. A. & JABLONSKI, K. A. 2014. A review of software for analyzing molecular sequences. *BMC Res Notes,* 7**,** 830.

NORTHFELT, D. W. 1989. Extrapulmonary pneumocystosis in patients taking aerosolised pentamidine. *Lancet,* 2**,** 1454.

O'DONNELL, W. J., PIECIAK, W., CHERTOW, G. M., SANABRIA, J. & LAHIVE, K. C. 1998. Clearance of Pneumocystis carinii cysts in acute P carinii pneumonia: assessment by serial sputum induction. *Chest,* 114**,** 1264-8.

ODUMERU, J., GAO, A., CHEN, S., RAYMOND, M. & MUTHARIA, L. 2001. Use of the bead beater for preparation of Mycobacterium paratuberculosis template DNA in milk. *Can J Vet Res,* 65**,** 201-5.

OKAHISA, M. & TOBINO, K. 2017. Pneumocystis Jirovecii Pneumonia in Immunocompetent Japanese Male. *CHEST,* 152**,** A205-A206.

OKIMOTO, N., HAYASHI, T., ISHIGA, M., NANBA, F., KISHIMOTO, M., YAGI, S., KURIHARA, T., ASAOKA, N. & TAMADA, S. 2010. Clinical features of Proteus mirabilis pneumonia. *J Infect Chemother,* 16**,** 364-6.

OLIN, A. C., ANDELID, K., VIKGREN, J., ROSENGREN, A., LARSSON, S., BAKE, B. & EKBERG-JANSSON, A. 2006. Single breath N2-test and exhaled nitric oxide in men. *Respir Med,* 100**,** 1013-9.

OLSSON, M., ELVIN, K., LOFDAHL, S. & LINDER, E. 1993. Detection of Pneumocystis carinii DNA in sputum and bronchoalveolar lavage samples by polymerase chain reaction. *J Clin Microbiol,* 31**,** 221-6.

OLSSON, M., LIDMAN, C., LATOUCHE, S., BJORKMAN, A., ROUX, P., LINDER, E. & WAHLGREN, M. 1998. Identification of Pneumocystis carinii f. sp. hominis gene sequences in filtered air in hospital environments. *J Clin Microbiol,* 36**,** 1737-40.

461

OLSSON, M., SUKURA, A., LINDBERG, L. A. & LINDER, E. 1996a. Detection of Pneumocystis carinii DNA by filtration of air. *Scand J Infect Dis,* 28**,** 279-82.

O'NEAL, C. B. & BALL, S. C. 2008. Splenic pneumocystosis: an atypical presentation of extrapulmonary Pneumocystis infection. *AIDS Read,* 18**,** 503-8.

OREN, I., HARDAK, E., FINKELSTEIN, R., YIGLA, M. & SPRECHER, H. 2011. Polymerase chain reaction-based detection of Pneumocystis jirovecii in bronchoalveolar lavage fluid for the diagnosis of pneumocystis pneumonia. *Am J Med Sci,* 342**,** 182-5.

WORLD HEALTH ORGANIZATION (WHO). 2015. Global tuberculosis report 2015, 20th ed. World Health Organization. http://www.who.int/iris/handle/10665/191102.

ORSI, C. F., BETTUA, C., PINI, P., VENTURELLI, C., LA REGINA, A., MORACE, G., LUPPI, M., FORGHIERI, F., BIGLIARDI, S., LUPPI, F., CODELUPPI, M., GIRARDIS, M. & BLASI, E. 2015. Detection of Pneumocystis jirovecii and Aspergillus spp. DNa in bronchoalveolar lavage fluids by commercial real-time PCr assays: comparison with conventional diagnostic tests. *New Microbiol,* 38**,** 75-84.

ORSI, C. F., GENNARI, W., VENTURELLI, C., LA REGINA, A., PECORARI, M., RIGHI, E., MACHETTI, M. & BLASI, E. 2012. Performance of 2 commercial real-time polymerase chain reaction assays for the detection of Aspergillus and Pneumocystis DNA in bronchoalveolar lavage fluid samples from critical care patients. *Diagn Microbiol Infect Dis,* 73**,** 138-43.

OST, D., FEIN, A. & FEINSILVER, S. H. 2017. Nonresolving pneumonia. *In:* BOND, S. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

ÖSTLING, J., VAN GEEST, M., VIKLUND, E., GUSTAFSSON, P., MIRGORODSKAYA, E. & OLIN, A.-C. 2017. Late Breaking Abstract - Exploring particles from exhaled air as a new source for protein biomarkers from the airways. *European Respiratory Journal,* 50**,** OA2933.

O'TOOLE, G. A. 2018. Cystic Fibrosis Airway Microbiome: Overturning the Old, Opening the Way for the New. *J Bacteriol,* 200.

OTSUKI, M., MAEDA, M., YUU, H., YAMASAKI, T. & OKANO, K. 1977. The nature and origin of hyperamylasemia following open-heart surgery with extracorporeal circulation. *Clin Chim Acta,* 77**,** 349-57.

OTSUKI, M., YUU, H., MAEDA, M., SAEKI, S. & YAMASAKI, T. 1977. Amylase in the lung. *Cancer,* 39**,** 1656-63.

OZ, H. S. & HUGHES, W. T. 2000. Search for Pneumocystis carinii DNA in upper and lower respiratory tract of humans. *Diagn Microbiol Infect Dis,* 37**,** 161-4.

PAN, S., GU, B., WANG, H., YAN, Z., WANG, P., PEI, H., XIE, W., CHEN, D. & LIU, G. 2013. Comparison of four DNA extraction methods for detecting Mycobacterium tuberculosis by real-time PCR and its clinical application in pulmonary tuberculosis. *J Thorac Dis,* 5**,** 251-7.

PANG, Y., LU, J., SU, B., ZHENG, H. & ZHAO, Y. 2017. Misdiagnosis of tuberculosis associated with some species of nontuberculous mycobacteria by GeneXpert MTB/RIF assay. *Infection,* 45**,** 677-681.

PAPINENI, R. S. & ROSENTHAL, F. S. 1997. The size distribution of droplets in the exhaled breath of healthy human subjects. *J Aerosol Med,* 10**,** 105-16.

PARADA, A. E., NEEDHAM, D. M. & FUHRMAN, J. A. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol,* 18**,** 1403-14.

PARIAN, M., FATA, A., NAJAFZADEH, M. J. & REZAEITALAB, F. 2015. Molecular detection of Pneumocystis jirovecii using polymerase chain reaction in immunocompromised patients with pulmonary disorders in northeast of Iran. *Curr Med Mycol,* 1**,** 13-18.

PARK, I. K., HSU, A. P., TETTELIN, H., SHALLOM, S. J., DRAKE, S. K., DING, L., WU, U. I., ADAMO, N., PREVOTS, D. R., OLIVIER, K. N., HOLLAND, S. M., SAMPAIO, E. P. & ZELAZNY, A. M. 2015. Clonal Diversification and Changes in Lipid Traits and Colony Morphology in Mycobacterium abscessus Clinical Isolates. *J Clin Microbiol,* 53**,** 3438-47.

PARKER, B. C., FORD, M. A., GRUFT, H. & FALKINHAM, J. O., 3RD 1983. Epidemiology of infection by nontuberculous mycobacteria. IV. Preferential aerosolization of Mycobacterium intracellulare from natural waters. *Am Rev Respir Dis,* 128**,** 652-6.

PATEL, N. & KOZIEL, H. 2004. Pneumocystis jiroveci pneumonia in adult patients with AIDS: treatment strategies and emerging challenges to antimicrobial therapy. *Treat Respir Med,* 3**,** 381-97.

PATIN, N. V., KUNIN, V., LIDSTROM, U. & ASHBY, M. N. 2013. Effects of OTU clustering and PCR artifacts on microbial diversity estimates. *Microb Ecol,* 65**,** 709-19.

PATTEN, H. E. & JOHNSON, A. J. 1919. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE LIQUEFACTION OF GELATIN. *Journal of Biological Chemistry,* 38**,** 179-190.

PATTERSON, B., MORROW, C., SINGH, V., MOOSA, A., GQADA, M., WOODWARD, J., MIZRAHI, V., BRYDEN, W., CALL, C., PATEL, S., WARNER, D. & WOOD, R. 2017. Detection of Mycobacterium tuberculosis bacilli in bio-aerosols from untreated TB patients. *Gates Open Res,* 1**,** 11.

PAVESI, L., SUBBURAJ, S. & PORTER-SHAW, K. 2001. Application and Validation of a Computerized Cough Acquisition System for Objective Monitoring of Acute Cough. *Chest,* 120**,** 1121-1128.

PAVLICA, F. 1962. The first observation of congenital pneumocystic pneumonia in a fully developed stillborn child. *Ann Paediatr,* 198**,** 177-84.

PEETERS, M. J. 2016. Practical significance: Moving beyond statistical significance. *Currents in Pharmacy Teaching and Learning,* 8**,** 83-89.

PEIRO-CABRERA, G., LERMA-PUERTAS, E., ESTEVA DE LLOBET, E., DE LAS HERAS-DURAN, P., MUNOZ-ANATOL, P. & CARRERAS-MARCER, A. 1994. Cytologic diagnosis of extrapulmonary Pneumocystis carinii infection in pleural and ascitic fluid. Report of a case in a patient with the acquired immunodeficiency syndrome. *Acta Cytol,* 38**,** 608-10.

PENN, C. C. & KLOTZ, S. A. 1997. Anthrax pneumonia. *Semin Respir Infect,* 12**,** 28-30.

PERALBO-MOLINA, A., CALDERON-SANTIAGO, M., JURADO-GAMEZ, B., LUQUE DE CASTRO, M. D. & PRIEGO-CAPOTE, F. 2017. Exhaled breath condensate to discriminate individuals with different smoking habits by GC-TOF/MS. *Sci Rep,* 7**,** 1421.

PETROFF, S. A. 1915. A New and Rapid Method for the Isolation and Cultivation of Tubercle Bacilli Directly from the Sputum and Feces. *J Exp Med,* 21**,** 38-42.

PHILLIPS, M. 1992. Breath tests in medicine. *Sci Am,* 267**,** 74-9.

PHIPPS, L. M., CHEN, S. C., KABLE, K., HALLIDAY, C. L., FIRACATIVE, C., MEYER, W., WONG, G. & NANKIVELL, B. J. 2011. Nosocomial Pneumocystis jirovecii pneumonia: lessons from a cluster in kidney transplant recipients. *Transplantation,* 92**,** 1327-34.

PIFER, L. L., HUGHES, W. T., STAGNO, S. & WOODS, D. 1978. Pneumocystis carinii infection: evidence for high prevalence in normal and immunosuppressed children. *Pediatrics,* 61**,** 35-41.

PITTMAN, J. E., WYLIE, K. M., AKERS, K., STORCH, G. A., HATCH, J., QUANTE, J., FRAYMAN, K. B., CLARKE, N., DAVIS, M., STICK, S. M., HALL, G. L., MONTGOMERY, G., RANGANATHAN, S., DAVIS, S. D., FERKOL, T. W. & AUSTRALIAN RESPIRATORY EARLY SURVEILLANCE TEAM FOR CYSTIC, F. 2017. Association of Antibiotics, Airway Microbiome, and Inflammation in Infants with Cystic Fibrosis. *Ann Am Thorac Soc,* 14**,** 1548-1555.

PONCE, C. A., GALLO, M., BUSTAMANTE, R. & VARGAS, S. L. 2010. Pneumocystis colonization is highly prevalent in the autopsied lungs of the general population. *Clin Infect Dis,* 50**,** 347-53.

POROLLO, A., MELLER, J., JOSHI, Y., JAISWAL, V., SMULIAN, A. G. & CUSHION, M. T. 2012. Analysis of current antifungal agents and their targets within the Pneumocystis carinii genome. *Curr Drug Targets,* 13**,** 1575-85.

POTTRATZ, S. T. & WEIR, A. L. 1995. Attachment of Pneumocystis carinii to primary cultures of rat alveolar epithelial cells. *Exp Cell Res,* 221**,** 357-62.

PRAGMAN, A. A., KIM, H. B., REILLY, C. S., WENDT, C. & ISAACSON, R. E. 2012. The lung microbiome in moderate and severe chronic obstructive pulmonary disease. *PLoS One,* 7**,** e47305.

PRICE, M. N., DEHAL, P. S. & ARKIN, A. P. 2010. FastTree 2--approximately maximum-likelihood trees for large alignments. *PLoS One,* 5**,** e9490.

PRUSSIN, A. J., 2ND, MARR, L. C. & BIBBY, K. J. 2014. Challenges of studying viral aerosol metagenomics and communities in comparison with bacterial and fungal aerosols. *FEMS Microbiol Lett,* 357**,** 1-9.

QIAN, J., HOSPODSKY, D., YAMAMOTO, N., NAZAROFF, W. W. & PECCIA, J. 2012. Size-resolved emission rates of airborne bacteria and fungi in an occupied classroom. *Indoor Air,* 22**,** 339-51.

RABODONIRINA, M., COTTE, L., BOIBIEUX, A., KAISER, K., MAYENCON, M., RAFFENOT, D., TREPO, C., PEYRAMOND, D. & PICOT, S. 1999. Detection of Pneumocystis carinii DNA in blood specimens from human immunodeficiency virus-infected patients by nested PCR. *J Clin Microbiol,* 37**,** 127-31.

RADOMSKI, N., KREITMANN, L., MCINTOSH, F. & BEHR, M. A. 2013. The critical role of DNA extraction for detection of mycobacteria in tissues. *PLoS One,* 8**,** e78749.

RALPH, A. P., ARDIAN, M., WIGUNA, A., MAGUIRE, G. P., BECKER, N. G., DROGUMULLER, G., WILKS, M. J., WARAMORI, G., TJITRA, E., SANDJAJA, KENAGALEM, E., PONTORORING, G. J., ANSTEY, N. M. & KELLY, P. M. 2010. A simple, valid, numerical score for grading chest x-ray severity in adult smear-positive pulmonary tuberculosis. *Thorax,* 65**,** 863-9.

RAMAKERS, C., RUIJTER, J. M., DEPREZ, R. H. & MOORMAN, A. F. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett,* 339**,** 62-6.

463

RAMANAN, P., BARRETO, J. N., OSMON, D. R. & TOSH, P. K. 2014. Rothia bacteremia: a 10-year experience at Mayo Clinic, Rochester, Minnesota. *J Clin Microbiol,* 52**,** 3184-9.

RAO, C. P. & GELFAND, E. W. 1983. Pneumocystis carinii pneumonitis in patients with hypogammaglobulinemia and intact T cell immunity. *J Pediatr,* 103**,** 410-2.

RAOULT, D. 2018. Clinical manifestations and diagnosis of Q fever. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

RATCLIFFE, H. L. 1960. Tuberculosis in captive wild birds: increased frequency with increased population density. *J Albert Einstein Med Cent (Phila),* 8**,** 138-42.

RATCLIFFE, H. L. & PALLADINO, V. S. 1953. Tuberculosis induced by droplet nuclei infection; initial homogeneous response of small mammals (rats, mice, guinea pigs, and hamsters) to human and to bovine bacilli, and the rate and pattern of tubercle development. *J Exp Med,* 97**,** 61-8.

RAVERTY, S. A., RHODES, L. D., ZABEK, E., ESHGHI, A., CAMERON, C. E., HANSON, M. B. & SCHROEDER, J. P. 2017. Respiratory Microbiome of Endangered Southern Resident Killer Whales and Microbiota of Surrounding Sea Surface Microlayer in the Eastern North Pacific. *Sci Rep,* 7**,** 394.

RAVIGLIONE, M. C. 1990. Extrapulmonary pneumocystosis: the first 50 cases. *Rev Infect Dis,* 12**,** 1127-38.

REBOLI, A. C. 2017. Erysipelothrix infection. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

REDHEAD, S. A., CUSHION, M. T., FRENKEL, J. K. & STRINGER, J. R. 2006. Pneumocystis and Trypanosoma cruzi: nomenclature and typifications. *J Eukaryot Microbiol,* 53**,** 2-11.

REEDER, J. & KNIGHT, R. 2010. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. *Nat Methods,* 7**,** 668-9.

REPLOEG, M. D., STORCH, G. A. & CLIFFORD, D. B. 2001. Bk virus: a clinical review. *Clin Infect Dis,* 33**,** 191-202.

REVATHY, M., THERESE, K. L., BAGYALAKSHMI, R., CHANDRASEKAR, C., KUMAR, S. & MADHAVAN, H. N. 2014. Application of real time polymerase chain reaction targeting kex 1 gene & its comparison with the conventional methods for rapid detection of Pneumocystis jirovecii in clinical specimens. *Indian J Med Res,* 140**,** 406-13.

RIBES, J. A., LIMPER, A. H., ESPY, M. J. & SMITH, T. F. 1997. PCR detection of Pneumocystis carinii in bronchoalveolar lavage specimens: analysis of sensitivity and specificity. *J Clin Microbiol,* 35**,** 830-5.

RICARD-BLUM, S. 2010. The collagen family. *Extracellular matrix biology***,** 19.

RICHARD, S., ALMEIDA, J., CISSE, O. H., LURASCHI, A., NIELSEN, O., PAGNI, M. & HAUSER, P. M. 2018. Functional and Expression Analyses of the Pneumocystis MAT Genes Suggest Obligate Sexuality through Primary Homothallism within Host Lungs. *MBio,* 9.

RIGAUD, G., GRANGE, T. & PICTET, R. 1987. The use of NaOH as transfer solution of DNA onto nylon membrane decreases the hybridization efficiency. *Nucleic Acids Res,* 15**,** 857.

RILEY, E. C., MURPHY, G. & RILEY, R. L. 1978. Airborne spread of measles in a suburban elementary school. *Am J Epidemiol,* 107**,** 421-32.

RILEY, R. L. 1974. Airborne infection. *Am J Med,* 57**,** 466-75.

RILEY, R. L., MILLS, C. C., NYKA, W., WEINSTOCK, N., STOREY, P. B., SULTAN, L. U., RILEY, M. C. & WELLS, W. F. 1959. Aerial dissemination of pulmonary tuberculosis. A two-year study of contagion in a tuberculosis ward. *American journal of epidemiology,* 70**,** 185-196.

RILEY, R. L., MILLS, C. C., O'GRADY, F., SULTAN, L. U., WITTSTADT, F. & SHIVPURI, D. N. 1962. Infectiousness of air from a tuberculosis ward. Ultraviolet irradiation of infected air: comparative infectiousness of different patients. *Am Rev Respir Dis,* 85**,** 511-25.

ROBBERTS, F. J., LIEBOWITZ, L. D. & CHALKLEY, L. J. 2007. Polymerase chain reaction detection of Pneumocystis jiroveci: evaluation of 9 assays. *Diagn Microbiol Infect Dis,* 58**,** 385-92.

ROBERT-GANGNEUX, F., BELAZ, S., REVEST, M., TATTEVIN, P., JOUNEAU, S., DECAUX, O., CHEVRIER, S., LE TULZO, Y. & GANGNEUX, J. P. 2014. Diagnosis of Pneumocystis jirovecii pneumonia in immunocompromised patients by real-time PCR: a 4-year prospective study. *J Clin Microbiol,* 52**,** 3370-6.

ROGER, P. M., VANDENBOS, F., PUGLIESE, P., DE SALVADOR, F., DURANT, J., LE FICHOUX, Y. & DELLAMONICA, P. 1998. Persistence of Pneumocystis carinii after effective treatment of P. carinii pneumonia is not related to relapse or survival among patients infected with human immunodeficiency virus. *Clin Infect Dis,* 26**,** 509-10.

ROGERS, G. B., CUTHBERTSON, L., HOFFMAN, L. R., WING, P. A., POPE, C., HOOFTMAN, D. A., LILLEY, A. K., OLIVER, A., CARROLL, M. P., BRUCE, K. D. & VAN DER GAST, C. J. 2013. Reducing bias in bacterial community analysis of lower respiratory infections. *ISME J,* 7**,** 697-706.

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ROHLF, F. 1972. An Empirical Comparison of Three Ordination Techniques in Numerical Taxonomy. *Systematic Zoology,* 21.

ROHNER, P., JACOMO, V., STUDER, R., SCHRENZEL, J. & GRAF, J. D. 2009. Detection of Pneumocystis jirovecii by two staining methods and two quantitative PCR assays. *Infection,* 37**,** 261-5.

ROSCH, C., KRATZ, F., HERING, T., TRAUTMANN, S., UMANSKAYA, N., TIPPKOTTER, N., MULLER-RENNO, C., ULBER, R., HANNIG, M. & ZIEGLER, C. 2017. Albumin-lysozyme interactions: Cooperative adsorption on titanium and enzymatic activity. *Colloids Surf B Biointerfaces,* 149**,** 115-121.

ROTH, V. R. 2005. Evaluation of N95 respirator use as a tuberculosis control measure in a resource-limited setting. *The International Journal of Tuberculosis and Lung Disease,* 9**,** 545-549.

ROTHS, J. B. & SIDMAN, C. L. 1993. Single and combined humoral and cell-mediated immunotherapy of Pneumocystis carinii pneumonia in immunodeficient scid mice. *Infect Immun,* 61**,** 1641-9.

ROUSSOS, A., PHILIPPOU, N. & GOURGOULIANIS, K. I. 2003. Helicobacter pylori infection and respiratory diseases: a review. *World Journal of Gastroenterology,* 9**,** 5-8.

ROY, C. J. & MILTON, D. K. 2004. Airborne transmission of communicable infection--the elusive pathway. *N Engl J Med,* 350**,** 1710-2.

RUBIN, B. E., GIBBONS, S. M., KENNEDY, S., HAMPTON-MARCELL, J., OWENS, S. & GILBERT, J. A. 2013. Investigating the impact of storage conditions on microbial community composition in soil samples. *PLoS One,* 8**,** e70460.

RUDRAMURTHY, S. M., SHARMA, M., SHARMA, M., RAWAT, P., GHOSH, A., VENKATESAN, L., AGGARWAL, R., SINGH, M. & CHAKRABARTI, A. 2018. Reliable differentiation of Pneumocystis pneumonia from Pneumocystis colonisation by quantification of Major Surface Glycoprotein gene using real-time polymerase chain reaction. *Mycoses,* 61**,** 96-103.

RUIJTER, J. M., RAMAKERS, C., HOOGAARS, W. M., KARLEN, Y., BAKKER, O., VAN DEN HOFF, M. J. & MOORMAN, A. F. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res,* 37**,** e45.

RUIZ-FUENTES, J. L., DIAZ, A., ENTENZA, A. E., FRION, Y., SUAREZ, O., TORRES, P., DE ARMAS, Y. & ACOSTA, L. 2015. Comparison of four DNA extraction methods for the detection of Mycobacterium leprae from Ziehl-Neelsen-stained microscopic slides. *Int J Mycobacteriol,* 4**,** 284-9.

RULE, A. M., EVANS, S. L. & SILBERGELD, E. K. 2008. Food animal transport: a potential source of community exposures to health hazards from industrial farming (CAFOs). *J Infect Public Health,* 1**,** 33-9.

RUSKIN, J. & REMINGTON, J. S. 1967. The compromised host and infection. I. Pneumocystis carinii pneumonia. *JAMA,* 202**,** 1070-4.

SADAGHDAR, H., HUANG, Z. B. & EDEN, E. 1992. Correlation of bronchoalveolar lavage findings to severity of Pneumocystis carinii pneumonia in AIDS. Evidence for the development of high-permeability pulmonary edema. *Chest,* 102**,** 63-9.

SAENZ, R. E., BROWN, W. D. & SANDERS, C. V. 2001. Allergic bronchopulmonary disease caused by Bipolaris hawaiiensis presenting as a necrotizing pneumonia: case report and review of literature. *Am J Med Sci,* 321**,** 209-12.

SAGE, E. K., NOURSADEGHI, M., EVANS, H. E., PARKER, S. J., COPAS, A. J., EDWARDS, S. G. & MILLER, R. F. 2010. Prognostic value of C-reactive protein in HIV-infected patients with Pneumocystis jirovecii pneumonia. *Int J STD AIDS,* 21**,** 288-92.

SAKAI, M., ISHIKAWA, S., YAMAMOTO, T., ONIZUKA, M., SAKAKIBARA, Y. & NOGUCHI, M. 2005. A direct measurement of serum amylase levels produced by lung cancer. *Ann Thorac Surg,* 79**,** 1409-11.

SAKRAN, W., RAZ, R., LEVI, Y., COLODNER, R. & KOREN, A. 1999. Campylobacter bacteremia and pneumonia in two splenectomized patients. *Eur J Clin Microbiol Infect Dis,* 18**,** 496-8.

SALAR, A., CARRATALA, J., FERNANDEZ-SEVILLA, A., MARIN, D. & GRANENA, A. 1997. Pneumonia caused by Micrococcus species in a neutropenic patient with acute leukemia. *Eur J Clin Microbiol Infect Dis,* 16**,** 546-8.

SALTER, S. J., COX, M. J., TUREK, E. M., CALUS, S. T., COOKSON, W. O., MOFFATT, M. F., TURNER, P., PARKHILL, J., LOMAN, N. J. & WALKER, A. W. 2014. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol,* 12**,** 87.

SAMANTA, S., PODDAR, B., AZIM, A., SINGH, R. K., GURJAR, M. & BARONIA, A. K. 2018. Significance of Mini Bronchoalveolar Lavage Fluid Amylase Level in Ventilator-Associated Pneumonia: A Prospective Observational Study. *Crit Care Med,* 46**,** 71-78.

SAMBROOK, J. & RUSSELL, D. W. 2001. *Molecular cloning : a laboratory manual,* Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press.

SAMUEL, C. M., WHITELAW, A., CORCORAN, C., MORROW, B., HSIAO, N. Y., ZAMPOLI, M. & ZAR, H. J. 2011. Improved detection of Pneumocystis jirovecii in upper and lower respiratory tract specimens from children with suspected pneumocystis pneumonia using real-time PCR: a prospective study. *BMC Infect Dis,* 11**,** 329.

SANO, M., ZENNAMI, S. & MASAOKA, A. 1986. Activities of some enzymes in tracheal and bronchial fluid. *Panminerva Med,* 28**,** 137-42.

SANTOS, C. R., DE ASSIS, A. M., LUZ, E. A., LYRA, L., TORO, I. F., SEABRA, J. C. C., DALDIN, D. H., MARCALTO, T. U., GALASSO, M. T., MACEDO, R. F., SCHREIBER, A. Z. & AOKI, F. H. 2017. Detection of Pneumocystis jirovecii by nested PCR in HIV-negative patients with pulmonary disease. *Rev Iberoam Micol,* 34**,** 83-88.

SARKAR, G. & SOMMER, S. S. 1990. Shedding light on PCR contamination. *Nature,* 343**,** 27.

SASSO, M., CHASTANG-DUMAS, E., BASTIDE, S., ALONSO, S., LECHICHE, C., BOURGEOIS, N. & LACHAUD, L. 2016. Performances of Four Real-Time PCR Assays for Diagnosis of Pneumocystis jirovecii Pneumonia. *J Clin Microbiol,* 54**,** 625-30.

SATAPATHY, P., DAS, D., MURMU, B. N. & KAR, S. K. 2014. Decontamination of sputum for longer time in sodium hydroxide for isolation of Mycobacterium tuberculosis. *Int J Mycobacteriol,* 3**,** 290-2.

SAVINI, V., GHERARDI, G., ASTOLFI, D., POLILLI, E., DICUONZO, G., D'AMARIO, C., FAZII, P. & D'ANTONIO, D. 2012. Insights into airway infections by enterococci: a review. *Recent Pat Antiinfect Drug Discov,* 7**,** 36-44.

SAX, P. E. 2016. Clinical presentation and diagnosis of Pneumocystis pulmonary infection in HIV-infected patients. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

SAX, P. E. 2018. Treatment and prevention of Pneumocystis infection in HIV-infected patients. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

SAX, P. E., KOMAROW, L., FINKELMAN, M. A., GRANT, P. M., ANDERSEN, J., SCULLY, E., POWDERLY, W. G. & ZOLOPA, A. R. 2011. Blood (1->3)-beta-D-glucan as a diagnostic test for HIV-related Pneumocystis jirovecii pneumonia. *Clin Infect Dis,* 53**,** 197-202.

SCADDEN, D. T., FREEDMAN, A. R. & ROBERTSON, P. 2018. Human T-lymphotropic virus type I: Virology, pathogenesis, and epidemiology. *In:* BLOOM, A. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

SCHAFER, M. P., FERNBACK, J. E. & JENSEN, P. A. 1998. Sampling and analytical method development for qualitative assessment of airborne mycobacterial species of the Mycobacterium tuberculosis complex. *Am Ind Hyg Assoc J,* 59**,** 540-6.

SCHAFER, M. P., MARTINEZ, K. F. & MATHEWS, E. S. 2003. Rapid detection and determination of the aerodynamic size range of airborne mycobacteria associated with whirlpools. *Appl Occup Environ Hyg,* 18**,** 41-50.

SCHEIDELER, L., MANKE, H. G., SCHWULERA, U., INACKER, O. & HAMMERLE, H. 1993. Detection of nonvolatile macromolecules in breath: a possible diagnostic tool? *Am Rev Respir Dis,* 148**,** 778-784.

SCHEIERMANN, J. & KLINMAN, D. M. 2017. Three distinct pneumotypes characterize the microbiome of the lung in BALB/cJ mice. *PLoS One,* 12**,** e0180561.

SCHICHT, M., KNIPPING, S., HIRT, R., BEILEKE, S., SEL, S., PAULSEN, F. & BRAUER, L. 2013. Detection of surfactant proteins A, B, C, and D in human nasal mucosa and their regulation in chronic rhinosinusitis with polyps. *Am J Rhinol Allergy,* 27**,** 24-29.

SCHILDGEN, V., MAI, S., KHALFAOUI, S., LUSEBRINK, J., PIEPER, M., TILLMANN, R. L., BROCKMANN, M. & SCHILDGEN, O. 2014. Pneumocystis jirovecii can be productively cultured in differentiated CuFi-8 airway cells. *MBio,* 5**,** e01186-14.

SCHLOSS, P. D. 2008. Evaluating different approaches that test whether microbial communities have the same structure. *Isme j,* 2**,** 265-75.

SCHLOSS, P. D. 2010. The effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16S rRNA gene-based studies. *PLoS Comput Biol,* 6**,** e1000844.

SCHLUGER, N., GODWIN, T., SEPKOWITZ, K., ARMSTRONG, D., BERNARD, E., RIFKIN, M., CERAMI, A. & BUCALA, R. 1992. Application of DNA amplification to pneumocystosis: presence of serum Pneumocystis carinii DNA during human and experimentally induced Pneumocystis carinii pneumonia. *J Exp Med,* 176**,** 1327-33.

SCHMOLDT, S., SCHUHEGGER, R., WENDLER, T., HUBER, I., SOLLNER, H., HOGARDT, M., ARBOGAST, H., HEESEMANN, J., BADER, L. & SING, A. 2008. Molecular evidence of nosocomial Pneumocystis jirovecii transmission among 16 patients after kidney transplantation. *J Clin Microbiol,* 46**,** 966-71.

466

SCHRADER, C., SCHIELKE, A., ELLERBROEK, L. & JOHNE, R. 2012. PCR inhibitors - occurrence, properties and removal. *J Appl Microbiol,* 113**,** 1014-26.

SCHRIEBER, R. & GAREIS, H. 2007. *Gelatine Handbook: Theory and Industrial Practice*, Wiley VCH.

SCHULZE, M. H., KELLER, C., MULLER, A., ZIEGLER, U., LANGEN, H. J., HEGASY, G. & STICH, A. 2011. Rickettsia typhi infection with interstitial pneumonia in a traveler treated with moxifloxacin. *J Clin Microbiol,* 49**,** 741-3.

SEAH, C., RICHARDSON, S. E., TSUI, G., YU, B., THORNBACK, J., MCTAGGART, L., BOGGILD, A., WENGENACK, N. L. & ZHANG, S. X. 2012. Comparison of the FXG: RESP (Asp+) real-time PCR assay with direct immunofluorescence and calcofluor white staining for the detection of Pneumocystis jirovecii in respiratory specimens. *Med Mycol,* 50**,** 324-7.

SEGAL, L. N., ALEKSEYENKO, A. V., CLEMENTE, J. C., KULKARNI, R., WU, B., CHEN, H., BERGER, K. I., GOLDRING, R. M., ROM, W. N., BLASER, M. J. & WEIDEN, M. D. 2013. Enrichment of lung microbiome with supraglottic taxa is associated with increased pulmonary inflammation. *Microbiome,* 1.

SEGAL, L. N. & BLASER, M. J. 2014. A Brave New World: The Lung Microbiota in an Era of Change. *Ann Am Thorac Soc,* 11**,** S21-7.

SEGAL, L. N., CLEMENTE, J. C., TSAY, J. C., KORALOV, S. B., KELLER, B. C., WU, B. G., LI, Y., SHEN, N., GHEDIN, E., MORRIS, A., DIAZ, P., HUANG, L., WIKOFF, W. R., UBEDA, C., ARTACHO, A., ROM, W. N., STERMAN, D. H., COLLMAN, R. G., BLASER, M. J. & WEIDEN, M. D. 2016. Enrichment of the lung microbiome with oral taxa is associated with lung inflammation of a Th17 phenotype. *Nat Microbiol,* 1**,** 16031.

SEINIGE, D., KRISCHEK, C., KLEIN, G. & KEHRENBERG, C. 2014. Comparative analysis and limitations of ethidium monoazide and propidium monoazide treatments for the differentiation of viable and nonviable campylobacter cells. *Appl Environ Microbiol,* 80**,** 2186-92.

SEXTON, D. J. & MCCLAIN, M. T. 2017. Other spotted fever group rickettsial infections. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

SEYAMA, K., NUKIWA, T., TAKAHASHI, K., TAKAHASHI, H. & KIRA, S. 1994. Amylase mRNA transcripts in normal tissues and neoplasms: the implication of different expressions of amylase isogenes. *J Cancer Res Clin Oncol,* 120**,** 213-20.

SHANKAR, E. M., KUMARASAMY, N., BELLA, D., RENUKA, S., KOWNHAR, H., SUNITI, S., RAJAN, R. & RAO, U. A. 2006. Pneumonia and pleural effusion due to Cryptococcus laurentii in a clinically proven case of AIDS. *Can Respir J,* 13**,** 275-8.

SHANNON, C. E. 1948. A Mathematical Theory of Communication. *Bell System Technical Journal,* 27**,** 379-423.

SHAW, K., SESARDIC, I., BRISTOL, N., AMES, C., DAGNALL, K., ELLIS, C., WHITTAKER, F. & DANIEL, B. 2008. Comparison of the effects of sterilisation techniques on subsequent DNA profiling. *Int J Legal Med,* 122**,** 29-33.

SHIMOYAMA, Y., UMEGAKI, O., OOI, Y., AGUI, T., KADONO, N. & MINAMI, T. 2017. Bacillus cereus pneumonia in an immunocompetent patient: a case report. *JA Clin Rep,* 3.

SHIN, S. & DAY, L. A. 1995. Separation and size determination of circular and linear single-stranded DNAs by alkaline agarose gel electrophoresis. *Anal Biochem,* 226**,** 202-6.

SHIOTA, T., YAMADA, M. & YOSHIDA, Y. 1986. Morphology, development and behavior of Pneumocystis carinii observed by light-microscopy in nude mice. *Zentralbl Bakteriol Mikrobiol Hyg A,* 262**,** 230-9.

SHOHAM, S., PIC-ALUAS, L., TAYLOR, J., CORTEZ, K., RINALDI, M. G., SHEA, Y. & WALSH, T. J. 2008. Transplant-associated Ochroconis gallopava infections. *Transpl Infect Dis,* 10**,** 442-8.

SHORT, W. R. 2009. Fatal herpes simplex virus type 2 pneumonia in a person with AIDS. *AIDS Read,* 19**,** 51-2, 60-1.

SHUKLA, S. D., BUDDEN, K. F., NEAL, R. & HANSBRO, P. M. 2017. Microbiome effects on immunity, health and disease in the lung. *Clin Transl Immunology,* 6**,** e133.

SIBLEY, C. D., GRINWIS, M. E., FIELD, T. R., ESHAGHURSHAN, C. S., FARIA, M. M., DOWD, S. E., PARKINS, M. D., RABIN, H. R. & SURETTE, M. G. 2011. Culture enriched molecular profiling of the cystic fibrosis airway microbiome. *PLoS One,* 6**,** e22702.

SIDORENKO, G. I., ZBOROVSKII, E. I. & LEVINA, D. I. 1980. [Surface-active properties of the exhaled air condensate (a new method of studying lung function)]. *Ter Arkh,* 52**,** 65-8.

SILLETTI, R. P., GLEZEROV, V. & SCHWARTZ, I. S. 1996. Pulmonary paracoccidioidomycosis misdiagnosed as Pneumocystis pneumonia in an immunocompromised host. *J Clin Microbiol,* 34**,** 2328-30.

SIMPSON, E. H. 1949. Measurement of Diversity. *Nature,* 163**,** 688.

SING, A., ROGGENKAMP, A., AUTENRIETH, I. B. & HEESEMANN, J. 1999a. Pneumocystis carinii carriage in immunocompetent patients with primary pulmonary disorders as detected by single or nested PCR. *J Clin Microbiol,* 37**,** 3409-10.

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SING, A., TREBESIUS, K., ROGGENKAMP, A., RUSSMANN, H., TYBUS, K., PFAFF, F., BOGNER, J. R., EMMINGER, C. & HEESEMANN, J. 2000. Evaluation of diagnostic value and epidemiological implications of PCR for Pneumocystis carinii in different immunosuppressed and immunocompetent patient groups. *J Clin Microbiol,* 38**,** 1461-7.

SING, A., WONHAS, C., BADER, L., LUTHER, M. & HEESEMANN, J. 1999b. Detection of Pneumocystis carinii DNA in the air filter of a ventilated patient with AIDS. *Clin Infect Dis,* 29**,** 952-3.

SINGER, C., ARMSTRONG, D., ROSEN, P. P. & SCHOTTENFELD, D. 1975. Pneumocystis carinii pneumonia: a cluster of eleven cases. *Ann Intern Med,* 82**,** 772-7.

SINGLA, N., KAISTHA, N., GULATI, N. & CHANDER, J. 2010. Morganella morganii could be an important Intensive Care Unit pathogen. *Indian J Crit Care Med,* 14**,** 154-5.

SKUDE, G. 1975. Sources of the serum isoamylases and their normal range of variation with age. *Scand J Gastroenterol,* 10**,** 577-84.

SMITH, J. A., ALIVERTI, A., QUARANTA, M., MCGUINNESS, K., KELSALL, A., EARIS, J. & CALVERLEY, P. M. 2012. Chest wall dynamics during voluntary and induced cough in healthy volunteers. *J Physiol,* 590**,** 563-74.

SMITH, R. L., EL-SADR, W. M. & LEWIS, M. L. 1988. Correlation of bronchoalveolar lavage cell populations with clinical severity of Pneumocystis carinii pneumonia. *Chest,* 93**,** 60-4.

SOMERVILLE, W., THIBERT, L., SCHWARTZMAN, K. & BEHR, M. A. 2005. Extraction of Mycobacterium tuberculosis DNA: a question of containment. *J Clin Microbiol,* 43**,** 2996-7.

SØRENSEN, T. 1948. {A method of establishing groups of equal amplitude in plant sociology based on similarity of species and its application to analyses of the vegetation on Danish commons}. *Biol. Skr.,* 5**,** 1-34.

SPANU, P. D. 2012. The genomics of obligate (and nonobligate) biotrophs. *Annu Rev Phytopathol,* 50**,** 91-109.

SPEKREIJSE, D., BOUMA, A., KOCH, G. & STEGEMAN, A. 2013. Quantification of dust-borne transmission of highly pathogenic avian influenza virus between chickens. *Influenza and other respiratory viruses,* 7**,** 132-138.

STENTON, C. 2008. The MRC breathlessness scale. *Occup Med (Lond),* 58**,** 226-7.

STILIANAKIS, N. I. & DROSSINOS, Y. 2010. Dynamics of infectious disease transmission by inhalable respiratory droplets. *J R Soc Interface,* 7**,** 1355-66.

STODDARD, S. F., SMITH, B. J., HEIN, R., ROLLER, B. R. & SCHMIDT, T. M. 2015. rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res,* 43**,** D593-8.

STRINGER, J. R. 2002. Pneumocystis. *Int J Med Microbiol,* 292**,** 391-404.

STRINGER, J. R., BEARD, C. B., MILLER, R. F. & WAKEFIELD, A. E. 2002. A new name (Pneumocystis jiroveci) for Pneumocystis from humans. *Emerg Infect Dis,* 8**,** 891-6.

STRINGER, J. R., CUSHION, M. T. & WAKEFIELD, A. E. 2001. New nomenclature for the genus Pneumocystis. *J Eukaryot Microbiol,* Suppl**,** 184S-189S.

SUDO, K. & KANNO, T. 1976. Properties of the amylase produced in carcinoma of the lung. *Clin Chim Acta,* 73**,** 1-12.

SUEISHI, K., HISANO, S., SUMIYOSHI, A. & TANAKA, K. 1977. Scanning and transmission electron microscopic study of human pulmonary pneumocystosis. *Chest,* 72**,** 213-6.

SUL, W. J., COLE, J. R., JESUS EDA, C., WANG, Q., FARRIS, R. J., FISH, J. A. & TIEDJE, J. M. 2011. Bacterial community comparisons by taxonomy-supervised analysis independent of sequence alignment and clustering. *Proc Natl Acad Sci U S A,* 108**,** 14637-42.

SUZUKI, K., NAKAMURA, A., FUJIEDA, A., NAKASE, K. & KATAYAMA, N. 2012. Pulmonary infection caused by Exophiala dermatitidis in a patient with multiple myeloma: A case report and a review of the literature. *Med Mycol Case Rep,* 1**,** 95-8.

SVERRILD, A., KIILERICH, P., BREJNROD, A., PEDERSEN, R., PORSBJERG, C., BERGQVIST, A., ERJEFALT, J. S., KRISTIANSEN, K. & BACKER, V. 2017. Eosinophilic airway inflammation in asthmatic patients is associated with an altered airway microbiome. *J Allergy Clin Immunol,* 140**,** 407-417.e11.

SWAIN, S. D., WRIGHT, T. W., DEGEL, P. M., GIGLIOTTI, F. & HARMSEN, A. G. 2004. Neither neutrophils nor reactive oxygen species contribute to tissue damage during Pneumocystis pneumonia in mice. *Infect Immun,* 72**,** 5722-32.

TAKAHASHI, S., TOMITA, J., NISHIOKA, K., HISADA, T. & NISHIJIMA, M. 2014. Development of a prokaryotic universal primer for simultaneous analysis of Bacteria and Archaea using next-generation sequencing. *PLoS One,* 9**,** e105592.

TAKANO, Y., IWAKIRI, T. & ICHIYASU, Y. 1993. [A case of lung cancer producing CA19-9 and amylase]. *Nihon Kyobu Shikkan Gakkai Zasshi,* 31**,** 267-70.

TANNER, M. A., GOEBEL, B. M., DOJKA, M. A. & PACE, N. R. 1998. Specific ribosomal DNA sequences from diverse environmental settings correlate with experimental contaminants. *Appl Environ Microbiol,* 64**,** 3110-3.

TASAKA, S., HASEGAWA, N., KOBAYASHI, S., YAMADA, W., NISHIMURA, T., TAKEUCHI, T. & ISHIZAKA, A. 2007. Serum indicators for the diagnosis of pneumocystis pneumonia. *Chest,* 131**,** 1173-80.

TAWFIK, O. W., PAPASIAN, C. J., DIXON, A. Y. & POTTER, L. M. 1989. Saccharomyces cerevisiae pneumonia in a patient with acquired immune deficiency syndrome. *J Clin Microbiol,* 27**,** 1689-91.

TAYLOR, M. J., BENTHAM, R. H. & ROSS, K. E. 2014. Limitations of Using Propidium Monoazide with qPCR to Discriminate between Live and Dead Legionella in Biofilm Samples. *Microbiol Insights,* 7**,** 15-24.

TEDJO, D. I., JONKERS, D. M., SAVELKOUL, P. H., MASCLEE, A. A., VAN BEST, N., PIERIK, M. J. & PENDERS, J. 2015. The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. *PLoS One,* 10**,** e0126685.

TELL, L. A., FOLEY, J., NEEDHAM, M. L. & WALKER, R. L. 2003. Comparison of four rapid DNA extraction techniques for conventional polymerase chain reaction testing of three Mycobacterium spp. that affect birds. *Avian Dis,* 47**,** 1486-90.

THAKUR, R., SARMA, S. & GOYAL, R. 2011. Comparison of DNA Extraction Protocols for Mycobacterium Tuberculosis in Diagnosis of Tuberculous Meningitis by Real-time Polymerase Chain Reaction. *J Glob Infect Dis,* 3**,** 353-6.

THIERRY, D., CAVE, M. D., EISENACH, K. D., CRAWFORD, J. T., BATES, J. H., GICQUEL, B. & GUESDON, J. L. 1990. IS6110, an IS-like element of Mycobacterium tuberculosis complex. *Nucleic Acids Res,* 18**,** 188.

THOMAS, C. F., JR. & LIMPER, A. H. 2004. Pneumocystis pneumonia. *N Engl J Med,* 350**,** 2487-98.

THOMAS, C. F., JR. & LIMPER, A. H. 2007. Current insights into the biology and pathogenesis of Pneumocystis pneumonia. *Nat Rev Microbiol,* 5**,** 298-308.

THOMAS, C. F. & LIMPER, A. H. 2017. Epidemiology, clinical manifestations, and diagnosis of Pneumocystis pneumonia in HIV-uninfected patients. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

THOMAS, C. F. & LIMPER, A. H. 2018. Treatment and prevention of Pneumocystis pneumonia in HIV-uninfected patients. *In:* MITTY, J. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

THOMPSON, A. B., BOHLING, T., PAYVANDI, F. & RENNARD, S. I. 1990. Lower respiratory tract lactoferrin and lysozyme arise primarily in the airways and are elevated in association with chronic bronchitis. *J Lab Clin Med,* 115**,** 148-58.

THOMPSON BASTIN, M. L., NEVILLE, N. R., PARSONS, R. E., FLANNERY, A. H., TENNANT, S. J. & JOHNSON, C. A. 2016. An unusual case of Salmonella Enteritidis causing pneumonia, septic shock and multiple organ failure in an immunocompetent patient. *IDCases,* 6**,** 85-89.

TIA, T., PUTAPORNTIP, C., KOSUWIN, R., KONGPOLPROM, N., KAWKITINARONG, K. & JONGWUTIWES, S. 2012. A highly sensitive novel PCR assay for detection of Pneumocystis jirovecii DNA in bronchoalveloar lavage specimens from immunocompromised patients. *Clin Microbiol Infect,* 18**,** 598-603.

TOMITA, N., MATSUURA, N., HORII, A., EMI, M., NISHIDE, T., OGAWA, M., MORI, T., DOI, O. & MATSUBARA, K. 1988. Expression of alpha-amylase in human lung cancers. *Cancer Res,* 48**,** 3292-6.

TOOP, L. J., THORPE, C. W. & FRIGHT, R. 1989. Cough Sound Analysis: A New Tool for the Diagnosis of Asthma? *Family Practice,* 6**,** 83-85.

TORRES, J., GOLDMAN, M., WHEAT, L. J., TANG, X., BARTLETT, M. S., SMITH, J. W., ALLEN, S. D. & LEE, C. H. 2000. Diagnosis of Pneumocystis carinii pneumonia in human immunodeficiency virus-infected patients with polymerase chain reaction: a blinded comparison to standard methods. *Clin Infect Dis,* 30**,** 141-5.

TRAMPUZ, A., PIPER, K. E., STECKELBERG, J. M. & PATEL, R. 2006. Effect of gamma irradiation on viability and DNA of Staphylococcus epidermidis and Escherichia coli. *J Med Microbiol,* 55**,** 1271-5.

TRAVIS, S. M., CONWAY, B. A., ZABNER, J., SMITH, J. J., ANDERSON, N. N., SINGH, P. K., GREENBERG, E. P. & WELSH, M. J. 1999. Activity of abundant antimicrobials of the human airway. *Am J Respir Cell Mol Biol,* 20**,** 872-9.

TRIPATHI, A., MIRANT-BORDE, M. C. & LEE, A. 2011. Amylase in bronchoalveolar lavage as a potential marker of oropharyngeal-to-pulmonary aspiration. *Am J Respir Crit Care Med,* 183**,** A4616.

TSENG, C.-C. & LI, C.-S. 2005. Collection efficiencies of aerosol samplers for virus-containing aerosols. *Journal of Aerosol Science,* 36**,** 593-607.

TSENG, C.-C., PAN, Z.-M. & CHANG, C.-H. 2016. Application of a quaternary ammonium agent on surgical face masks before use for pre-decontamination of nosocomial infection-related bioaerosols. *Aerosol Science and Technology,* 50**,** 199-210.

TSUKAWAKI, M., IZAWA, M., YOSHIDA, M., ARAKI, N., HASHIBA, Y., NAKAGAWA, H., MOMOSE, M., KAWASE, S., TOMINAGA, J. & KAWAI, S. 1992. A case of amylase-producing lung cancer. *Intern Med,* 31**,** 60-3.

TUNCER, S., ERGUVEN, S., KOCAGOZ, S. & UNAL, S. 1998. Comparison of cytochemical staining, immunofluorescence and PCR for diagnosis of pneumocystis carinii on sputum samples. *Scand J Infect Dis,* 30**,** 125-8.

TURNER, R., REPOSSI, A., MATOS, S., BIRRING, S. & BOTHAMLEY, G. 2014. Cough Prevalence And Frequency In Pulmonary Tuberculosis [abstract]. *Thorax,* 69**,** A43-A44.

TURNER, R. D., BIRRING, S. S., DARMALINGAM, M., HOOPER, R. L., KUNST, H., MATOS, S. & BOTHAMLEY, G. H. 2018. Daily cough frequency in tuberculosis and association with household infection. *Int J Tuberc Lung Dis,* 22**,** 863-870.

TURNER, R. D. & BOTHAMLEY, G. H. 2015. Cough and the transmission of tuberculosis. *J Infect Dis,* 211**,** 1367-72.

TURNER, R. D., CHIU, C., CHURCHYARD, G. J., ESMAIL, H., LEWINSOHN, D. M., GANDHI, N. R. & FENNELLY, K. P. 2017. Tuberculosis Infectiousness and Host Susceptibility. *J Infect Dis,* 216**,** S636-S643.

UNNEWEHR, M., FRIEDERICHS, H., BARTSCH, P. & SCHAAF, B. 2016. High Diagnostic Value of a New Real-Time Pneumocystis PCR from Bronchoalveolar Lavage in a Real-Life Clinical Setting. *Respiration,* 92**,** 144-9.

VALDEBENITO, C., BONACIC, M., MATAMALA, J. & WOLFF, M. 2015. [Extrapulmonary pneumocystosis: a case report]. *Rev Chilena Infectol,* 32**,** 344-9.

VALERO, C., BUITRAGO, M. J., GITS-MUSELLI, M., BENAZRA, M., STURNY-LECLERE, A., HAMANE, S., GUIGUE, N., BRETAGNE, S. & ALANIO, A. 2016. Copy Number Variation of Mitochondrial DNA Genes in Pneumocystis jirovecii According to the Fungal Load in BAL Specimens. *Front Microbiol,* 7**,** 1413.

VALWAY, S. E., SANCHEZ, M. P., SHINNICK, T. F., ORME, I., AGERTON, T., HOY, D., JONES, J. S., WESTMORELAND, H. & ONORATO, I. M. 1998. An outbreak involving extensive transmission of a virulent strain of Mycobacterium tuberculosis. *N Engl J Med,* 338**,** 633-9.

VAN DEN BOSCH, E. & GIELENS, C. 2003. Gelatin degradation at elevated temperature. *Int J Biol Macromol,* 32**,** 129-38.

VAN DER ZANDEN, A. G., HOENTJEN, A. H., HEILMANN, F. G., WELTEVREDEN, E. F., SCHOULS, L. M. & VAN EMBDEN, J. D. 1998. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis complex in paraffin wax embedded tissues and in stained microscopic preparations. *Mol Pathol,* 51**,** 209-14.

VAN DROOGENBROECK, C., VAN RISSEGHEM, M., BRAECKMAN, L. & VANROMPAY, D. 2009. Evaluation of bioaerosol sampling techniques for the detection of Chlamydophila psittaci in contaminated air. *Vet Microbiol,* 135**,** 31-7.

VAN EMBDEN, J. D., CAVE, M. D., CRAWFORD, J. T., DALE, J. W., EISENACH, K. D., GICQUEL, B., HERMANS, P., MARTIN, C., MCADAM, R., SHINNICK, T. M. & ET AL. 1993. Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol,* 31**,** 406-9.

VARGAS, S. L., HUGHES, W. T., SANTOLAYA, M. E., ULLOA, A. V., PONCE, C. A., CABRERA, C. E., CUMSILLE, F. & GIGLIOTTI, F. 2001. Search for primary infection by Pneumocystis carinii in a cohort of normal, healthy infants. *Clin Infect Dis,* 32**,** 855-61.

VARGAS, S. L., HUGHES, W. T., WAKEFIELD, A. E. & OZ, H. S. 1995. Limited persistence in and subsequent elimination of Pneumocystis carinii from the lungs after P. carinii pneumonia. *J Infect Dis,* 172**,** 506-10.

VARGAS, S. L., PONCE, C. A., GIGLIOTTI, F., ULLOA, A. V., PRIETO, S., MUNOZ, M. P. & HUGHES, W. T. 2000. Transmission of Pneumocystis carinii DNA from a patient with P. carinii pneumonia to immunocompetent contact health care workers. *J Clin Microbiol,* 38**,** 1536-8.

VARGAS, S. L., PONCE, C. A., SANCHEZ, C. A., ULLOA, A. V., BUSTAMANTE, R. & JUAREZ, G. 2003. Pregnancy and asymptomatic carriage of Pneumocystis jiroveci. *Emerg Infect Dis,* 9**,** 605-6.

VENKATARAMAN, A., BASSIS, C. M., BECK, J. M., YOUNG, V. B., CURTIS, J. L., HUFFNAGLE, G. B. & SCHMIDT, T. M. 2015. Application of a neutral community model to assess structuring of the human lung microbiome. *MBio,* 6.

VERNON, M., LEIDY, N. K., NACSON, A. & NELSEN, L. 2009. Measuring cough severity: Perspectives from the literature and from patients with chronic cough. *Cough,* 5**,** 5.

VERREAULT, D., MOINEAU, S. & DUCHAINE, C. 2008. Methods for sampling of airborne viruses. *Microbiol Mol Biol Rev,* 72**,** 413-44.

VERWEIJ, P. E., VAN KASTEREN, M., VAN DE NES, J., DE HOOG, G. S., DE PAUW, B. E. & MEIS, J. F. 1997. Fatal pulmonary infection caused by the basidiomycete Hormographiella aspergillata. *J Clin Microbiol,* 35**,** 2675-8.

VITORINO, R., LOBO, M. J., FERRER-CORREIA, A. J., DUBIN, J. R., TOMER, K. B., DOMINGUES, P. M. & AMADO, F. M. 2004. Identification of human whole saliva protein components using proteomics. *Proteomics,* 4**,** 1109-15.

WADE, W., THOMPSON, H., RYBALKA, A. & VARTOUKIAN, S. 2016. Uncultured Members of the Oral Microbiome. *J Calif Dent Assoc,* 44**,** 447-56.

WADE, W. G. 2013. The oral microbiome in health and disease. *Pharmacol Res,* 69**,** 137-43.

WAGNER MACKENZIE, B., WAITE, D. W. & TAYLOR, M. W. 2015. Evaluating variation in human gut microbiota profiles due to DNA extraction method and inter-subject differences. *Front Microbiol,* 6**,** 130.

WAKEFIELD, A. E. 1994. Detection of DNA sequences identical to Pneumocystis carinii in samples of ambient air. *J Eukaryot Microbiol,* 41**,** 116S.

WAKEFIELD, A. E. 1996. DNA sequences identical to Pneumocystis carinii f. sp. carinii and Pneumocystis carinii f. sp. hominis in samples of air spora. *J Clin Microbiol,* 34**,** 1754-9.

WAKEFIELD, A. E. 2002. Pneumocystis carinii. *Br Med Bull,* 61**,** 175-88.

WAKEFIELD, A. E., GUIVER, L., MILLER, R. F. & HOPKIN, J. M. 1991. DNA amplification on induced sputum samples for diagnosis of Pneumocystis carinii pneumonia. *Lancet,* 337**,** 1378-9.

WAKEFIELD, A. E., PIXLEY, F. J., BANERJI, S., SINCLAIR, K., MILLER, R. F., MOXON, E. R. & HOPKIN, J. M. 1990. Detection of Pneumocystis carinii with DNA amplification. *Lancet,* 336**,** 451-3.

WALKER, A. W., MARTIN, J. C., SCOTT, P., PARKHILL, J., FLINT, H. J. & SCOTT, K. P. 2015. 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. *Microbiome,* 3**,** 26.

WALZER, P. D. 1999. Immunological features of Pneumocystis carinii infection in humans. *Clin Diagn Lab Immunol,* 6**,** 149-55.

WALZER, P. D. 2013. The ecology of pneumocystis: perspectives, personal recollections, and future research opportunities. *J Eukaryot Microbiol,* 60**,** 634-45.

WANG, H. & WU, Q. 2016. A case of amylase-producing small cell lung cancer. *Clin Biochem,* 49**,** 613-6.

WANG, J., GIGLIOTTI, F., MAGGIRWAR, S., JOHNSTON, C., FINKELSTEIN, J. N. & WRIGHT, T. W. 2005. Pneumocystis carinii activates the NF-kappaB signaling pathway in alveolar epithelial cells. *Infect Immun,* 73**,** 2766-77.

WANG, Q., GARRITY, G. M., TIEDJE, J. M. & COLE, J. R. 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology,* 73**,** 5261-5267.

WANG, X., LIM, H. J. & SON, A. 2014. Characterization of denaturation and renaturation of DNA for DNA hybridization. *Environ Health Toxicol,* 29.

WANG, Z., BAFADHEL, M., HALDAR, K., SPIVAK, A., MAYHEW, D., MILLER, B. E., TAL-SINGER, R., JOHNSTON, S. L., RAMSHEH, M. Y., BARER, M. R., BRIGHTLING, C. E. & BROWN, J. R. 2016. Lung microbiome dynamics in COPD exacerbations. *Eur Respir J,* 47**,** 1082-92.

WARREN, R., DE KOCK, M., ENGELKE, E., MYBURGH, R., GEY VAN PITTIUS, N., VICTOR, T. & VAN HELDEN, P. 2006. Safe Mycobacterium tuberculosis DNA extraction method that does not compromise integrity. *J Clin Microbiol,* 44**,** 254-6.

WATANABE, J. M., CHINCHINIAN, H., WEITZ, C. & MCIVANIE, S. K. 1965. Pneumocystis Carinii Pneumonia in a Family. *JAMA,* 193**,** 685-6.

WATANABE, W., THOMAS, M., CLARKE, R., KLIBANOV, A. M., LANGER, R., KATSTRA, J., FULLER, G. G., GRIEL, L. C., FIEGEL, J. & EDWARDS, D. 2007. Why inhaling salt water changes what we exhale. *J Colloid Interface Sci,* 307**,** 71-8.

WEISS, C. H., MOAZED, F., DIBARDINO, D., SWAROOP, M. & WUNDERINK, R. G. 2013. Bronchoalveolar lavage amylase is associated with risk factors for aspiration and predicts bacterial pneumonia. *Crit Care Med,* 41**,** 765-73.

WEISS, M. J., EDMONDSON, H. A. & WERTMAN, M. 1951. Elevated serum amylase associated with bronchogenic carcinoma; report of case. *Am J Clin Pathol,* 21**,** 1057-61.

WEISS, S., AMIR, A., HYDE, E. R., METCALF, J. L., SONG, S. J. & KNIGHT, R. 2014. Tracking down the sources of experimental contamination in microbiome studies. *Genome Biol,* 15**,** 564.

WELLER, P. F. & LEDER, K. 2018a. Toxocariasis: Visceral and ocular larva migrans. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

WELLER, P. F. & LEDER, K. 2018b. Trichinellosis. *In:* BARON, E. L. (ed.) *UpToDate.* Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on August 11, 2018.).

WELLS, W. F. 1934. ON AIR-BORNE INFECTION: STUDY II. DROPLETS AND DROPLET NUCLEI. *American Journal of Epidemiology,* 20**,** 611-618.

WELLS, W. F. 1948. On the mechanics of droplet nuclei infection; apparatus for the quantitative study of droplet nuclei infection of animals. *Am J Hyg,* 47**,** 1-10.

WELLS, W. F., RATCLIFFE, H. L. & GRUMB, C. 1948. On the mechanics of droplet nuclei infection; quantitative experimental air-borne tuberculosis in rabbits. *Am J Hyg,* 47**,** 11-28.

WENDT, S. L., GEORGE, K. L., PARKER, B. C., GRUFT, H. & FALKINHAM, J. O., 3RD 1980. Epidemiology of infection by nontuberculous Mycobacteria. III. Isolation of potentially pathogenic mycobacteria from aerosols. *Am Rev Respir Dis,* 122**,** 259-63.

WESTCOTT, S. L. & SCHLOSS, P. D. 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. *PeerJ,* 3**,** e1487.

WEYRICH, L. S., FARRER, A. G., EISENHOFER, R., ARRIOLA, L. A., YOUNG, J., SELWAY, C. A., HANDSLEY-DAVIS, M., ADLER, C., BREEN, J. & COOPER, A. 2019. Laboratory contamination over time during low-biomass sample analysis. *Mol Ecol Resour*.

WHITELOCK-JONES, L., CARSWELL, J. & RASMUSSEN, K. C. 1989. Listeria pneumonia. A case report. *S Afr Med J,* 75**,** 188-9.

WILFINGER, W. W., MACKEY, K. & CHOMCZYNSKI, P. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques,* 22**,** 474-6, 478-81.

WILLIAMS, C., HALDAR, P. & BARER, M. 2013. P33 Mask sampling in pulmonary tuberculosis. *Thorax,* 68**,** A89-A89.

WILLIAMS, C. M., ABDULWHHAB, M., BIRRING, S. S., DE KOCK, E., GARTON, N. J., STOLTZ, A., HALDAR, P. & BARER, M. R. 2018. Twenty-four hour face mask sampling in pulmonary tuberculosis reveals three distinct patterns of bacterial aerosol production dissociated from conventional markers of transmission risk. *bioRxiv*.

WILLIAMS, C. M., CHEAH, E. S., MALKIN, J., PATEL, H., OTU, J., MLAGA, K., SUTHERLAND, J. S., ANTONIO, M., PERERA, N., WOLTMANN, G., HALDAR, P., GARTON, N. J. & BARER, M. R. 2014. Face mask sampling for the detection of Mycobacterium tuberculosis in expelled aerosols. *PLoS One,* 9**,** e104921.

WILLIAMS, N. 2017. The MRC breathlessness scale. *Occup Med (Lond),* 67**,** 496-497.

WILLS, T. S., DEGRYSE, A., LAVINA, J. & SINNOTT, J. T. 2004. Blastoschizomyces capitatus pneumonia in an immunocompetent male. *South Med J,* 97**,** 702-4.

WILSON, J. W., LIMPER, A. H., GRYS, T. E., KARRE, T., WENGENACK, N. L. & BINNICKER, M. J. 2011. Pneumocystis jirovecii testing by real-time polymerase chain reaction and direct examination among immunocompetent and immunosuppressed patient groups and correlation to disease specificity. *Diagn Microbiol Infect Dis,* 69**,** 145-52.

WINSLOW, C. E. & ROBINSON, E. A. 1910. An Investigation of the Extent of the Bacterial Pollution of the Atmosphere by Mouth-Spray. *Am J Public Hygiene,* 20**,** 566-9.

WONG, K. K., FISTEK, M. & WATKINS, R. R. 2013. Community-acquired pneumonia caused by Yersinia enterocolitica in an immunocompetent patient. *J Med Microbiol,* 62**,** 650-1.

WOOD, B. R., KOMAROW, L., ZOLOPA, A. R., FINKELMAN, M. A., POWDERLY, W. G. & SAX, P. E. 2013. Test performance of blood beta-glucan for Pneumocystis jirovecii pneumonia in patients with AIDS and respiratory symptoms. *Aids,* 27**,** 967-72.

WOOD, R., MORROW, C., BARRY, C. E., 3RD, BRYDEN, W. A., CALL, C. J., HICKEY, A. J., RODES, C. E., SCRIBA, T. J., BLACKBURN, J., ISSAROW, C., MULDER, N., WOODWARD, J., MOOSA, A., SINGH, V., MIZRAHI, V. & WARNER, D. F. 2016. Real-Time Investigation of Tuberculosis Transmission: Developing the Respiratory Aerosol Sampling Chamber (RASC). *PLoS One,* 11**,** e0146658.

WOODWORTH, B. A., LATHERS, D., NEAL, J. G., SKINNER, M., RICHARDSON, M., YOUNG, M. R. & SCHLOSSER, R. J. 2006. Immunolocalization of surfactant protein A and D in sinonasal mucosa. *Am J Rhinol,* 20**,** 461-5.

WOOTTEN, C. T., LABADIE, R. F., CHEN, A. & LANE, K. F. 2006. Differential expression of surfactant protein A in the nasal mucosa of patients with allergy symptoms. *Arch Otolaryngol Head Neck Surg,* 132**,** 1001-7.

WU, J. Y., JIANG, X. T., JIANG, Y. X., LU, S. Y., ZOU, F. & ZHOU, H. W. 2010. Effects of polymerase, template dilution and cycle number on PCR based 16 S rRNA diversity analysis using the deep sequencing method. *BMC Microbiol,* 10**,** 255.

WU, Y., SHEN, F. & YAO, M. 2010. Use of gelatin filter and BioSampler in detecting airborne H5N1 nucleotides, bacteria and allergens. *Journal of Aerosol Science,* 41**,** 869-879.

WYDER, M. A., RASCH, E. M. & KANESHIRO, E. S. 1994. Assessment of Pneumocystis carinii DNA content. *J Eukaryot Microbiol,* 41**,** 120s.

WYLIE, K. M. 2017. The Virome of the Human Respiratory Tract. *Clin Chest Med,* 38**,** 11-19.

XIE, X., LI, Y., SUN, H. & LIU, L. 2009. Exhaled droplets due to talking and coughing. *J R Soc Interface,* 6 Suppl 6**,** S703-14.

XU, Z., SHEN, F., LI, X., WU, Y., CHEN, Q., JIE, X. & YAO, M. 2012. Molecular and Microscopic Analysis of Bacteria and Viruses in Exhaled Breath Collected Using a Simple Impaction and Condensing Method. *PLOS ONE,* 7**,** e41137.

YACOUB, A. T., VELEZ, A. P., KHWAJA, S. I., SANDIN, R. L. & GREENE, J. 2014. Streptomyces Pneumonia in an Immunocompromised Patient: A Case Report and a Review of Literature. *Infectious Diseases in Clinical Practice,* 22**,** e113-e115.

YANAGITANI, N., KAIRA, K., SUNAGA, N., NAITO, Y., KOIKE, Y., ISHIHARA, S., ISHIZUKA, T., SAITO, R. & MORI, M. 2007. Serum amylase is a sensitive tumor marker for amylase-producing small cell lung cancer? *Int J Clin Oncol,* 12, 231-3.

YANG, B., WANG, Y. & QIAN, P. Y. 2016. Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinformatics,* 17, 135.

YAO, M. & MAINELIS, G. 2006. Effect of physical and biological parameters on enumeration of bioaerosols by portable microbial impactors. *Journal of Aerosol Science,* 37, 1467-1483.

YAO, M., WU, Y., ZHEN, S. & MAINELIS, G. 2009a. A comparison of airborne and dust-borne allergens and toxins collected from home, office and outdoor environments both in New Haven, United States and Nanjing, China. *Aerobiologia,* 25, 183-192.

YAO, M., ZHU, T., LI, K., DONG, S., WU, Y., QIU, X., JIANG, B., CHEN, L. & ZHEN, S. 2009b. Onsite infectious agents and toxins monitoring in 12 May Sichuan earthquake affected areas. *J Environ Monit,* 11, 1993-2001.

YAZAKI, H., GOTO, N., UCHIDA, K., KOBAYASHI, T., GATANAGA, H. & OKA, S. 2009. Outbreak of Pneumocystis jiroveci pneumonia in renal transplant recipients: P. jiroveci is contagious to the susceptible host. *Transplantation,* 88, 380-5.

YEGHEN, T., FENELON, L., CAMPBELL, C. K., WARNOCK, D. W., HOFFBRAND, A. V., PRENTICE, H. G. & KIBBLER, C. C. 1996. Chaetomium pneumonia in patient with acute myeloid leukaemia. *J Clin Pathol,* 49, 184-6.

YOKOUCHI, H., YAMAZAKI, K., ASAHINA, H., SHIGEMURA, M., MORIYAMA, T., TAKAOKA, K., MORIYA, J., ITOH, T., KINOSHITA, I., DOSAKA-AKITA, H., TSUTSUMI, Y. & NISHIMURA, M. 2006. Establishment and characterization of amylase-producing lung adenocarcinoma cell line, IMEC-2. *Anticancer Res,* 26, 2821-7.

YONEDA, K. & WALZER, P. D. 1980. Interaction of Pneumocystis carinii with host lungs: an ultrastructural study. *Infect Immun,* 29, 692-703.

YOSHIDA, Y., MATSUMOTO, Y., YAMADA, M., OKABAYASHI, K., YOSHIKAWA, H. & NAKAZAWA, M. 1984. Pneumocystis carinii: electron microscopic investigation on the interaction of trophozoite and alveolar lining cell. *Zentralbl Bakteriol Mikrobiol Hyg A,* 256, 390-9.

YOSHIOKA, H., MORI, Y., TSUKIKAWA, S. & KUBOTA, S. 1998. Thermoreversible gelation on cooling and on heating of an aqueous gelatin–poly(N-isopropylacrylamide) conjugate. *Polymers for Advanced Technologies,* 9, 155-158.

YPMA-WONG, M. F., FONZI, W. A. & SYPHERD, P. S. 1992. Fungus-specific translation elongation factor 3 gene present in Pneumocystis carinii. *Infect Immun,* 60, 4140-5.

YU, G., GAIL, M. H., CONSONNI, D., CARUGNO, M., HUMPHRYS, M., PESATORI, A. C., CAPORASO, N. E., GOEDERT, J. J., RAVEL, J. & LANDI, M. T. 2016. Characterizing human lung tissue microbiota and its relationship to epidemiological and clinical features. *Genome Biol,* 17, 163.

ZAKHARKINA, T., HEINZEL, E., KOCZULLA, R. A., GREULICH, T., RENTZ, K., PAULING, J. K., BAUMBACH, J., HERRMANN, M., GRUNEWALD, C., DIENEMANN, H., VON MULLER, L. & BALS, R. 2013. Analysis of the airway microbiota of healthy individuals and patients with chronic obstructive pulmonary disease by T-RFLP and clone sequencing. *PLoS One,* 8, e68302.

ZAKHARKINA, T., KOCZULLA, A. R., MARDANOVA, O., HATTESOHL, A. & BALS, R. 2011. Detection of microorganisms in exhaled breath condensate during acute exacerbations of COPD. *Respirology,* 16, 932-8.

ZHANG, J., ZHANG, L., PAN, S., GU, B., ZHEN, Y., YAN, J. & ZHOU, Y. 2013. Amylase: sensitive tumor marker for amylase-producing lung adenocarcinoma. *J Thorac Dis,* 5, E167-9.

ZHANG, Z. Q. & ISHAQUE, M. 1997. Evaluation of methods for isolation of DNA from slowly and rapidly growing mycobacteria. *Int J Lepr Other Mycobact Dis,* 65, 469-76.

ZHAO, Y., AARNINK, A. J., WANG, W., FABRI, T., GROOT KOERKAMP, P. W. & DE JONG, M. C. 2014a. Airborne virus sampling: Efficiencies of samplers and their detection limits for infectious bursal disease virus (IBDV). *Ann Agric Environ Med,* 21, 464-71.

ZHAO, Y., AARNINK, A. J. A., DOORNENBAL, P., HUYNH, T. T. T., KOERKAMP, P. W. G. G., LANDMAN, W. J. M. & DE JONG, M. C. M. 2011. Investigation of the Efficiencies of Bioaerosol Samplers for Collecting Aerosolized Bacteria Using a Fluorescent Tracer. II: Sampling Efficiency and Half-Life Time. *Aerosol Science and Technology,* 45, 432-442.

ZHENG, Y., CHEN, H., YAO, M. & LI, X. 2018. Bacterial pathogens were detected from human exhaled breath using a novel protocol. *Journal of Aerosol Science,* 117, 224-234.

ZUO, Z., KUEHN, T. H., BEKELE, A. Z., MOR, S. K., VERMA, H., GOYAL, S. M., RAYNOR, P. C. & PUI, D. Y. 2014. Survival of airborne MS2 bacteriophage generated from human saliva, artificial saliva, and cell culture medium. *Appl Environ Microbiol,* 80, 2796-803.

ZWADYK, P., JR., DOWN, J. A., MYERS, N. & DEY, M. S. 1994. Rendering of mycobacteria safe for molecular diagnostic studies and development of a lysis method for strand displacement amplification and PCR. *J Clin Microbiol,* 32**,** 2140-6.