# Interaction of phages with outer membrane vesicles:

# Immunological aspect

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By

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# Statement of Originality

This accompanying thesis submitted for the degree of PhD entitled **"Interaction of phages with outer membrane vesicles: Immunological aspect"** is based on work conducted by the author in the Department of respiratory science at University of Leicester during the period between January 2017 and December 2019.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of this work has been submitted for another degree in this or any other University.

Signed……..... Date……

**Abstract**

Bacteriophages (phages) are viruses that infect bacteria. Although it is clear that phages may help the host immune system to kill bacterial pathogens, many details of bacteria-phage-host immune interactions remain poorly understood. Bacterial outer membrane vesicles (OMVs) are one of the virulence factors of Gram-negative organisms. OMVs are spherical membrane-enclosed microparticles produced during bacterial growth. During bacterial infections, OMVs are recognized by the host immune system and participate in the elicitation of immune responses. OMVs have been found to interact with bacteriophages in a host-specific manner. The effects of such interactions on the biological functions of OMVs remain largely unknown. In this project, research into the immunological consequences of bacteriophages interacting with OMVs was assessed by investigating whether OMV-phage interaction affects the inflammatory response elicited by OMVs alone.

An important methodological development from this project was to optimise a protocol to isolate and purify OMVs from *E. coli*. OMVs were isolated in a highly pure form. They were interacted with purified *E. coli* specific phages. OMVs, phages and OMV-phage mixtures were then interacted with macrophage cells, and it was found that the OMV-phage interaction led to a reduction of the pro-inflammatory response elicited by OMVs alone. This suggests that phages may impact bacterial-host immune system interactions not only by killing the pathogens, but also by altering host responses elicited by the conserved bacterial virulence factor, OMVs.

Furthermore, preliminary results revealed that interactions of a bacterial lysogen with macrophages or whole blood samples resulted in the induction of prophages from lysogenized bacteria. This may suggest that during the interaction of bacteria with the host immune system, some bacteria may be killed not by the immune assault per se, but by the induced prophages.

Three bacteriophages infecting *K. pneumoniae* were isolated from sewage samples by the traditional method of phage isolation. Genomic and biological characterisations were performed on two of these phages. These phages were used in this current research and may be used in the future to investigate their impact on the immunological responses elicited by *Klebsiella* OMVs, once a methodology to isolate sufficient quantities of OMVs from this pathogen is developed.

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**Abbreviation**

| | |
|---|---|
| **%** | Percent |
| **µl** | Micro-litre |
| **µm** | Micro-metre |
| **°C** | Degrees centigrade |
| **bp** | Base pair(s) |
| **CFU** | Colony forming unit |
| **CIM®** | Convective Interaction Media |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxynucleotide |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EOMV** | Explosive outer membrane vesicles |
| **EU** | Endotoxin Unit |
| **FPLC** | Fast Protein Liquid Chromatography |
| **H2O** | Water |
| **His-tag** | Histidine Tag |
| **ICTV** | International Committee on Taxonomy of Viruses |
| **kDa** | Kilo Daltons |
| **l** | Litre |
| **LA** | Luria agar |
| **LAL** | Limulus Amebocyte Lysate |
| **LB** | Luria broth |
| **log** | Logarithmic |
| **LPS** | Lipopolysaccharides |
| **M** | Molar |
| **MDR** | Multi-drug resistant |
| **ml** | Millilitre |
| **mM** | Milli-Molar |
| **NaCl** | Sodium chloride |
| **NTA** | Nanoparticle Tracking Analysis |
| **OD** | Optical density |
| **OIMV** | Outer-inner membrane vesicles |
| **OMV** | Outer Membrane Vesicle |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PFU** | Plaque Forming Unit |
| **RCF** | Relative Centrifugal Force |
| **Rpm** | Round per minute |
| **SDS-PAGE** | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| **SEM** | Standard Error of the Mean |
| **SM** | Sodium chloride - Magnesium sulphate |
| **TAE** | Tris acetic acid EDTA |
| **TBS** | Tris-Buffered Saline |
| **TEM** | Transmission Electron Microscopy |
| **TEMED** | Tetramethylethylenediamine |
| **v/v** | Volume per volume |
| **w/v** | Weight per volume |

Table of Contents

Statement of Originality ..... i

Abstract ..... ii

Acknowledgements ..... iii

Scientific contribution ..... v

Abbreviation ..... vi

List of figures ..... xii

List of tables ..... xiv

1 . Introduction ..... 1

1.1 Outer membrane vesicles (OMVs) ..... 1

1.1.1 Discovery of OMVs ..... 1

1.1.2 OMVs structure and biogenesis ..... 2

1.1.3 OMVs function ..... 5

1.1.4 Types of OMVs ..... 7

1.1.5 Role of OMVs in biofilm formation ..... 8

1.1.6 OMVs and the immune system ..... 9

1.1.7 Anti-inflammatory role of OMVs ..... 10

1.1.8 OMVs in vaccination ..... 10

1.1.9 Bacteria of interest in this project ..... 11

1.1.10 *Klebsiella pneumoniae (K. pneumoniae)* ..... 11

1.1.11 *Escherichia coli (E. coli)* ..... 12

1.2 Bacteriophages ..... 14

1.2.1 Definition of bacteriophages ..... 14

1.2.2 Bacteriophage history ..... 14

1.2.3 Bacteriophage classification ..... 14

1.2.4 Bacteriophage life cycles ..... 19
1.2.5 Applications of phages ..... 22
1.2.6 Phages and immunomodulation ..... 26
1.3 Interaction of phages and OMVs ..... 27
1.4 Focus of this project ..... 28
1.5 Aims and objectives ..... 28
1.6 Project story schematic ..... 30
2 . General methods ..... 31
2.1 Environmental sample collection ..... 32
2.2 Bacterial media used in this study ..... 32
2.2.1 Luria Bertani Broth (LB) ..... 32
2.2.2 Lauria Agar (LA) ..... 32
2.2.3 Semi-solid Agar ..... 32
2.3 Buffers ..... 32
2.3.1 Phosphate Buffered Saline (PBS) ..... 32
2.3.2 SM buffer ..... 33
2.3.3 50X Tris-acetic acid EDTA (TAE) ..... 33
2.3.4 10X SDS buffer ..... 33
2.3.5 10X Western blot buffer (transfer buffer) ..... 33
2.3.6 Colloidal Coomassie stain ..... 33
2.4 Bacterial strains and growth conditions ..... 33
2.5 Bacteriophage used in this project ..... 34
2.6 Plaque assay ..... 35
2.7 Spot test ..... 36
2.8 Phage Purification ..... 37
2.9 Phage propagation ..... 37

2.10 Transmission electron microscopy (TEM) ..... 38
2.11 Nanoparticle Tracking Analysis (NTA) ..... 38
3 . Isolation and characterizations of *K. pneumoniae* phages. ..... 39
3.1 Introduction: ..... 40
3.2 Aim and objective: ..... 41
3.3 Methods ..... 42
3.3.1 Transmission electron microscope (TEM) ..... 42
3.3.2 Phage DNA extraction ..... 42
3.3.3 Phage whole-genome sequencing ..... 42
3.3.4 Bioinformatics Analysis ..... 43
3.3.5 One-step growth curve ..... 43
3.3.6 Phage thermal and pH stability ..... 44
3.3.7 Determination of the phage host range ..... 44
3.4 Results ..... 45
3.4.1 Phage Kp2811 and phage Kp3151 characterization ..... 45
3.5 Discussion ..... 57
4 . Extraction and purification of OMVs from *K. pneumoniae* and *E. coli* ..... 60
4.1 Introduction: ..... 61
4.2 Methods ..... 63
4.2.1 Bacterial strains identification confirmation by 16S RNA PCR ..... 63
4.2.2 Gel electrophoresis ..... 63
4.2.3 Outer membrane vesicles (OMVs) extraction and purification from *K. pneumoniae*: ..... 64
4.3 Results: ..... 67
4.3.1 *K. pneumoniae* strain identification confirmation ..... 67
4.3.2 Outer membrane extraction and purification ..... 68

4.3.3 Assessment of the OMVs isolation from *K. pneumoniae* strains ..... 69

4.3.4 Optimized protocol for extraction of OMVs from *E. coli* DH5α ..... 71

4.3.5 Assessment of the OMVs isolation from *E. coli* strain..... 73

4.4 Discussion: ..... 80

4.5 Conclusion: ..... 82

5 . Interaction of phages with bacterial outer membrane vesicles: immunological aspects. ..... 83

5.1 Introduction ..... 84

5.2 Aim and objectives..... 86

5.2.1 Objectives ..... 86

5.3 Methods..... 88

5.3.1 Endotoxin removal kit ..... 88

5.3.2 Endotoxin removal..... 88

5.3.3 Phage purification using anion-exchange chromatography and CIM® monoliths: ..... 89

5.3.4 Phage and OMVs interaction and assessment ..... 90

5.3.5 Cell line..... 90

5.3.6 Thawing cells..... 90

5.3.7 Cell line subculture (passage) ..... 91

5.3.8 Cell counting..... 91

5.3.9 Freezing J774 cells..... 91

5.3.10 Tissue culture plate layout and the interaction of J774 cell lines with the conditions..... 92

5.3.11 Murine TNF alpha ELISA ..... 93

5.4 Results..... 95

5.4.1 Phage purification using CIM® monolithic column ..... 95

5.4.2 Phage titration after purification ..... 96

5.4.3 Endotoxin level of purified and un-purified phage lysate ..... 96

5.4.4 Visualization of OMVs and OMV-phage complex ..... 97

5.4.5 Investigation of the effect of the interaction of OMVs with phage ..... 101

5.5 Discussion ..... 103

6 . Induction of prophages upon bacterial interactions with macrophages and preliminary investigation of phages in whole blood. ..... 106

6.1 Introduction ..... 107

6.2 Aim and objectives ..... 109

6.3 Methods ..... 110

6.3.1 Bacterial strain preparation ..... 110

6.3.2 Prophage induction ..... 110

6.3.3 Spontaneous induction ..... 110

6.3.4 Mitomycin C induction ..... 111

6.3.5 Lambda phage induction by Macrophages ..... 111

6.3.6 Ethical Statement ..... 111

6.3.7 Spot test ..... 111

6.4 Result ..... 113

6.4.1 Comparison of induction methods of phage Lambda from *E. coli* NM522 n4 113

6.4.2 Survival and recovery of Lambda phage from whole blood ..... 114

6.5 Discussion ..... 116

7 . General discussion ..... 118

General discussion ..... 119

7.1 Future work ..... 124

Supplementary ..... 126

Bibliography ..... 128

**List of figures**

Figure 1.1: The formation of OMVs..... 2

Figure 1.2: Route of OMVs formation in Gram-Negative bacteria..... 8

Figure 1.3: Highlights of the main Caudovirales families..... 16

Figure 1.4: different families of Pleomorphic group. ..... 17

Figure 1.5: Different families of polyhedral group..... 18

Figure 1.6: Different family members of *Filamentous* phages..... 19

Figure 1.7*: Bacteriophage life cycles* ..... 22

Figure 2.1: A diagram illustrating the plaque assay. ..... 36

Figure 2.2: Diagram illustrates the spot test. ..... 37

Figure 3.1. Linear annotated genomic map of phage Kp2811..... 46

Figure 3.2: Circular annotated genomic map of bacteriophage Kp3153..... 47

Figure 3.3: Transmission electron microscopy analysis pf the purified and propagated phages. ..... 48

Figure 3.4: Proteomic tree showing the most related phages to phage Kp2811 and phage Kp3153..... 50

Figure 3.5. Alignment of phage Kp2811 with other *K. pneumoniae* phages. ..... 51

Figure 3.6: Alignment of Kp3153 phage with other *K. pneumoniae* phages ..... 52

Figure 3.7: One-step growth curve of phage Kp2811 and phage Kp3153. ..... 53

Figure 3.8: Phage Kp2811L and phage Kp3153 thermal and PH stabilities. ..... 56

Figure 4.1. Agarose gel electrophoresis analysis of the 16S rRNA genes amplified from six bacterial strains: ..... 67

Figure 4.2: A schematic diagram illustrates the steps were followed to verify the quality of the OMV lysate..... 68

Figure 4.3: Protein profiles of OMVs extracted from different *K. pneumoniae* strains: 69

Figure 4.4: A diagram illustrating the optimised protocol for the extraction and purification of OMVs..... 73

Figure 4.5: Confirmation of the presence of outer membrane protein GroE at the OMVs extracted from *E. coli* DH5α. ..... 74

Figure 4.6: OMVs protein profile in mass spectrometry. ..... 76

Figure 4.7: Representative NanoSight size distribution and concentration of E. coli DH5α OMVs. ..... 77

Figure 4.8: TEM assessments of *E. coli* OMVs using the centrifugation-based method. ..... 78

Figure 4.9: TEM assessments of crude *E. coli* OMVs. ..... 79

Figure 4.10: TEM assessment of purified *E. coli* OMVs. ..... 79

Figure 5.1: A diagram illustrating the conditions that are involved in this study. ..... 87

Figure 5.2. Endotoxin concentration standard curve. ..... 89

Figure 5.3: Tissue culture plate layout. ..... 92

Figure 5.4: Murine TNF α standard curve. ..... 94

Figure 5.5: Linear gradient purification of EL phage. ..... 95

Figure 5.6: Comparison of endotoxin level in phage lysate before and after purification. ..... 97

Figure 5.7: TEM images showing *E. coli* OMVs before interaction with EL phages. ..... 98

Figure 5.8: TEM analysis of the interaction between phage EL and OMVs of *E. coli*. ..... 99

Figure 5.9. TEM analysis of the *E. coli* phage EL interacted with OMVs from *P. aeruginosa*. ..... 100

Figure 5.10: Quantifying the amount of TNFα induced from macrophage cells J774 upon interacting with either phages, OMVs or phage and OMVs complexes. ..... 102

Figure 6.1: Temperate phage lifecycle. ..... 108

Figure 6.2: Comparisons of Lambda phage induction from *E. coli* NM522 n4. ..... 113

Figure 6.3: Induction and survival of Lambda phage from whole blood sample. ..... 115

**List of tables**

Table 2.1: Bacterial strains used in this study. ..... 34

Table 2.2: List of the bacteriophage used in this study. The table illustrates the bacterial host and the source of each phage. ..... 35

Table 3.1: A summary of the host-range of phage Kp2811 against and lab strains of *Klebsiella Pneumoniae* and *E.coli.* ..... 54

Table 4.1. Forward and reverse primers used for the 16S rRNA gene amplification .... 63

Table 5.1: Titration of phage EL after purification via anion-exchange chromatography. ..... 96

# Chapter one

## 1. Introduction

## 1.1 Outer membrane vesicles (OMVs)

### 1.1.1 Discovery of OMVs

The production of outer membrane vesicles (OMVs) is one of the virulence-related features of Gram-negative organisms. In 1959, before the first discovery of OMVs, it was noticed that a cell-free filtrate of *Vibrio cholerae* had the ability to elicit rabbits' immune response (De 1959). A few years later in 1965, it was suggested that vesicles found in the bacterial culture were a result of the bacterial cell lysis after centrifugation. An *E. coli* culture was grown under a lysine limiting growth condition; lipopolysaccharide particles were detected in the supernatant using electron microscopy, and were assumed to be traces of bacterial lysis (Bishop and Work 1965). A couple of years later, under normal conditions, *V. cholerae* was found to produce spherical membrane vesicles that bulged away from the membrane (Chatterjee and Das 1967).

Subsequently, it was discovered that different species of Gram-negative organisms such as *Bacteroides melaninogenicus* (Shah *et al.* 1976), *Pseudomonas* fragi (Dutson *et al.* 1971), *Haemophilus influenzae* (Deich and Hoyer 1982), *Brucella melitensis* (Gamazo and Moriyon 1987), *Campylobacter jejuni* (Elmi *et al.* 2012) and *Salmonella* species (Elhenawy *et al.* 2016) produced similar vesicles, ranging in size from 20 to 250 nm (Kulp and Kuehn 2010). In addition, membrane vesicles were detected in Gram-positive bacteria, *Bacillus subtilis* and *Bacillus cereus*; the first was reported in 1990 (Dorward and Garon 1990).

Following this, several Gram-positive organisms releasing MVs were identified, e.g., *Bacillus anthracis* and *Staphylococcus aureus* (Lee *et al.* 2009; Rivera *et al.* 2010; Klimentová and Stulík 2015). However, the way in which MVs are produced and released from the surface of Gram-positive bacteria remains unclear (MacDonald and Kuehn 2012a; Schwechheimer and Kuehn 2015), due to difference in structure between the Gram-positive and Gram-negative cell envelope, such as the latter's lack of OM (Klimentová and Stulík 2015). Thus, it is not surprising that the biogenesis of OMVs has been mainly investigated in Gram-negative bacteria (MacDonald and Kuehn 2012a).

### 1.1.2 **OMVs structure and biogenesis**

OMVs are spherical, ranging between 20-250 nm in diameter, and are naturally produced during bacterial growth (Hoekstra *et al.* 1976; Schwechheimer and Kuehn 2015). As shown in (Figure 1.1), OMVs form as small lumps on the outer membrane of the bacterial cell, eventually pinch off from the membrane and are subsequently released away. OMVs can scatter around the bacterial cells and can define certain biological functions (Kulp and Kuehn 2010).

OMVs are produced in all phases of bacterial growth, although their amount and composition may be dependent on the growth conditions (Kulkarni and Jagannadham 2014). However, OMVs are not products of cell lysis or cell death (Kuehn and Kesty 2005). They have been reported to be produced under various *in vitro* conditions, by bacteria growing in liquid media, on solid media and in biofilms (Schooling and Beveridge 2006; Klimentová and Stulík 2015). Importantly, the production of OMVs has been reported *in vivo* during intracellular infections (Namork and Brandtzaeg 2002); e.g., OMVs were detected in the cerebrospinal fluid of an infant with an *N. meningitides* infection (Stephens *et al.* 1982).

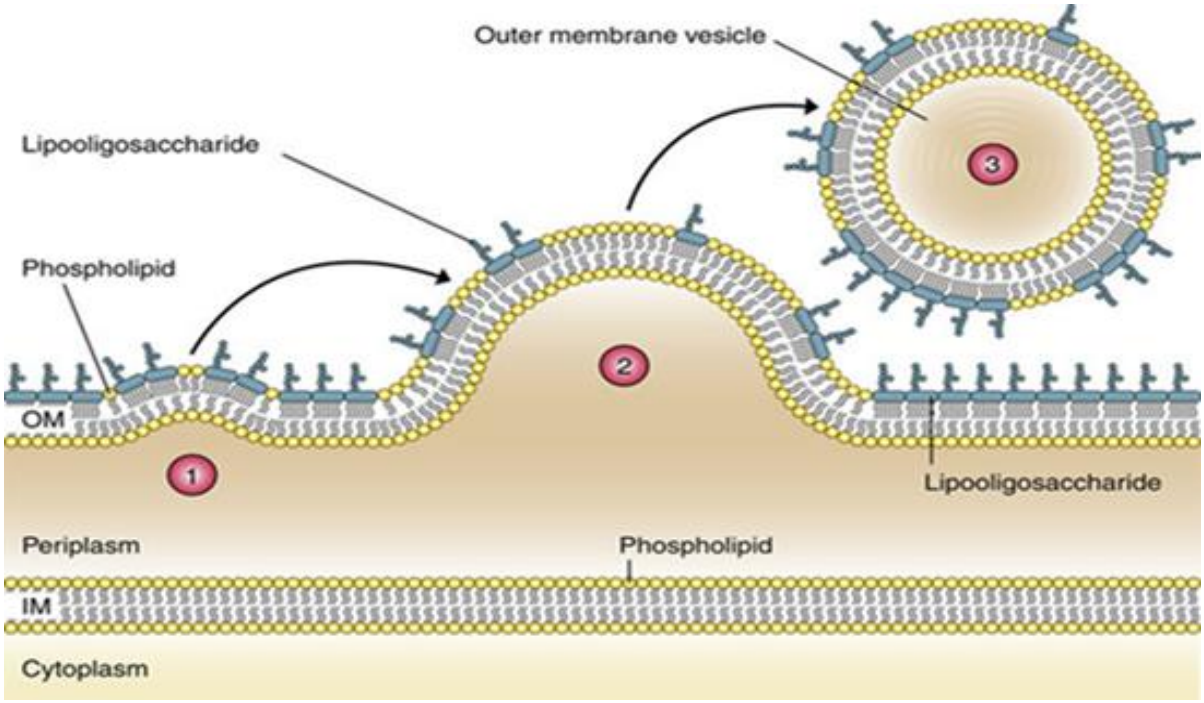



**Figure 1.1: The formation of OMVs**
A small lump on the OM of the bacterial cell is formed which increases in size and eventually bulges away from the bacterial cell. Adapted from Roier *et al*. (2016).

In order to understand the structure of OMVs, it is vital to understand where they originate from. Gram-negative bacteria possess two membranes, the cytoplasmic membrane and the outer membrane. In between these two membranes is the periplasmic layer, and this periplasmic space contains the peptidoglycan layer (Silhavy *et al.* 2010; Seltmann and Holst 2013). The outer membrane from the inside of the bacterium is composed of phospholipids, and lipopolysaccharide is present on the outside. However, the cytoplasmic membrane is composed of two layers of phospholipids that act as an electrochemical barrier (Silhavy *et al.* 2010). The periplasmic space lacks nucleotides as a source of energy; at the same time, it is considered as an oxidative environment that allows protein folding (Kojer and Riemer 2014). The bacterium cell maintains its shape due to the presence of the peptidoglycan layer that exists in the periplasmic space. In addition to that, the peptidoglycan membrane imparts extra protection against osmotic changes and sheer stress (Kojer and Riemer 2014). OMVs are produced due to the separation of the outer membrane from the peptidoglycan layer in some areas, followed by the formation of a small lump which does not compromise the bacterial cell integrity. Vesiculation levels can be affected by different stressors and several factors such as temperature, nutrient, quorum sensing, oxidation and by antibiotics that target the cell envelope (McBroom and Kuehn 2007).

In many cases, OMVs contain periplasmic enzymes and virulence factors (Beveridge 1999; Mashburn-Warren *et al.* 2008). For instance, *P. aeruginosa* has several virulence factors which are important for their pathogenesis; some of them have been detected within *P. aeruginosa* OMVs, e.g., phospholipase C, proteases, alkaline phosphatases, and hemolysins (Kadurugamuwa and Beveridge 1995) In addition, OMVs may contain nucleic acids (Mashburn-Warren *et al.* 2008; Koeppen *et al.* 2016).

#### 1.1.2.1 **Proteomic studies of OMVs**

The OMVs composition reflects the bacterial outer membrane and periplasmic components of the bacteria from which they originated. Many proteomics studies using classical and advanced proteomics techniques such as Western blotting and mass spectrophotometry have been used to understand the protein contents of OMVs. It has been found that the protein contents of OMVs are different from that of other subcellular fragments (Wensink and Witholt 1981; Beveridge 1999; Choi *et al.* 2011; Jan 2017). It

has been shown that the protein constituents of OMVs are sourced from the outer membrane and periplasmic space of the bacterial cell. Nevertheless, other protein components of the OMVs were also found to be similar to some proteins found in the cytosolic and inner membrane of the cell. Proteins that exist in OMVs are from different functional groups, such as OM structural proteins, ion exchange channels, transporter proteins, stress response proteins and periplasmic and cytoplasmic enzymes such as proteases, peptidases, nucleases and β lactamases. (Lee *et al.* 2007; Lee *et al.* 2008; Lee *et al.* 2012; Schaar *et al.* 2012; Schertzer and Whiteley 2012; Schaar *et al.* 2013; Aguilera *et al.* 2014).

#### 1.1.2.2 **Phospholipids of OMVs**

Other studies were conducted on the phospholipid contents and the similarity between OMVs and OM of the parent cell. Earlier studies showed that both OMVs and OM have a similar phospholipid profile (Hoekstra *et al.* 1976). A few years later, it was found that some of the OM phospholipid contents were found in the OMVs. However, some other lipids were present in the OMVs but not in the parent cells OM. Moreover, it was found that the quantity of OMVs phospholipid is far higher than the amount of the phospholipids in the OM. From that, it was concluded that the OMVs' phospholipid content and fatty chains can be characterised differently to that found in the OM (Tashiro *et al.* 2011). This may lead to an increased rigidity of the OMVs, and leave the OM with higher fluid contents that would allow optimum growth conditions for the parent cell (Tashiro *et al.* 2012).

#### 1.1.2.3 **Lipopolysaccharides (LPS) of OMVs**

One of the main macromolecule structure element and the most abundant component of the bacterial OM are the lipopolysaccharides (LPS), also known as endotoxins (Roper and Kirkpatrick 2010). LPS are located on the cell surface and constitutes about three quarters of the surface components (Rezania *et al.* 2011). They mediate the bacterial cell to interact with its surrounding environments, acting as a main receptor that facilitates the attachment to various host cells. The main three components of LPS are: lipid A, oligosaccharides and O-antigens. Both Lipid A and oligosaccharides are highly conserved in most Gram-negative bacteria (Sperandeo *et al.* 2016). Lipid A is anchored into the membrane and plays a role in the endotoxin activity, while the oligosaccharides are

assembled on Lipid A. The O antigen carbohydrate varies between species and is used for bacterial serotyping (Erridge *et al.* 2002). Furthermore, while it is not essential for bacterial viability, it plays an important role in virulence and host specificity. Any minor changes in the sugar type or arrangement can lead to a major change in virulence (Roper and Kirkpatrick 2010).

LPS are one of the main components of OMVs (Work *et al.* 1966), but its profile in OMVs may differ from that of the parent cell profile (Chowdhury and Jagannadham 2013).

### 1.1.3 **OMVs function**

OMVs act as transporters for molecules and contribute to bacterial virulence and survival *in vivo* (Kulp and Kuehn 2010). Upon their release into the environment, OMVs disseminate bacterial products and act as a secretion and delivery system. Unlike other bacterial secretion systems, OMVs can facilitate the release of different bacterial lipids, bacterial membrane proteins and other insoluble molecules. Soluble molecules present in OMVs are either surrounded by, or even complexed with, other insoluble molecules (Grenier and Mayrand 1987; Kesty and Kuehn 2004; Inagaki *et al.* 2006). Some proteins secreted by the bacterial cells need to be delivered efficiently to their targets, meaning that they need to be delivered at high concentrations and in close proximity to their targets. OMVs as delivery vehicles help bacteria to deliver high concentrations of proteins to targets distant from the bacterium itself, but also to nearby targets to assure efficient functionality for secreted molecules. Moreover, OMVs are able to deliver to specific targeted distal sites by binding specifically to ligands or receptors present on the target sites. Furthermore, OMVs can deliver their content either by self-lysis, allowing for their contents to diffuse to the target, or by connecting with the recipient membrane, allowing delivery either by proximal lysis, internalization or fusion (Kadurugamuwa and Beveridge 1996). In a study conducted by the Beveridge lab, OMVs derived from either *S. flexneri* or *P. aeruginosa* were incubated with intact *S. typhi* or *E. coli* cells; this resulted in a significant fusion and the incorporation of the OMVs with the intact *S. typhi* or *E. coli*. Each recipient bacterium acquired surface antigens of *S. flexneri* or *P. aeruginosa* when mixed with the OMVs. This fusion was confirmed with the immunogold labelling of the fused OMVs and subsequent visualisation via electron microscopy. Furthermore, the LPS antigens of the OMVs integrated with the bacterial

cells were analysed via ELISA and Western blot assays. The newly acquired antigens that were a result of the fusion were found to be stable and were able to withstand several freeze and thaw cycles (Kadurugamuwa and Beveridge 1999). In a related approach, the autolysin enzymes activity of OMVs, which were derived from *P. aeruginosa*, were investigated on other bacteria. It was found that OMVs were able to fuse into the outer membrane of other Gram-negative bacteria, resulting in the hydrolysis of several glycyl peptides present on the recipient bacterium (Kadurugamuwa and Beveridge 1996). It can be concluded that OMVs can fuse with bacterial outer membranes in order to deliver contents directly into the target cells (Kulp and Kuehn 2010). To sum up, by having all these characteristics, OMVs can be considered as effective and efficient secretion and delivery molecules (Kulp and Kuehn 2010).

OMVs participate in bacterial survival, especially under environmental stress when the integrity of the bacterial envelope is jeopardised. Moreover, OMV production is increased when the cell is faced with protein aggregates, or other potential surface-damaging agents. The OMVs are released by the cell in order to act as decoys, neutralise any damaging agents and provide relief from the stressors. OMVs can be induced by OM targeting antibacterial agents, the OMVs can bind to them, leading to their inactivation. OMVs contribute to biofilm formation and to the antibacterial resistance mechanism of such bacterial communities. (Thompson *et al.* 1985; Ciofu *et al.* 2000; McBroom and Kuehn 2007). It is believed that OMVs act as a short term solution to antibacterial stressors by allowing the bacteria to survive the first onset of the attack, and provide time to find more long-term solutions to counter these lethal antibiotic attacks (Kulp and Kuehn 2010).

OMVs can also participate in parent cell nutrient acquisition, additionally they can act as cargo for degradative enzymes and receptors. OMVs can help to release amino acids that are crucial to bacterial growth (Bauman and Kuehn 2006). In addition, OMVs can contribute to bacterial iron acquisition. An example of this is the attachment of OMVs with PQS, which is a hydrophilic molecule associated with *P. aeruginosa.* PQS has a role leading iron to the PQS-$Fe^{++}$-OMVs molecule that would be absorbed into OM by fusion. After absorption into the cell, the OMVs will release the PQS-bound iron into the cell, allowing for iron uptake (Dubern and Diggle 2008).

### 1.1.4 **Types of OMVs**

Depending on the manner in which the vesicles are formed, OMVs can be different in their composition and structure, and can therefore be categorized differently (Toyofuku *et al.* 2019). It has been suggested that OMVs can be formed by two main routes, as shown in Figure 1.2. The first is the formation of the classical OMVs via the blebbing of the cell membrane. The second route occurs during endolysin-triggered bacterial cell lysis, resulting in the formation of outer-inner membrane vesicles (OIMVs) and explosive outer membrane vesicles (EOMVs) (Toyofuku *et al.* 2019).

The classical OMVs are spherical vesicles derived from the outer membrane of the parent Gram-negative cell (Orench-Rivera and Kuehn 2016). They are produced as a result of outer membrane blebbing, and thus, are composed of LPS in the outer leaflet, phospholipid in the inner leaflet and enriched with outer membrane proteins (Schwechheimer and Kuehn 2015).

The other two types of vesicles are formed due to the activity of endolysins, which are mainly derived from phages and can degrade bacterial peptidoglycan (Turnbull *et al.* 2016). As a result of this degradation, vesicles that are formed contain cytoplasmic contents such as DNA, protein, endolysin residues and phage particles. Vesicles that contain DNA in the double-bilayers are referred to as OIMVs, while vesicles that contain DNA with a single-bilayer are referred to as EOMVs (Pï *et al.* 2013) (Toyofuku *et al.* 2019).

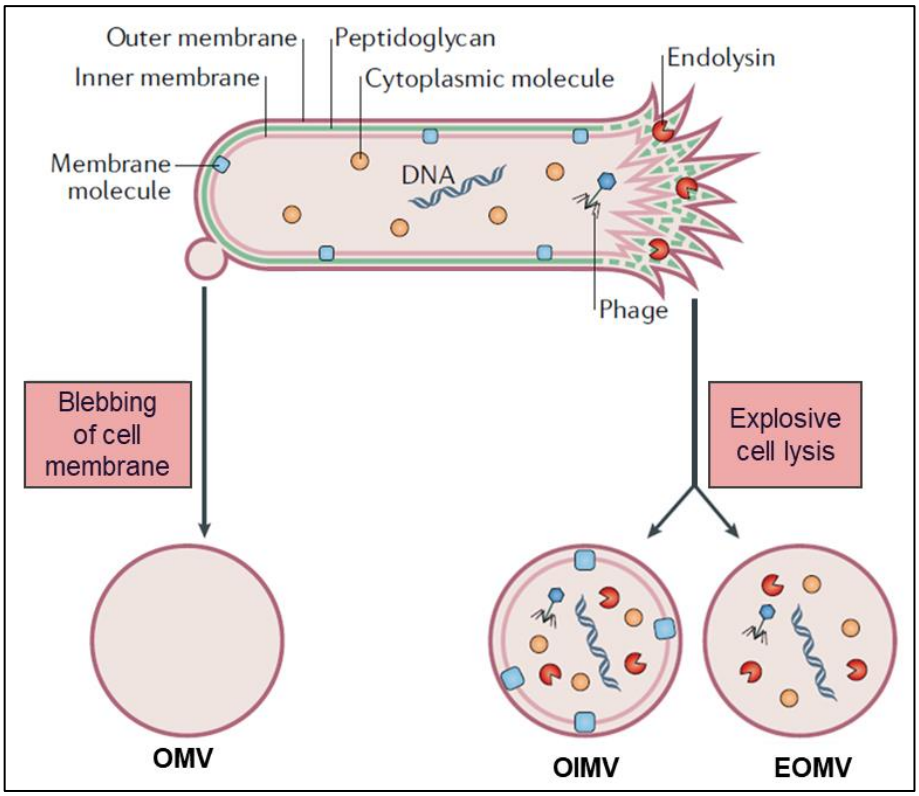



**Figure 1.2. Route of OMV formation in Gram-negative bacteria.**

This figure shows the different pathways for the formation of membrane vesicles. The classical OMVs are formed by the blebbing of the cell membrane. The other types of vesicles are formed as a result of endolysin activity, these being the OIMVs (double-bilayer) and EOMVs (single bilayer). This figure is adopted from (Toyofuku *et al.* 2019) with licenced permission from Springer Nature.

### 1.1.5 **Role of OMVs in biofilm formation**

It has been found that OMVs can account for more than half of the LPS present in a *P. aeruginosa* biofilm, indicating that OMVs may have a significant role in biofilm formation. These biofilm-associated OMVs, have been categorised based on density into 2 groups. First are the high-density OMVs that are larger than planktonic culture OMVs. Second are the low-density OMVs which are similar in size to OMVs found in planktonic cultures, but with more LPS and less protein contents (Schooling and Beveridge 2006). This suggests that OMVs are definite components of the biofilm matrix and may contribute to biofilm formation. A study by (Yonezawa *et al.* 2009) suggests that OMVs can act as a stimulator for biofilm formation, as biofilm production was induced after the stimulation of *H. pylori* cultures by the addition of OMVs. To date, the influence of

OMVs on biofilm formation in other bacterial species in which biofilms are implicated in virulence has not been studied and elucidated in detail.

### 1.1.6 **OMVs and the immune system**

OMVs of different Gram-negative bacterial species were found to elicit pro-inflammatory responses upon interacting with host cells because of their LPS content (Section 1.1.2.3). LPS can stimulate the release of pro-inflammatory cytokines upon the binding of lipid A with the TLR4 receptor (Kuehn and Kesty 2005; Park *et al.* 2010). However, the LPS of *B. abortus* was found to not stimulate a potent pro-inflammatory response, and is not considered to be as endotoxic as the LPS from *E. coli* (Kianmehr *et al*. 2015). However, in a mouse model, lethal sepsis was induced by OMVs, when LPS alone was added at a similar concentration as the OMVs in a different group, the sepsis was not observed. Even when the LPS amount was doubled in comparison to the OMVs, no sepsis was observed. Therefore, this may suggest that OMVs can elicit a potent immune response, and that LPS is not the only causative element of this elicitation. In a different mouse model, *E. coli* OMVs were injected intraperitoneally; this was enough to result in fatal systemic inflammatory response syndrome (SIRS) without the presence of the bacterial cells (Park *et al.* 2010). *H. pylori* OMVs play a role in the host inflammatory response activation, cell toxicity and interleukin 8 (IL-8) production (Ismail *et al.* 2003). Moreover, *S. typhimurium* OMVs were found to stimulate antigen presenting cells (APCs), macrophages, dendritic cells and enhance the production of pro-inflammatory cytokines such as TNFα and IL-12 (Alaniz *et al.* 2007). Furthermore, the OMVs of *Vibrio anguillarum* were found to stimulate the production of TNFα, IL-1β and IL-6 (Hong *et al.* 2009). Other cytokines were elicited by *N. meningitidis* OMVs (Durand *et al.* 2009). Similar to most Gram-negative pathogens, OMVs of *K. pneumoniae* elicit the expression of the pro-inflammatory cytokines IL-8 and IL-1β in epithelial cells (Lee *et al.* 2012). Beyond the pro-inflammatory effect, OMVs have been found to be immunogenic. OMVs can potentially be used as a nonviable vaccine for *Salmonella*, due to their ability to prime protective B and T cell responses *in vivo* (Alaniz *et al.* 2007). Naturally produced OMVs of *P. aeruginosa* elicit innate immune responses, and both the LPS and protein contents of OMVs are needed to produce an immune response that is specific for a bacterial strain via macrophages. This response is more distinct than the response that is triggered by individual and purified vesicle components (Ellis *et al.* 2010).

### 1.1.7 **Anti-inflammatory role of OMVs**

In addition to the pro-inflammatory properties of OMVs, some studies indicate that OMVs may play an anti-inflammatory role that can useful to the parent bacterium during infection. As an example, OMVs from *H. pylori* can limit the inflammation elicited during infection, increasing the chances of bacterial survival. This is achieved by stimulating human peripheral blood mononuclear cells to induce the production of the immunosuppressive cytokine IL-10 (Winter *et al.* 2014). Similarly, OMVs from *B. abortus* have been found to inhibit the response of TLR2, TLR4 and TLR5, which lessen IFN-γ, increase the expression of MHC class II and facilitate bacterial internalization via THP-1 cells (Pollak *et al.* 2012). Furthermore, OMVs from *P. gingivalis* can leave macrophages unresponsive to secondary infections caused by *E. coli* after facilitating the loss of CD14 expression (Duncan *et al.* 2004).

### 1.1.8 **OMVs in vaccination**

The human vaccine is a pharmaceutical product that should resemble a pathogen without causing the associated disease of the pathogen when delivered. Any vaccine should have the ability to stimulate immune responses to prevent pathogens from causing disease, usually by eliciting a broad and long-lasting immune response that involves both the innate and adaptive immune systems (van der Pol *et al.* 2015).

Infections induce the host immune response. However, infections per se and excessive immune responses may lead to tissue damage. As explained in Section 1.1.3, OMVs play an important role in transporting toxins and virulence factors to host cells. This transportation modulates the immune responses of the host. Therefore, OMVs could be used to prepare the immune system to combat pathogens, if they can be administrated to the host in a controlled manner (Wang *et al.* 2019).

OMVs have been studied and tested in humans and animals as potential vaccine candidates for decades. The use of OMVs to develop vaccines against various pathogens has been studied thoroughly. They have been developed specifically against different pathogens, particularly against *N. meningitides* type B. Meningococcal OMV vaccines have been produced for the control of an outbreak caused by a single clone, and was produced in a multivalent form to control outbreaks that might be caused by different

strains (Gerritzen *et al.* 2019). In addition, OMV-based vaccines have been developed to combat infections caused by other pathogens such as *B. pertussis*. OMVs from *B. parapertussis* have been used to prevent infections caused by *B. parapertussis* and interestingly, have been found to be cross-protective against infections caused by *B. parapertussis* and *B. pertussis* (Bottero *et al.* 2013; Luu *et al.* 2020).

OMVs from *A. baumannii* have been used in vaccination to protect against pneumonia and meningitis caused by multidrug resistant strains of *A. baumannii*. This vaccine has been shown to induce a strong humoral immune response, resulting in the production of specific IgM and IgG antibodies (McConnell *et al.* 2011).

### 1.1.9 Bacteria of interest in this project

#### 1.1.10 *Klebsiella pneumoniae (K. pneumoniae)*

*K. pneumoniae* is a Gram-negative bacterium and one of the main causative agents of nosocomial infections. It can cause several infections such as, but not limited to, urinary tract infections, respiratory tract infections, blood stream infections that can start as bacteraemia and lead to septicaemia, wound infections and other community acquired infections (Ko 2002; Robin *et al.* 2012). Such infections can be complicated by being caused by multidrug resistant (MDR) strains (Anderson *et al.* 2007).

*K. pneumonia* has emerged as one of the most antibiotic resistant pathogens and is responsible for major outbreaks in health care settings (Uz Zaman *et al.* 2014). Infections caused by multidrug-resistant *K. pneumoniae* and other *Enterobacteriaceae* are usually treated with carbapenems. However, with the increase of carbapenem resistance and the fact that *K. pneumoniae* strains that are resistant to carbapenem confer resistance to other antibiotics families, due to the presence of the KPC enzyme that provides resistance to all β- lactams agents including the penicillins, cephalosporins and monbactams. This results in a limited number of antimicrobial options to treat MDR strains (Anderson *et al.* 2007).

Pathogens face different environmental stresses and defence mechanisms during an infection. *K. pneumoniae* has several virulence factors that aid it to invade host cells, such as the capsular polysaccharide, pili or fimbriae, type IV secretion system,

hypermucoviscosity, siderophore and Iron-Uptake Systems, and biofilm formation (Clegg and Murphy 2016). In addition, the production of OMVs and their involvement in pathogenicity can be considered as another virulence factor of *K. pneumoniae*. OMVs from *K. pneumoniae* have been found to elicit pro-inflammatory responses (Martora *et al.* 2019), and thus led me to selecting OMVs from *K. pneumoniae* to investigate their effect when interacting with bacteriophages and to assess the immune response elicited upon exposure of the OMV-phage mixture to immune cells.

### 1.1.11 *Escherichia coli (E. coli)*

*Escherichia coli* is a Gram-negative bacterium that can either be harmless or pathogenic. Since the first identification of this organism in 1885, it has become the most studied species due to the ease with which it can be grown and manipulated in the laboratory setting. Additionally, the natural ability of *E. coli* to acquire genetic elements has made it a favorable agent to study (Clements *et al.* 2012). Although they are part of the human microbiome, *E. coli* strains can cause various infections depending on the pathotype and the site of infection. They generally cause three clinical syndromes, these being diarrhoeal/enteric disease, urinary tract infections (UTIs) or sepsis/meningitis (Cha *et al.* 2019; Koksal *et al.* 2019; Trecarichi *et al.* 2019).

At least six pathotypes are associated with intestinal diseases: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). Extraintestinal *E. coli* (ExPEC) infections such as UTIs are mainly caused by uropathogenic *E. coli* (UPEC), while meningitis and sepsis are caused by meningitis-associated *E. coli* (MNEC) (Kaper *et al.* 2004). The ability of *E. coli* to cause infections depends on several virulence factors such as, biofilm formation, adhesion, attachment, nutrients acquirements, competition with other bacteria, toxin production and avoidance of host defense mechanisms (Raimondi *et al.* 2019).

Depending on the type and site of infection, different antibiotics are used to treat severe *E. coli* infections. However, the extensive use of antibiotics stimulates bacteria to resist antibiotic activity, leading to the development and spread of MDR *E. coli* (Vila *et al.* 2016). *E. coli* was the most common cause of bloodstream infections in England; it rose

from 65.7 cases per 100,000 people in 2014 to 76.0 cases per 100.000 in 2018. The susceptibility data that was obtained from the Public Health England, English surveillance programme for antimicrobial utilization and resistance (ESPAUR, 2018) indicated that the proportion of resistant isolates was higher in hospital-onset compared to community-onset cases, and that carbapenems resistance in hospital-onset cases were 76 % higher than in community-onset cases (Public Health England, 2018). According to the WHO, 3rd generation cephalosporin-resistant and carbapenem-resistant *E. coli* are 'critical' priority pathogens that require an urgent solution (Alvarez-Uria *et al.* 2018; Organization 2019). Similar to other Gram-negative bacteria, *E. coli* produces OMVs, and their production has been found to be involved in the communication between different bacterial cells and in bacterial pathogenicity (Cañas *et al.* 2018).

## 1.2 Bacteriophages

### 1.2.1 Definition of bacteriophages

Bacteriophages, or phages for short, are viruses that infect bacteria by making use of bacterial resources. The word bacteriophage translates to “bacteria eater”. Virulent bacteriophages are likely to cause full lysis to a susceptible bacterial culture (Orlova 2012). Bacteriophages are considered to be the most abundant biological entities on earth. Their population is estimated to be $10^{32}$ phages in the world (O'Flaherty *et al.* 2009; Haq *et al.* 2012). They can be found in soil, sewage water, food products, and other different sources; generally speaking, they are found in the same environment as their bacterial host.

### 1.2.2 Bacteriophage history

In 1896, British bacteriologist Ernest Hankin was the first to notice small particles that could pass through very small filter pores in the water of rivers of India; these had unexpected antibacterial properties against cholera (Summers 2005). In 1915, another British bacteriologist, Frederick Twort, discovered small particles that killed bacterial culture colonies. However, these particles were not named bacteriophage until 1917 by the Canadian French bacteriologist Felix d’Herelle, who noticed zones of clearance in bacterial cultures spread on agar plates (Summers 2005).

### 1.2.3 Bacteriophage classification

Phages are classified based on their morphology and nucleic acid properties. The majority of them contain double-stranded DNA, while smaller groups of phages contain single-stranded DNA. Other phage groups contain double stranded-RNA or single stranded-RNA. Phages can be classified according to their morphology and can be grouped to be either filamentous phages, icosahedral phages without tails, phages with tails, and phages that may possess a lipid-containing envelope or contain lipids in the particle shell (Ackermann, 2009).

#### 1.2.3.1 *Caudovirales* order of phages

The tailed phages classed under the *Caudovirales* order (Figure 1.3) are the most predominant, representing 96 % of known phages. They are the most studied group of

phages and are the easiest to find and purify. They are generally between 24 to 400 nm in length and can be recognised by their double-stranded DNA genome, and their capsid and tail. The *Caudovirales* order can be further divided into three phage families; *Myoviridae*, with contractile tails consisting of a sheath and a central tube (25 % of tailed phages), *Siphoviridae*, with long non-contractile tails (61 %) and *Podoviridae*, with short tails (14 %). Most of the phages within each of the three families have similar morphologies. The phage capsid is attached to the tail through a connector which plays many fundamental roles during the phage infection cycle, including packaging dsDNA into the capsid and participating in the release of phage DNA into the host bacterium. The phage DNA is delivered to the host bacterial cell through the tail, which has tail fibres at its end so that it is able to bind to its specific bacterial receptor (Ackermann, 2009). The remaining phages are the pleomorphic and filamentous phages. These are further classified into ten small families and make up approximately 4 % of the phage population studied to date (Ackermann, 2009).

With the evolvement and improvement of genomic analysis technologies, phages have been sequenced and analysed via various bioinformatics-based tools. For example, the *Ackermannviridae* and *Herelleviridae* families used to be classified within the *Viunalikevirus* and *Spounavirinae* subfamilies as part of the *Myoviridae* family (Adriaenssens *et al.* 2018; Barylski *et al.* 2020). Moreover, these three families were classified under one family due to the similarity of their morphology of possessing tail fibers and contractile tails attached to either elongated or icosahedral heads (Barylski *et al.* 2018; Barylski *et al.* 2020). However, genomic sequencing analysis revealed that there are differences in their genomic homology and phylogenic clustering.

The *Ackermanniviridae* phage possesses capsids approximately 90 nm in diameter and a tail approximately 110 nm in length. These features make the morphology of this phage different from the morphology of the traditional *Myoviridae* morphology (Day *et al.* 2018). The same morphological features were observed in the *Viunalikevirus* genus, therefore they were classified as part of the *Ackermanniviridae* family (King *et al.* 2018). To conclude, genomic analysis alongside morphological analyses are required to characterise phages. Nowadays, the Bacterial and Archaeal Viruses Subcommittee of the ICTV are considering reorganizing the phage taxonomy of this family due to the genomic diversity among its members (Barylski *et al.* 2020).

The other popular member of the *Caudovirales* order are the *Siphoviridae* phages, which are characterized by their long, thin, non-contractile tails. The other family is the *Podoviridae* family, characterized by their relatively short tails (Ackermann, 2009). On the other hand, the non-tailed phages can be classified into three different groups, as described below.

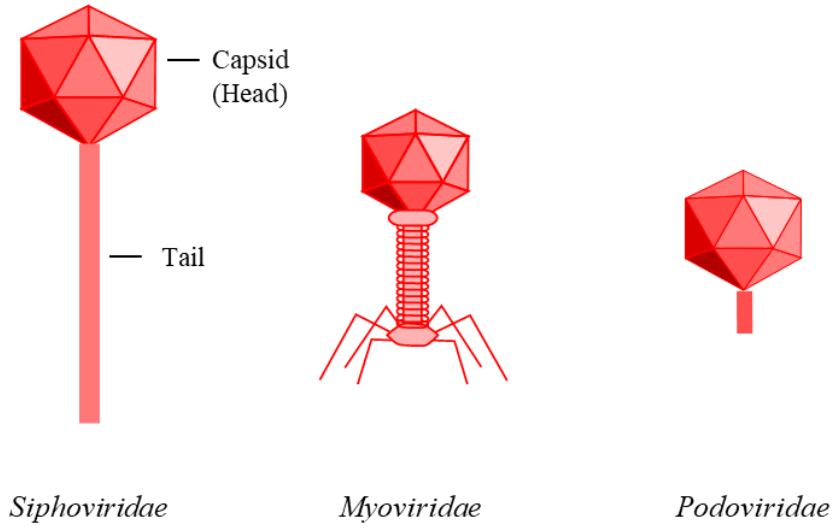



**Figure 1.3: Structure of the main *Caudovirales* families.**

The three commonly isolated phages are the *Siphoviridae*, with a simple non-contractile tail. *Myoviridae*, which are phages with a tail that consists of neck, a contractile tail and central tube. Finally are the *Podoviridae* which are phages with a short non-contractile tail.

#### 1.2.3.2 **Pleomorphic phages**

The families of pleomorphic phages present with different morphological structures but all contain dsDNA. This group includes seven known families: *Plasmaviridae*, *Fuselloviridae*, *Guttaviridae*, *Bicaudaviridae*, *Ampullaviridae*, *Salterprovirus* and *Globuloviridae*. The morphologies of these phages are illustrated in Figure 1.4.

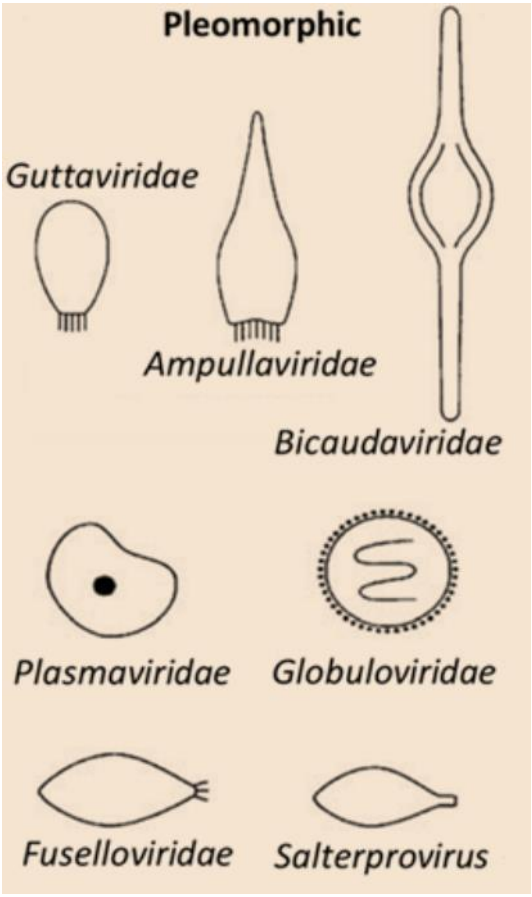



**Figure 1.4. Different families of the Pleomorphic group of phages.**

The figure shows the different families from the Pleomorphic phages. The *Guttaviridae* family has a droplet-shape, the *Ampullaviridae* have a bottle-shape mantle, cone-shape body and a helical nucleocapsid and the *Bicaudaviridae* have a tail-like appendage. The *Plasmaviridae* phages have an envelope and nucleoprotein without a capsid, whereas the *Globuloviridae* have a round envelope that contains lipid and helical nucleoprotein. The *Fuselloviridae* are dsDNA spindle-shaped viruses with no capsid, the *Salterprovirus* have a spindle-shaped family that differ from the *Fuselloviridae* by their linear DNA, terminal protein and lytic activity. This figure was adapted from (Ackermann, 2009).

#### 1.2.3.3 **Polyhedral phages**

This group of phages contains five different families. The first is the *Microviridae* family, which are characterized according to their icosahedral capsids that contain ssDNA genomes ranging between 1.4 to 8.5 kb in size (Zhong *et al.* 2015). The second family is the *Corticoviridae* phages, which are characterized by capsids that are composed of two protein shells and a protein layer in between (Ackermann, 2006; Ackermann, 2009). Thirdly, the *Tectiviridae* family is composed of phages that have dsDNA protected by a lipoprotein vesicle inside the proteinic capsid. The vesicle acts as a DNA injecting device

by forming a tail-like shape in the case of infection and DNA release. Fourth are the *Levivirida* family, consisting of ssRNA viruses. Some of the phage members of this family are capable of infecting *Enterobacteria*, *Pseudomonads*, *Acinetobacters*, and *Caulobacters* (Ackermann, 2009). Finally, the *Cystoviridae* family are phages with dsRNA located inside a double-layered protein capsid. These phages are capable of infecting the plant pathogen *Pseudomonas syringae* (Figure 1.5) (Ackermann, 2009; Poranen and Mäntynen 2017).

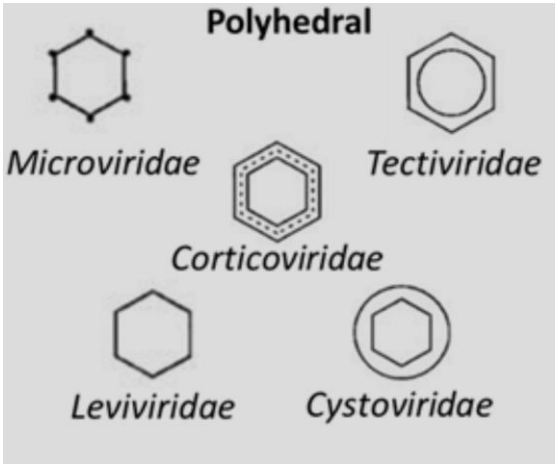



**Figure 1.5: Different families of the polyhedral group of phages.**

The families of this group are generally characterised by their small size, lack of tail and knob-like capsids with no envelope, except for the *Cystoviridae* viruses that have an envelope. This figure was adapted from (Ackermann, 2009).

#### 1.2.3.4 **Filamentous phages**

This group of phages contains three taxonomic families, the *Inoviridae*, *Lipothrixviridae* and *Rudiviridae* (Figure 1.6). The first family is *Inoviridae*, consisting of phages that are simple and small, with circular ssDNA genomes approximately 9 kb in size encoding for 14 open reading frames (ORFs) (Addy *et al.* 2012). Their size and simplicity has made these viruses a useful model for genetic manipulation studies (Hemminga *et al.* 2010). The two other families are the *Lipothrixviridae* phages, which are long rods with a lipoprotein envelope and the *Rudiviridae* phages, which are straight rods without envelopes. Both of these phage families consist of dsDNA genomes and are capable of infecting hyperthermophilic Archea (*Sulfolobus*) (Ackermann, 2009).

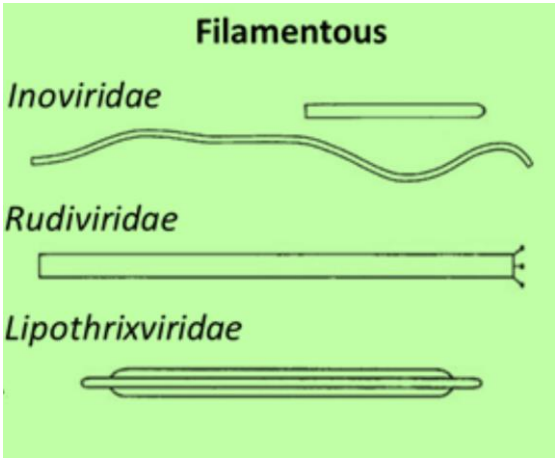



**Figure 1.6: Different family members of the Filamentous phages.**

A schematic illustration of the filamentous phage families. The *Inoviridae* are long filamentous phage, the *Rudiviridae* virions have straight rods without an envelope and the *Lipothrixviridae* virions have long rods with an envelope. This figure was adapted from (Ackermann, 2009).

### 1.2.4 **Bacteriophage life cycles**

Bacteriophages need to maintain their population by proliferation; to achieve this purpose, they require the presence of a sensitive bacterial host. Additionally, this proliferation is achieved by the employment of several life cycles.

#### 1.2.4.1 **Lytic cycle**

In this cycle, phages attach to the host cell by recognizing bacterial receptors such as lipopolysaccharides, pili or flagella, on the outside of the bacterial host. After that, they introduce their genetic materials either by injecting it into the bacterial host (Molineux and Panja 2013) or via endocytosis mechanisms (Romantschuk *et al.* 1988). The viral genome then uses the bacterial machinery to produce the phage-encoded proteins and replicate the phage genetic material. Afterwards, the viral proteins are self-assembled to form capsids to package the viral genetic materials and tail structures. The bacterial cell is lysed after the generation of 50-200 new phages on average; thereafter, the viral progeny are released (Figure 1.7). This process is mediated by two key components, the holin, which creates pores within the cytoplasmic membrane, and the endolysin, which is one of the hydrolytic enzymes capable of cleaving the bacterial cell wall. This combination of events depletes the resources of the host cell, weakening it and causing it

to lyse and release the progeny phages. The released phages start another cycle by infecting other susceptible bacterial cells (Guo *et al.* 2020).

#### 1.2.4.2 **Lysogenic cycle**

In the lysogenic cycle, phages integrate their genetic material into the bacterial genome, and once inserted, they can be referred to as prophages (Leigh 2019). Phages in this cycle can undergo both the lysogenic and lytic cycles, and in this case, are called temperate phages. The integrated genome can replicate within the host during normal bacterial division. Prophages are normally stable within the host. They can enter the lytic cycle by being spontaneously induced. Prophage induction can be initiated by either DNA-damaging bacterial agents such as UV or antibiotics, via stressful conditions such as temperature or via hydrogen peroxide (Łoś *et al.* 2010; Clokie *et al.* 2011) (Figure 1.7). Prophages can lose their ability to disjoin the host genome, at which point they can be referred to as cryptic phages. Prophages and cryptic phages can be useful to their host, increase their fitness, aid resilience under stressful conditions, and increase their virulence and ability to infect eukaryotic cells (Wang *et al.* 2010; Fortier and Sekulovic 2013).

#### 1.2.4.3 **Pseudolysogenic state**

The third life cycle that the phages can undergo is the pseudolysogenic state, in which the phages neither integrate their genomes within the bacterial genome nor enter the lytic cycle. This state can be described as a peaceful relationship between the host and the un-integrated phage genome. The phage in this state is not reproducible or lysogenic, and does not cause host cell lysis (Hyman and Abedon 2010). This status is mainly believed to be caused by nutrient starvation conditions and can change, resulting in either lysogeny or lysis when this condition is resolved (Abedon 2009). The pseudolysogenic state is referred to as such because it is not clear whether it is an actual cycle or just an interruption in phage activity as a result of the cell's reduction in activity because of starvation. In this state, phages are not eliminated due to the lack of bacterial growth; rather, they maintain and persist until the bacterial cells exit the starvation mode. After the starvation issue is resolved, the phage can enter either the lytic or lysogenic life cycle (Díaz-Muñoz and Koskella 2014).

#### 1.2.4.4 **Carrier state**

Similar to the pseudolysogenic state, in the carrier state, phages neither integrate into the host genome nor induce cell lysis. Instead, they establish a chronic infection within the bacterial host. Phages maintain such persistence where progeny virions are routinely released from the cells by budding or asymmetrically transferred down to daughter cells upon division (Cenens *et al.* 2013). The difference between the carrier state and the pseudolysogenic state is that the carrier state is not caused by starvation as with pseudolysogeny; in contrast, the carrier state can be established and maintained under rich nutrient conditions, and phages can persist during the exponential growth of the host (Cuppels *et al.* 1979). Because of this main difference, the carrier state has been described as a host resistance mechanism. Moreover, this state can represent a coexistence mechanism of bacteria and phages. Though the phage and bacteria co-exist with each other peacefully, that does not necessarily mean that phage production does not affect the bacterial cell's physiology and fitness (Rakonjac *et al.* 2011).

Although the carrier state has been under-studied and is not well-understood, it was found that this state can slow the bacterial growth rate. This was observed in *E. coli*; when infected with a filamentous phage, its growth rate was altered (Rakonjac *et al.* 2011). In a similar study, this state was found to affect bacteria-phage interaction in nature. This was found when *C. jejuni* biofilms were exposed to a phage that eventually entered a carrier state in some host cells. These persistent phages helped in providing their host cells with extra environmental tolerance outside the chicken gut. At the same time, these phages reduced the existent *C. jejuni* population in the guts as efficiently as pure phage lysates (Siringan *et al.* 2014).

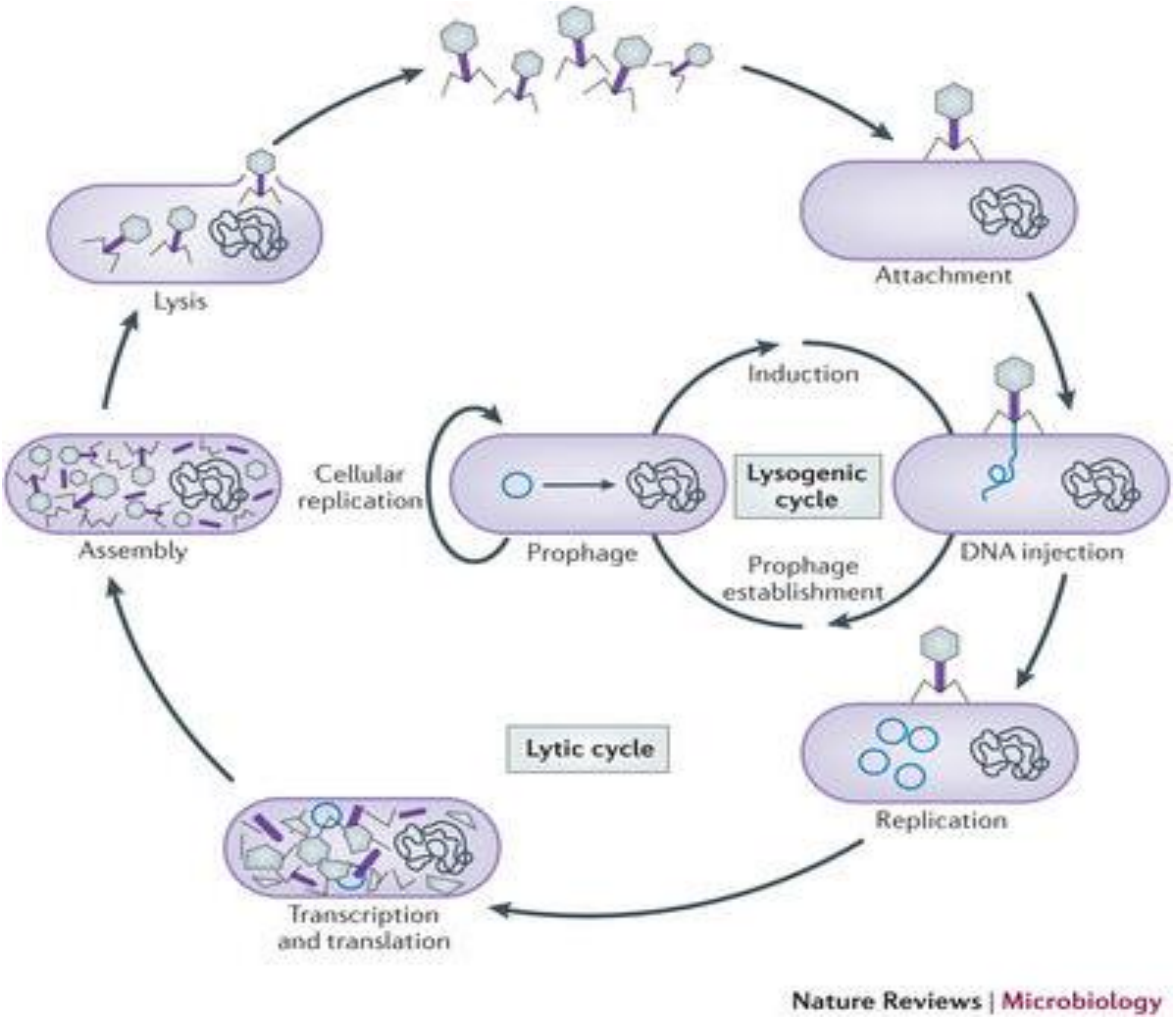



**Figure 1.7. Bacteriophage life cycles**

Lytic phages attach to the host cells and eject their genome inside, before forming multiple progeny phages leading to cell lysis. The lysogenic cycle involves the integration of the phage DNA into the host cell genome (prophage). This is replicated along with the host bacterial genome, resulting in daughter bacteria inheriting viral DNA. This process can continue for many generations with no consequences for the bacterial host. The cycle will eventually return back to the lytic cycle where phages multiply and can be released. (O'Flaherty *et al.* 2009; Clokie *et al.* 2011; Orlova 2012).

### 1.2.5 Applications of phages

#### 1.2.5.1 Phage therapy

The treatment of bacterial infections with bacteriophages started at least ten years before the discovery of antibiotics. However, due to the discovery of antibiotics in the mid-20$^{th}$

century, with a better understanding of the infectious diseases and a lack of good understanding of phage biology, the use of phage therapy was not as high as that of antibiotics for many years (Chanishvili, 2012). Antibiotics were indispensable medical tools that were involved in improving life quality and expectancy. However, bacterial species' acquisition of resistance to common antibiotics, such as β-lactams, aminoglycosides, chloramphenicol, and tetracycline, makes the treatment of bacterial infections a challenging task (Zhang *et al.* 2009; Laxminarayan *et al.* 2013). Antibiotic-resistant bacteria have been described as the greatest and most urgent global risk, according to the United Nations General assembly. As a result, scientists have been looking for alternative strategies to fight bacterial infections. One of the most promising ideas is phage therapy (MacGowan and Macnaughton 2017; WHO 2017).

Many bacterial infections have been treated with phage therapy, including skin ulcers, surgical site infections (LaVergne *et al.* 2018), pancreatitis (Schooley *et al.* 2017), wound and burn infections (Jault *et al.* 2019), eye infections (Kudrin *et al.* 2017), respiratory tract infections, urinary tract infections (Khawaldeh *et al.* 2011) and otitis media (Wright *et al.* 2009). Several reports have shown the effectiveness of phage therapy. For example, in a case that has been reported by (Duplessis *et al.* 2018), a two-year-old patient with congenital heart disease presented with bacteraemia caused by a multidrug resistance *P. aeruginosa* after multiple surgeries. The patient was treated with antibiotics that seemed to control the infection initially, but eventually failed to clear the infection. The patient reacted to the cephalosporins and fluoroquinolones antibiotics. Thereafter, the patient was treated with a phage cocktail of two phages with a titre of $3.5 \times 10^5$ PFU/ml four times a day for almost two days. The treatment was paused due to the inability of the patient's heart to maintain adequate blood circulation. Phage therapy was continued again after nine days. The bacteraemia infection was monitored by blood cultures, which showed the ability of the phage to eliminate the infection, and sterilize the blood alongside the anti-pseudomonal antibiotics (Duplessis *et al.* 2018).

Phage therapy has potential advantages in comparison to antibiotics. The normal microbiota can be significantly disrupted with antibiotics; on the other hand, treatment with phages specifically attacks pathogens and leaves the occupying microbiota undisturbed, thus preventing pathogenic strains from invading the host (Theriot and Young 2015). The phages that are typically chosen for phage therapy are lytic phages.

The use of antibiotics may prompt antibiotic resistance mechanisms and allow for bacterial persistence. Lytic phages always attack specific bacterial cells and use all the bacterial resources, eventually lysing the cell at the completion of its replication cycle (Loc-Carrillo and Abedon 2011). Phages are capable of propagating at the site of infection; this may allow for the use of a lower dosage of phage compared to the application of antibiotics that need to be delivered regularly until the infection is cleared. Phages will be cleared from the human body once the host bacteria are depleted. Finally, while the discovery of new antibiotics has declined in the past few years, the discovery of new phages is still an active research area due to the their vast bio-diversity (Loc-Carrillo and Abedon 2011; Fair and Tor 2014).

On the other hand, phage therapy has some potential limitations in comparison with antibiotics. Phages do not possess a reliable method to enter eukaryotic cells, while antibiotics have the ability to treat intracellular bacterial infection which has been proven. Additionally, the interaction of antibiotics with the immune system has been excessively studied, whereas there is still a lot to be known about the interaction of phages with immune cells (Roach *et al.* 2017). Phages attack bacterial cells, which results in entire cell lysis, leading to the release of endotoxins and maybe other antigens that could be dangerous. Additionally, one of the main current concerns with phage therapy is the need to have purified phage lysates, especially lysates free from endotoxins (Kortright *et al.* 2019).

#### 1.2.5.2 **Phage therapy and cancer**

Alongside treating bacterial infections, phage therapy has been suggested as a potential cancer therapy agent. Phage therapy may contribute towards cancer therapy in two ways. The first is in a direct manner via phage display (Robson and Ghatage 2011). Bacteriophages can be genetically modified in order to be used to deliver certain peptides and proteins to targeted cells. The most common used phage in this technique is the M13 filamentous bacteriophage, which possesses a minor coat protein pIII that is capable of fusing 5 protein copies, and a major coat pVIII protein that is capable of fusing up to 2,800 protein copies of peptides (Mimmi *et al.* 2019). In a simplified form, genetic modification in phage display is the insertion of genes encoding the desired peptides onto the capsid of the phage. Once inserted, the phage will “display” the peptide or protein on

the outer surface while carrying the encoding gene for this protein within the virion. When these peptides are delivered to the targeted cells by the phage, they will launch their therapeutic ability (Goracci *et al.* 2020). The phage display technique has been used to deliver various types of biologically active molecules, for example, in colorectal cancer (Murer *et al.* 2020), ovarian cancer (Robson and Ghatage 2011) and breast cancer (Arab *et al.* 2020).

The other indirect way of using phages within cancer therapy is by targeting bacterial pathogens that might lead to cancer, such as *Helicobacter pylori*, which can cause gastric cancer, and *Fusobacterium nucleatum*, which can cause colorectal cancer (Shen *et al.* 2020) (Kannen *et al.* 2019). Therefore, by targeting the bacterial pathogens that might lead to cancer, some of the main risk factors of certain cancers could be eliminated. Thus, it can be concluded that phages can contribute towards cancer therapy.

#### 1.2.5.3 **Phages in the food industry**

Foodborne diseases are mainly caused by bacteria that are present in the food, and overcoming this problem in the food industry is an expensive process (Cof fey *et al.* 2010). Using chemical agents such as disinfectants, biocides and antibiotics has shown to be effective in eliminating foodborne pathogens (Huang *et al.* 2018). On the other hand, using such chemicals may leave remnants in the food which might jeopardise its taste, smell and structure. More importantly, it may even pressure certain bacterial populations and encourage the generation of new antibiotic-resistant bacterial strains, and eventually lead to the insufficient sanitation of food products (Cof fey *et al.* 2010; Beier *et al.* 2011; Safavieh *et al.* 2015; Huang *et al.* 2018)

Bacteriophages are a potentially effective solution to reduce foodborne bacterial pathogens. Phage biocontrol can be a beneficial approach in treating foodborne diseases and in preserving food from being contaminated with pathogens (Cof fey *et al.* 2010). Bacteria have different phage resistance mechanisms in comparison to the resistance mechanisms seen with antibiotics. (Torres-Barceló and Hochberg 2016). Examples of such resistance mechanisms are the enzymatic degradation of antibiotics, alteration of antibiotics targets by altering bacterial proteins, changing bacterial cell wall permeability, pumping antibiotics out of the cell via efflux pump systems and manipulating the host

signalling mechanisms. On the other hand, the phage resistance mechanisms of bacteria include the inhibition of the injection of phage DNA, changing of bacterial receptors to prevent phage attachment, the use of restriction modification system and the induction of abortive infections (Dever and Dermody 1991; Reyes-Robles *et al.* 2018; Lim *et al.* 2020). Furthermore, phages are natural antimicrobial agents, unlike antiseptic agents, which are synthetic. Phages are able to self-replicate as long as the sensitive bacterial host exists (Huang *et al.* 2018). Given the advantages of phages in combating foodborne pathogens in comparison to antibiotics, bacteriophages garner a lot of attention in the food industry. One of the first European initiatives was launched in 2013, giving rise to the "phagoburn" project funded by the European Union, which was established to study the use of phages in the food industry (Matsuzaki *et al.* 2014).

Phage biocontrol has been used against several foodborne pathogens, such as *Listeria monocytogenes* in dairy, poultry and meat production (Reinhard 2020; Zhou *et al.* 2020), *Salmonella* spp. in poultry production (Connerton *et al.* 2020), *E. col*i O157:H7 in dairy and vegetables production (Duc *et al.* 2020), and *C. jejuni* in poultry production (Ushanov *et al.* 2020).

### 1.2.6 **Phages and immunomodulation**

Many studies have been conducted to determine whether phages stimulate pro-inflammatory responses in humans and mice. The vast majority of these studies indicate that neither phages nor their proteins stimulate inflammatory mediators or the production of reactive oxygen species (ROS) *in vitro* or *in vivo (Górski et al. 2017a).* In contrast, phages have shown the ability to exert strong anti-inflammatory actions (Górski *et al.* 2017a). The tail fibre protein of the T4 phage (gp12), which is responsible for binding the phage with the bacterial cell by binding surface protein or LPS recombinant protein, was expressed *in vitro* in a bacteria expression system is responsible. After expression, the proteins functionality was confirmed by testing its ability to attach LPS *in vitro*. The protein was tested *in vivo* by injecting it into mice; no harmful effects on the mice were observed. Furthermore, the protein was combined with LPS and assessed in a murine model. Interestingly, the protein decreased the inflammatory effect of the LPS. This was assessed by measuring the level of IL-1α and IL-6 in the serum and via histopathological analysis of the liver, spleen, kidney and lungs (Miernikiewicz *et al.* 2016). A similar effect

was found with the whole T4 phage in transplant mice (Górski *et al.* 2006). It was shown, firstly, that the administration of phage therapy to uninfected mice did not change the level of IL-1β, IL-6 and TNF-α cytokines produced. Secondly, the phages administered significantly decreased the level of the same cytokines when delivered to infected mice. This suggests that phages do not stimulate pro-inflammatory cytokines production and might have the ability to exert anti-inflammatory action by attenuating inflammation induced by bacterial pathogens (Wang *et al.* 2016).

Such anti-inflammatory activity may be useful to the host. In a study conducted by Lepage *et al*, it was suggested that phages may contribute to abnormal immune responses that occur in patients suffering from Crohn's disease, an inflammatory bowel disease. In this study, it was found that the concentration of phages in the gut intestinal mucosa is higher in patients with the disease than the healthy control. Additionally, when comparing ulcerated mucosa with un-ulcerated mucosa, it was found that the phage concentration was lower in ulcerated mucosa. This data suggests that phages may have a beneficial role in protecting patients' mucosa (Lepage *et al.* 2008). In a similar context, phages and the immune system of the metazoan hosts are said to be in a symbiotic relationship; the phages provide protection to the intestinal mucosal surface from bacterial invasion by carrying out antimicrobial defence activities (Barr *et al.* 2013). This is achieved by the adherence of phages to the mucosal surface, eventually leading to the reduction of microbial colonization and pathology (Barr *et al.* 2013).

## 1.3 Interaction of phages and OMVs

A study conducted by (Manning and Kuehn 2011) showed that the interaction of *E. coli* OMVs with phage T4, an *E. coli* phage, resulted in the reduction of phage T4 infectivity. This study shows the ability of OMVs to bind with bacteriophages, leading to the inactivation of these phages upon interaction with the OMVs. In a more recent study, it was shown that OMVs from *V. cholera* act as a defence mechanism by protecting parent bacterial cells against infection from three different phages (Reyes-Robles *et al.* 2018). This interaction is suggested to be dependent on the presence of phage receptors on the surface of the OMVs. Both studies describe efficient interactions between OMVs and bacteriophages. What is more, these studies have revealed the ability of OMVs to act as decoys that can neutralize phages.

In another approach, the interaction of phages with OMVs was assessed with SPPI phage which is able to infect *B. subtilis*. This phage-susceptible bacterium releases MVs that carry phage receptors. When these MVs fuse with phage-resistant strains, they make the cells sensitive to the phage. This mechanism enables phages to infect resistant strains that lack its binding receptor, promotes the spread of phages and enables phages to invade in mixed natural communities. This phenomenon facilitates transduction and horizontal gene transfer among different bacterial species. This eventually might lead to expedited bacterial evolution (Tzipilevich *et al.* 2017).

## 1.4 Focus of this project

This project was designed to investigate whether the presence of bacteriophages can alter OMV-induced immune responses in macrophages, and assess whether the interaction of lysogenized bacteria with macrophages can induce prophage release. In particular, since several studies have shown that OMVs from Gram-negative bacteria are capable of stimulating host pro-inflammatory cytokines production, this project assessed whether interacting OMVs from Gram-negative bacteria (*K. pneumoniae* or *E. coli*) with specifically isolated phages can alter the production of OMV-induced pro-inflammatory cytokines (TNFα) in immortalized macrophages. In addition, since it is known that the exposure of lysogenized bacteria to stressors and oxidizing agents can induce prophages *in vitro*, and as macrophages use such agents to kill bacteria, several pilot experiments were performed to assess whether the interaction of lysogenized *E. coli* with macrophages results in prophage induction.

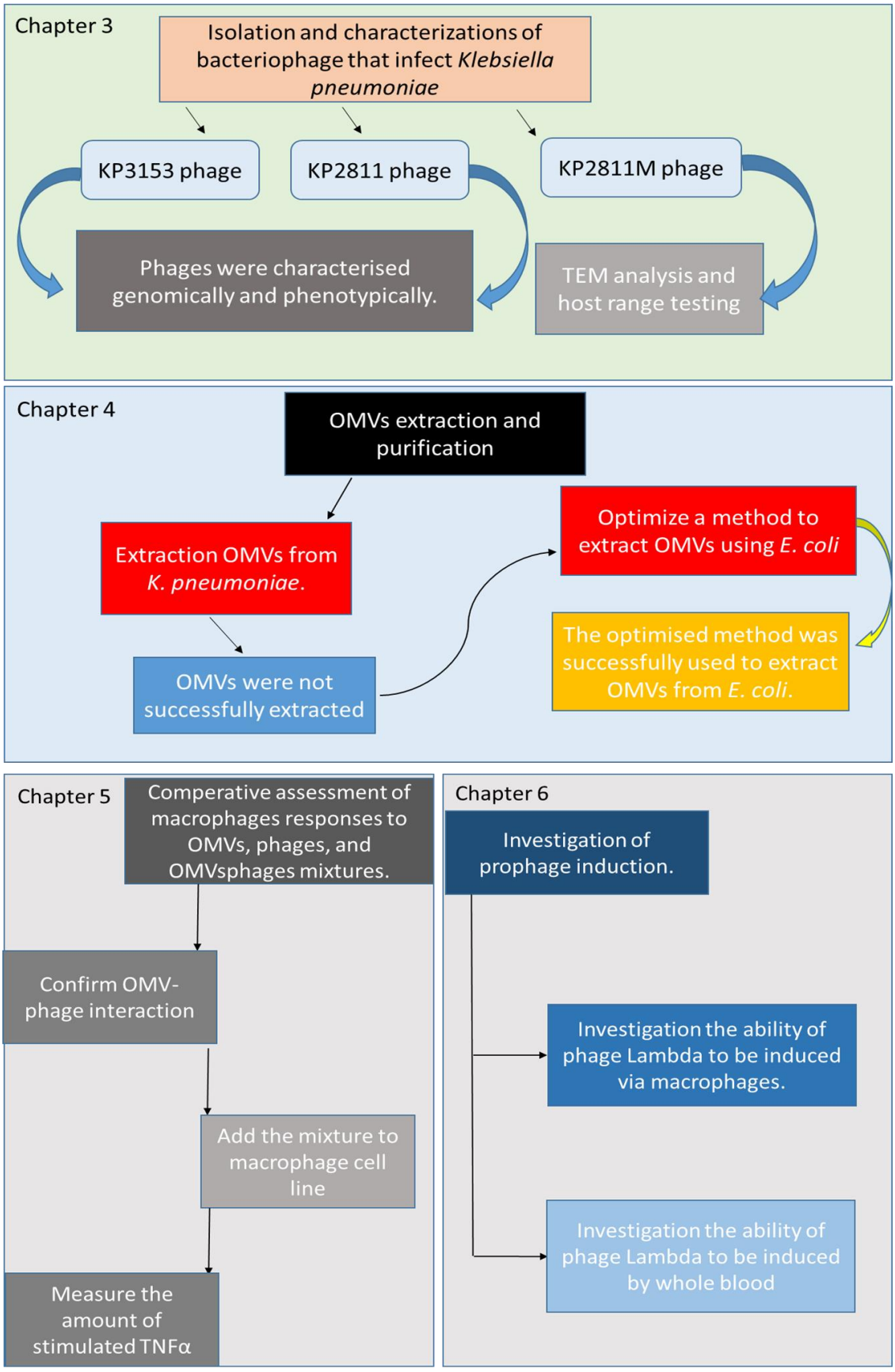
## 1.5 Aims and objectives

This research aimed to study the interaction between bacteriophages and OMVs from Gram-negative bacteria, mainly *K. pneumoniae* and *E. coli*, and to assess whether their interaction affects the inflammatory host response elicited by OMVs. In addition, it seeked to assess whether the interaction of lysogenized bacteria with macrophages results in the induction of prophages. The research objectives were as follows:

- To isolate and characterise phages capable of infecting *K. pneumoniae* from environmental samples.

- To define an optimal method to extract and purify OMVs from Gram-negative organisms.
- To interact purified OMVs with bacteriophages, and to assess the effect of this interaction on the immune response.
- To run pilot experiments to assess potential prophage induction upon the interaction of lysogenized *E. coli* with macrophages.

## 1.6 **Project story schematic**



# Chapter Two

## 2. General methods

## 2.1 Environmental sample collection

Two types of sewage samples were collected from the Seven-Trent water treatment plant in Wanlip. The samples were collected in DURAN® glass transparent bottles. The first sample was 1 L of untreated sewage water. The second sample was half-way sludge water. Before screening the sample for phages, the samples were filter-sterilized by using 0.22 µm filters (MF-Millipore™ Membrane Filter, 0.22 µm pore size).

## 2.2 Bacterial media used in this study

### 2.2.1 Luria Bertani Broth (LB)

LB was made by following the manufacturer's recommendation of adding 10 g of LB medium (Oxoid, Ref# 13274842) to 400 ml of distilled water; this was then sterilized using a bench top autoclave. LB consists of 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl.

### 2.2.2 Lauria Agar (LA)

The 1 % solid agar was prepared by mixing 4 gm of bacterial agar (Bacteriological Agar Powder, HiMedia Laboratories, 500 g, catalogue number GRM026-500G) with 10 g LB medium in 400 ml of distilled water, and then autoclaved using a benchtop autoclave.

### 2.2.3 Semi-solid Agar

0.4 % semi-solid agar was prepared by adding 1.6 g bacterial agar with 10 g LB medium in 400 ml of distilled water and then sterilized by autoclaving.

## 2.3 Buffers

### 2.3.1 Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving one PBS tablet (Oxoid™, catalogue number BR0014G) in 100 ml of distilled water. The final buffer contained 0.01 M phosphate buffer (pH 7.4), 0.0027 M potassium chloride and 0.137 M sodium chloride. After that, the solution was autoclaved at 121 °C for 30 minutes.

### 2.3.2 SM buffer

This a widely used buffer in molecular biology, particularly in the study field of bacteriophage. It was prepared by mixing the following recipe (5.8 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50 ml 1 M Tris-HCl pH 7.4, in 1L $dH_2O$). The solution was then sterilized either by using 0.22 µm filters or by autoclaving.

### 2.3.3 50X Tris-acetic acid EDTA (TAE)

This buffer was made by dissolving 48.4 g of Tris base in 800 ml of distilled water, and then adding 11.4 ml of glacial acetic acid (17.4 M) and 3.7 g of ethylene-diamine-tetra acetic acid (EDTA). The solution was adjusted to 1 litre with distilled water, after which it was diluted 1 in 10 (10X) with distilled grade water, which was distilled before use.

### 2.3.4 10X SDS buffer

SDS buffer was prepared by dissolving 144 g of glycine, 10 g of SDS and 30.3 g of Tris base in 1 litre of distilled water. For the running gel, 1X SDS was prepared by mixing 100 ml of 10X SDS with 900 ml of distilled water.

### 2.3.5 10X Western blot buffer (transfer buffer)

The Western blot buffer was prepared by adding 144 g of glycine and 30.3 g of Tris base to 1 litre of distilled water.

### 2.3.6 Colloidal Coomassie stain

A stock solution was prepared by mixing 0.1 % (w/v) Coomassie Brilliant Blue G-250, 10 % (w/v) ammonium sulphate and 2 % (w/v) phosphoric acid. For the working solution, 80 ml of Colloidal Coomassie stain solution was added to 20 ml of methanol.

## 2.4 Bacterial strains and growth conditions

In this study, six different bacterial strains were used. For *K. pneumoniae*, six different strains were kindly provided from the strains collection of Dr. Kumar Rajakumar (University of Leicester, UK). The seventh strain used was *E. coli* DH5α, which was obtained from Dr. Edouard Galyov's laboratory collection. All the bacterial strains were used in this study for the isolation of the phage and to extract OMVs. The details of the

strains used in this study are listed in the table below. All the strains were grown on Lauria-Bertani (LB) agar plates at 37 °C for 16 hours. An isolated colony was then inoculated in LB broth and incubated again at 37 °C for 16 hours at 200 RPM in the shaking incubator to make liquid bacterial cultures.

**Table 2.1: Bacterial strains used in this study.**

| Strain catalogue | Species | Alternative designation | **Source** |
|---|---|---|---|
| ***KR 640*** | *K. pneumoniae* | MGH 78578/3792 | Dr K. Rajakumar |
| ***KR2813*** | *K. pneumoniae* | HS11286 | |
| ***KR2811*** | *K. pneumoniae* | HS04160 | |
| ***KR4010*** | *K. pneumoniae* | HS11286-RR2 | |
| ***KR3153*** | *K. pneumoniae* | MG 559842W | |
| ***DH5α*** | *E. coli* | Unknown | Lab strain |

## 2.5 Bacteriophage used in this project

Three *K. pneuminiae* phages were isolated during this study from the sewage sample. One previously isolated phage was obtained from Dr. Edouard Galyov's laboratory collection.

**Table 2.2: List of the bacteriophages used in this study.** The table illustrates the bacterial host and the source of each phage.

| Bacteriophage | Bacterial host | Bacterial strains* | Source | Reference |
|---|---|---|---|---|
| **Kp3153** | *K. pneumoniae* | KP3153 | Sewage sample | Isolated in this study |
| **Kp2811** | *K. pneumoniae* | KP2811 | Sewage sample | Isolated in this study |
| **Kp2811M** | *K. pneumoniae* | KP2811 | Sewage sample | Isolated in this study |
| **MiEL** | *E. coli* | DH5α | Horse manure | Dr M. Imam |

*Bacterial strain used to isolate the bacteriophages

## 2.6 Plaque assay

The plaque assay was used to either screen environmental samples for the presence of phages, to purify phage population, or to check the purity of phages by assessing plaque morphology. Briefly, 300 µl of a mid-log phase culture was mixed with 100 µl of environmental sample or phage lysate; this was added to 5 ml of molten semi-solid agar, and then poured onto LA plates and left to solidify. The plates were then incubated overnight at 37 °C, and plaques morphology and counts were assessed. This protocol was adopted and modified from (Kropinski *et al.* 2009) and is shown in Figure 2.1.

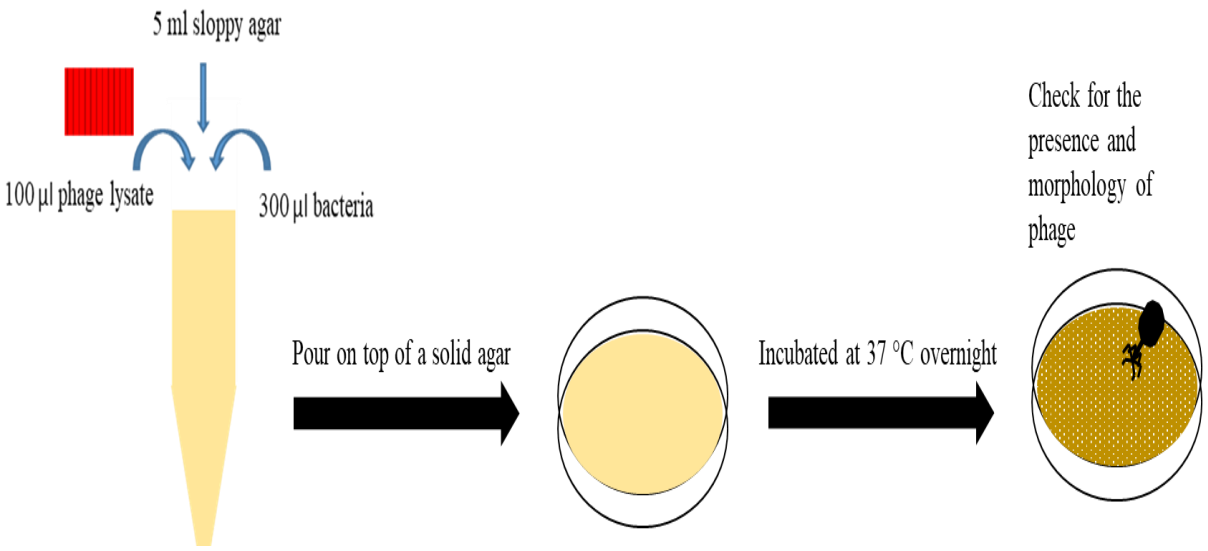



**Figure 2.1: A diagram illustrating the plaque assay.**

## 2.7 Spot test

The bacteriophage titre, or number of plaque forming units/mL (PFU/mL), can be determined by using the spot test as described in (Bonilla *et al.* 2016). To perform the spot test, as illustrated in Figure 2.2, 8 ml of semi-solid agar was mixed with 300 µl of bacterial culture and poured on top of LA plates. Once solidified, 10-fold serial dilutions of the phage sample to be tested were made, after which 10 µl of each dilution was spotted onto the bacterial lawn. The plates were incubated overnight at 37 °C. The next day, the PFU/ml was determined by multiplying the number of plaques by the dilution factor and 10, given that the volume of phage spotted was 10 µl.

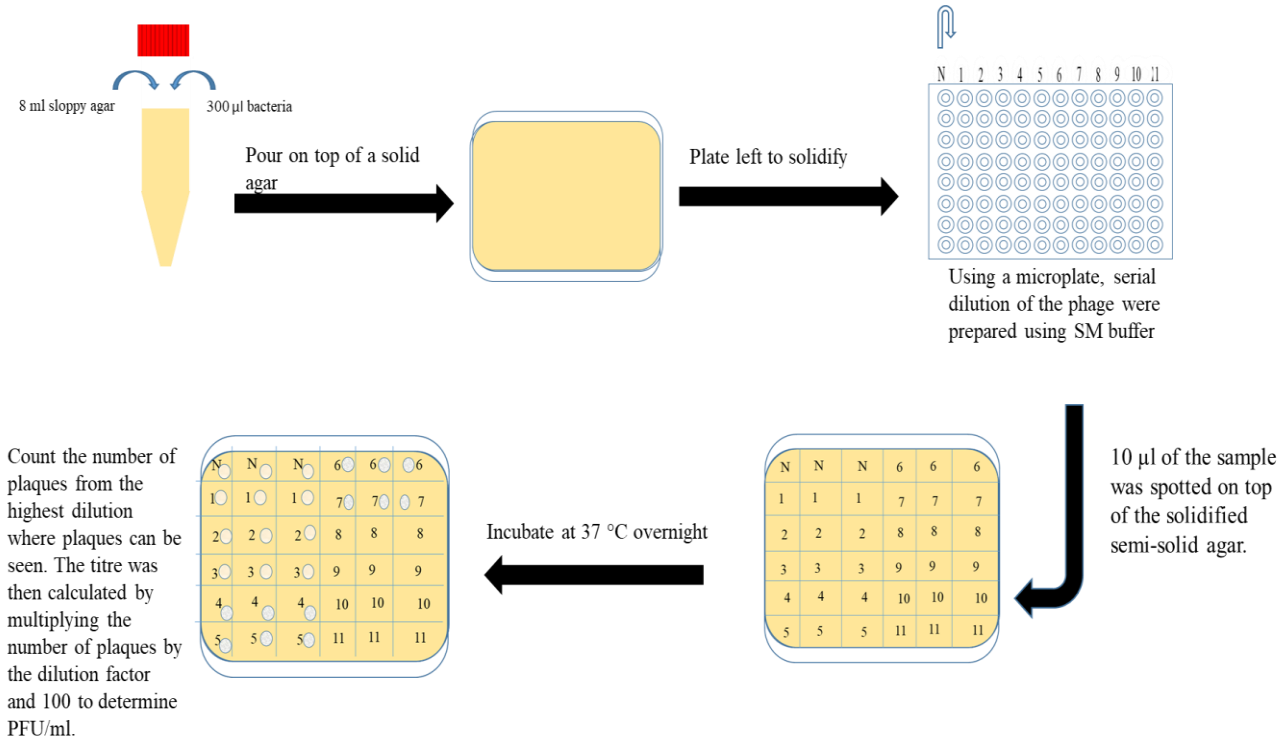



**Figure 2.2: Diagram illustrating the spot test.**

## 2.8 Phage Purification

A single plaque was selected based on distinct morphology, and re-suspended in 500µl of SM buffer (5.8 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50 ml 1 M Tris-HCl pH 7.4, dissolved in 1L $dH_2O$). After incubation for 60 minutes at 37 °C, the solution was centrifuged, and the supernatant was filtered through 0.22 µm filters (Bonilla *et al.* 2016). To produce a homogenous phage stock, five rounds of plaque picking and plaque assays were performed.

## 2.9 Phage propagation

Phages were propagated by performing plaque assays (under conditions resulting in a nearly complete lysis of the bacterial lawn) using large square petri dish plates. After incubating the plates overnight, 6 ml of SM buffer was poured on top of each propagation plate; the plates were then placed on a rocking platform for 3 to 5 hours. By using an L-shape loop, the top agar was then harvested with the SM buffer into a 50 ml sterile tube. The tubes were then centrifuged at 4,500 g for 45 minutes to remove all bacterial and agar residue. The supernatant was collected into different tubes and then filtered twice through 0.22 µm filters. The phage lysate was then stored at 4°C for subsequent spot testing to determine the phage titre (Bonilla *et al.* 2016).

## 2.10 Transmission electron microscopy (TEM)

TEM was used to visualize phages, OMVs and their interaction. A copper grid with carbon film coating was used for TEM. The grid was discharged by glow using (Q150V, Quorum®) carbon coater for 10 minutes. 4 µl of the sample was applied onto a glow discharged copper grid and incubated for 2 minutes, after which the grid was negatively stained twice with 5 µl of 1 % uranyl acetate (R1260A, Agar scientific®) and washed with 2 drops of distilled water. The grid was left to dry before being inserted into the electron microscope (JEOL 1400). The SIS Megaview software version III was used to process and acquire digital images. This protocol was adopted from (Manning and Kuehn 2011) and by following the instruction of the Electron microscopic facility providers at the University of Leicester.

## 2.11 Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis is a technique that allows for the measuring of the size and concentration of nanoparticles, such as viruses and OMVs, using laser emissions. A NanoSight LM10 instrument (Malvern Panalytical) equipped with the NTA 2.2 analytical software was used to determine the size and concentration of the extracted OMVs. This analytical tool was used as described in (Nordin *et al.* 2015). Briefly, samples were diluted in PBS between 1:100 and 1:100,000 to achieve the optimal particle count for the instrument to be analysed, which is between $1 \times 10^8$ and $1 \times 10^9$ particle/ml. The system settings used for all recordings were: camera level of 13 or 15, a detection threshold of 5, and the automatic function for all other post-acquisition sittings. Diluted samples were loaded in the sample chamber, and the camera focus was adjusted to make the particles appear as sharp, bright dots according to the description in the instrument manual. Videos were recorded for lengths of 60 or 90 seconds at room temperatures, and the measurements were analysed using the batch process function. The default outcome results and multigraph set files were exported to Microsoft Excel to be analysed (Nordin *et al.* 2015).

# Chapter Three

## 3. Isolation and characterizations of *K. pneumoniae* phages.

## 3.1 **Introduction:**

Among the creatures present in the environment, bacteriophages (phages) are the most abundant entities in the world. Phages exists in the environment with a great diversity in their characteristics such as morphology, structure, sizes and genomic sequences (Clokie *et al.* 2011). Studying the characteristic features of bacteriophages is essential to identify them, differentiate them from each other and potentially, to exploit them in different applications. To study the interaction between bacteriophages and outer membrane vesicles, phages were isolated from sewage samples.

This chapter will focus mainly on the characterisations of phages isolated from sewage samples. First of all, the isolation of phages started with a journey to the Severn-Trent Water Treatment Plant to collect the samples. Phages were then isolated by performing plaque assays and picking up the plaques according to their morphology. Each phage was subjected to at least 5 rounds of purification also by plaque assay. After purifying, each phage was propagated using the solid media propagation method. Isolated and propagated phages were then characterised. The characterisation of these phages includes a morphological characterisation by observing the phages by TEM. Phages were studied biologically by the determination of the host range specificity, by performing the one-step growth curve experiment; the stability of the phage was assessed in variant temperatures and different pH. More importantly, the genomic characteristics of the phages were studied by sequencing their genomes and performing bioinformatics analysis to reveal their genomic features.

Three phages were studied out of the nine different plaques picked initially. Two out of the three phages were *Siphoviridae* viruses, while the third one was a Myovirus. The two Siphovirus phages were successfully sequenced while the Myovirus was not, at the time of writing this thesis. Further proteomic and bioinformatics analysis was performed on the two sequenced phages, which is discussed below in this chapter. Another sequencing attempt is currently being performed for the *Myoviridae* virus that was isolated and purified in this project.

## 3.2 **Aim and objective:**

The main aim of the work outlined in this chapter was to characterise the isolated phages based on their morphology, genome and biology.

To achieve this, the following objectives were assigned:

1- To study the individual phage morphology for each phage.
2- To extract the genomic DNA of the viruses, sequence them and carry out bioinformatics and proteomics analysis.
3- To study some of the biological features of the phages.

## 3.3 Methods

### 3.3.1 Transmission electron microscope (TEM)

TEM was used to visualize the phage diversity of the isolated phages from the environmental sample. TEM was performed as described in section 2.10.

### 3.3.2 Phage DNA extraction

Phage DNA was extracted and used for sequencing. The following protocol was adopted and modified from (Rihtman *et al.* 2016). Briefly, phage lysate was mixed with 30 mg/ml DNAase and 100 mg/ml RNAase to clear the sample from any free DNA and/or RNA and incubated at 37 °C overnight. PCR was then performed using 16s rRNA universal primers to confirm for removal of bacterial host DNA from the sample, before extracting the nucleic acids from each of the phages. The following day, the lysate was mixed with 12.5 µl of 1 M $MgCl_2$. To degrade the phage capsid, 40 µl of 0.5 M EDTA, 5 µl of proteinase K (10 mg/ml), and 50 µl of 10 % w/v SDS were added. The mixture was then incubated at 55 °C for 60 minutes with interval vortexing every 20 minutes. The sample was then aliquoted into 4 chloroform-resistant tubes with volumes of 800 µl. Then, the samples were mixed with an equal amount of phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) and centrifuged at 21,000 g, at 4°C for 15 minutes. The top aqueous layer was then removed and placed into new tubes, and the same step was repeated. The DNA was then precipitated by adding 1/10 volume of 3 M Sodium acetate, and 2 volumes of ice-cold absolute ethanol, and then incubated at -20 °C overnight. The next morning, the tubes were centrifuged for 20 minutes at 21,000 g at 4 °C. Without disturbing the pellets, the top liquid was removed and 1 ml of 70 % ethanol was added to wash the pellet by centrifugation for 10 minutes; this was repeated twice. After the second centrifugation, the top liquid layer was removed by pipetting, and the pellet was left to dry for about 15 minutes. The DNA in the pellet was then dissolved in 50 µl of 5 mM TrisCl (pH 8.0 to 8.5). The DNA concentration was then determined and measured using the Qubit flourometer (Thermo scientific), and then at -20 °C, ready to be sent for sequencing.

### 3.3.3 Phage whole-genome sequencing

Genomic libraries were prepared, and whole-genome sequencing for the genomic DNA of phage Kp2811 was performed at the NUCLEUS Genomics facility at the University of Leicester, UK by Dr Nathan Brown. On the other hand, the genomic library and whole-genome

sequencing for the genomic DNA of phage Kp3153 was performed with the help of Lucy Gannon. The genomic libraries of phage Kp2811 DNA were prepared using Illumina's TruSeq® DNA Library Prep Kit (300 bp, FC-121-2003, Illumina) and Nextera XT version 2 kit from Illumina (>300 bp, FC-131-1024, Illumina). The whole-genome sequencing for both phages was performed using MiSeq® FGx system (Illumina). The resulting FASTQ files were assembled using Megahit version 1.2.1 (Li *et al.* 2015; Li *et al.* 2016) for phage Kp2811 and SPAdes version 3.13.1for phage Kp3153 (Bankevich *et al.* 2012; Nurk *et al.* 2013). The average coverage depth of the sequenced genome of phage Kp2811 was 252x, while the average coverage of phage Kp 3153 Genome was 234x.

Once the genomic assembly was complete, the final contigs of both genomes were annotated with Prokka version 1.12 using the protein model databases HAMAP vog and Bacteria Viruses, in addition to manual gene prediction using all the complete viral genomes within the NCBI database and the European Nucleotide Archive (Seemann 2014). Rapid Annotation using the Subsystem Technology (RAST) pipeline was used as a second method for annotation (McNair *et al.* 2018).

### 3.3.4 **Bioinformatics Analysis**

The Genome map was visualized and created using the SnapGene Viewer 3.3.4 software (from GSL Biotech: available at http://www.snapgene.com). To compare between the annotated genomes and similar phages, a proteomic tree was generated using the ViPTree server (Viral proteomic tree server) version 1.9 (Mihara *et al.* 2016; Nishimura *et al.* 2017). Once the tree was generated, a genomic alignment was made between the sequenced phage genomes and closely related genomes from the VIPTree database.

### 3.3.5 **One-step growth curve**

The one-step growth curve is one of the fundamental experiments that is used to characterise newly isolated phages. It is essential to understand key phenotypic characteristics of isolated phages. This curve defines the latent period and the burst size of the phage by determining the time needed for the phage to complete one replicative cycle. This protocol was adopted from (Clokie 2018). In brief, the experiment started by pipetting 9.9 ml of a mid-log phase bacterial culture ($OD_{600}$ ~0.5) into a sterile flask, then 100 µl of lysate at a concentration of $10^7$ phage was added to the same flask (adsorption flask). Immediately afterward, the flask was incubated

for 5 minutes to allow the adsorption of the phage to the bacterial cells. After that, the phage-bacterial mixture was diluted to three different tubes, namely A, B and C, by taking 100 µl aliquots from the adsorption tube to tube A, which contained 9.9 ml of media, by taking 1 ml from tube A to tube B and taking 1 ml from tube B to tube C. This point was considered as time zero of the growth curve. Starting from zero minute and for every 5 minutes after, until 90 minutes; 100 µl was collected from each of the three tubes, A, B and C and then mixed with 300 µl of mid-log phase bacterial culture and 7 ml of 0.4 % LB agar (semi-solid agar). This was then poured on top of LB agar plates (plaque assay). As an adsorption control, 1 ml of the sample from flask A was transferred into a tube with a few drops of chloroform, to confirm the adsorption of the phages to the bacteria and to identify the burst size at the end of the experiment. The plates for each time point were incubated overnight at 37 ºC. After that, the plaques were counted for each plate and plotted, and the burst size was calculated accordingly.

### 3.3.6 **Phage thermal and pH stability**

This experiment was performed to investigate the ability of isolated phages to survive in various temperatures and pH mediums. Multiple aliquots of the phage lysates were incubated at different temperatures, namely 40 ºC, 50 ºC, 60 ºC, 70 ºC and 80 ºC for 1 hour. After incubation, a spot test was performed for each aliquot to determine whether the phages were able to survive and tolerate the various temperatures, and if so, to what extent. To study the stability of the phages in different pH, SM buffer was prepared with different pH values ranging from 1 to 10. The pH of the buffer was adjusted by using 1M HCL and/or 1M NaOH. 50 µl of phage lysate at a concentration of $10^7$ was transferred into each pH tube; the final phage titre in each tube was $10^6$. The phage mixtures were then incubated at 37 ºC for 2 hours. After that, the survival rate of the phages was determined via spot test (Ahmadi *et al.* 2016).

### 3.3.7 **Determination of the phage host range**

The plaque assay was used to define the phages' ability to infect different bacterial hosts. In a 15 ml falcon tube, 100 µl of the phage lysate at a concentration of $10^7$ was mixed with 300 µl of a mid-log phase bacterial culture and topped with 7 ml of 0.4 % agar (semi-solid agar). The tube was inverted 5 times before pouring the mixture on top of agar plates. The poured mixture on the plates was left to dry for 5 minutes, then incubated overnight at 37 ºC. The next day, the plates were checked for any formed plaques.

## 3.4 Results

### 3.4.1 Phage Kp2811 and phage Kp3151 characterization

#### 3.4.1.1 General features of phage Kp2811 and phage Kp3153

Phage Kp2811, phage Kp3153 and phage Kp2811M were isolated from a sewage sample that was collected from the Severn-Trent water treatment plant in Wanlip (Leicester). The phages were picked according to their plaque morphology. All phages were purified through five rounds of purification, and were propagated to a concentration of 1 x $10^9$. For two of the phages, phage Kp2811 and phage Kp3153, phage DNA was successfully extracted and sequenced as described above (Sections 3.3.2 and 3.3.3).

After sequencing analysis, phage Kp2811 was found to have a genome of 49.131 bp, (Figure 3.1). After sequencing, the DNA sequence was blasted against the NCBI database using the BLASTn tool to find similarities between Kp2811 and the other. The blast results showed a homology of phage Kp2811 (49,131bp) with *Klebsiella* phage TAH8 (49,344 bp), Klebsiella phage Shelby (49,045 bp), Klebsiella phage NJS3 (49,387 bp) and other *Klebsiella* phages.

In a similar manner, phage Kp3153 genomic DNA analysis was performed after DNA was extracted and sequenced succesfully. The sequence analysis revealed that this phage possesses a genome size of 51,695 bp (Figure 3.2). The DNA sequence was blasted against the NCBI database using BLASTn to find similarities between the sequenced phage and the other similar phages in the NCBI database. The blast results showed homology between phage Kp3153 (51,695 bp) and *Klebsiella* phage KOX1 (50526 bp), *Klebsiella* phage Vb_KpnS_KpV522 (51,099 bp), *Klebsiella* phage Vb_KpnS_Penguinator (51,678 bp), and other *Klebsiella* phages.

All these phages belong to the *Caudovirales* order, *Siphoviridae* family, *Tunavirinae* Genus and *Webervirus* species. According to this, and as shown in (Figure 3.3A and Figure 3.3B) below in the TEM analysis, both phages, phage Kp2811 and phage Kp3153, belong to the *Siphoviridae* family, and have the same taxonomy as the other similar phages. According to the phage morphology in Figure 3.3C, phage Kp2811M belongs to the *Myoviridae* family.

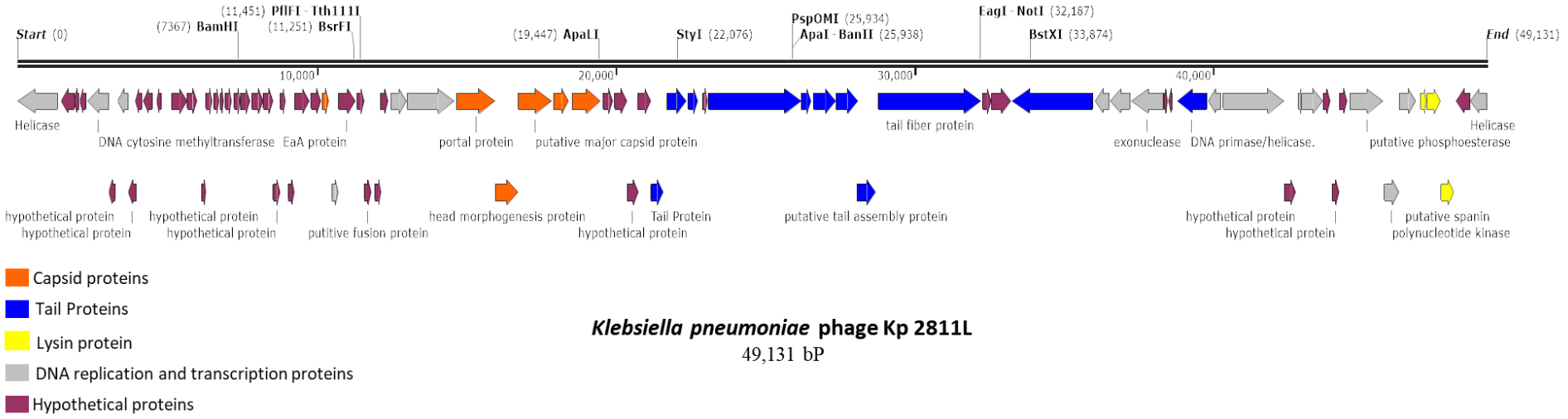



**Figure 3.1. Linear annotated genomic map of phage Kp2811**.

Shown are the various different proteins which are encoded within the genome of phage Kp2811. Highlighted are genes relating to the virion capsid structure (orange), tail protein structure (blue), lysis proteins (yellow), DNA replications and transcription (grey), and other hypothetical proteins with unknown involvements (purple). The Genome map was generated using the snap gene viewer software.

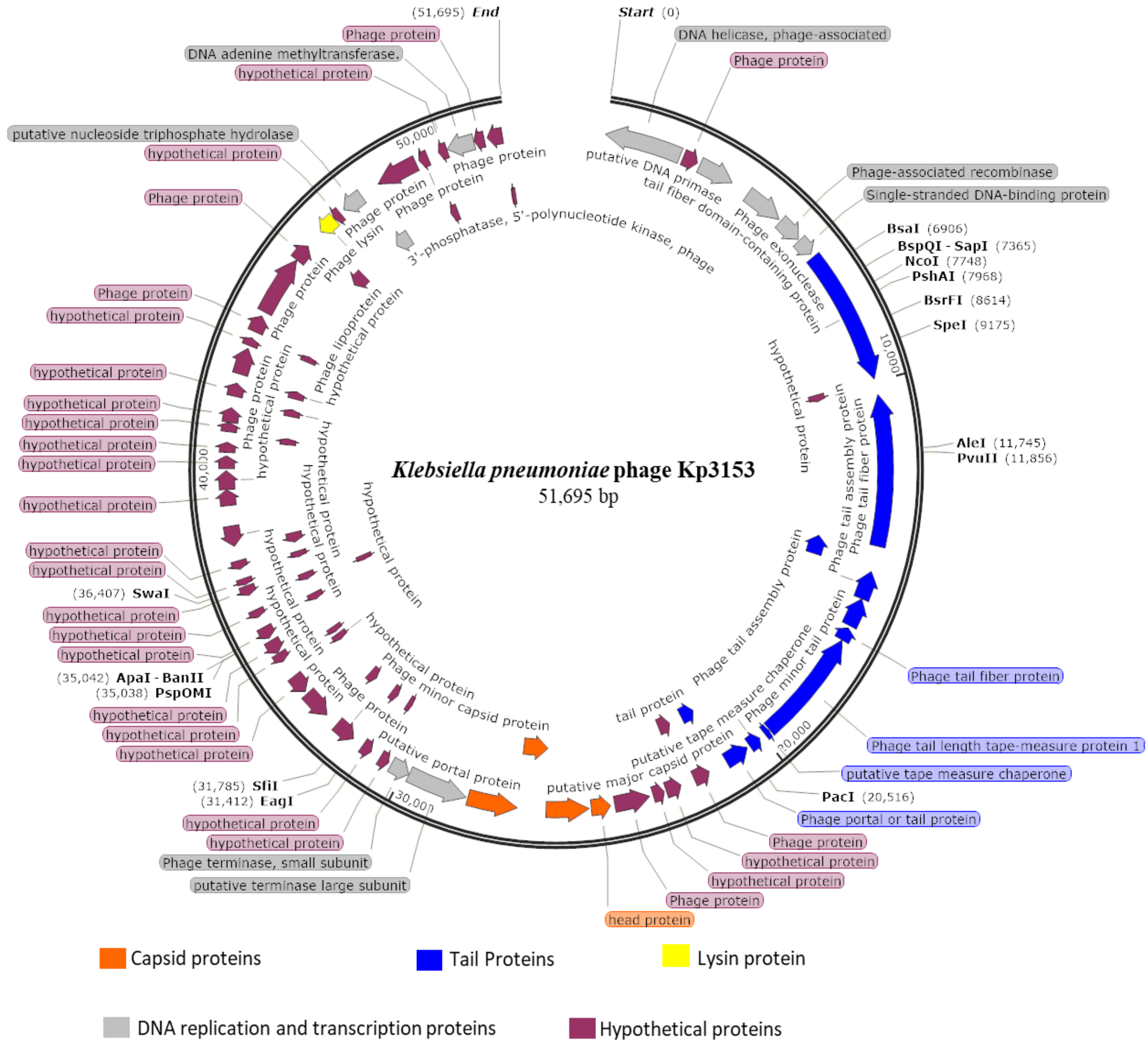



**Figure 3.2: Circular annotated genomic map of bacteriophage Kp3153.**

Highlighted are the proteins involved in the virion capsid structure (orange), tail protein structure (blue), lysis proteins (yellow), DNA replications and transcriptional proteins and other hypothetical proteins with unknown involvements (dark pink). The map was generated using the snap gene viewer software.

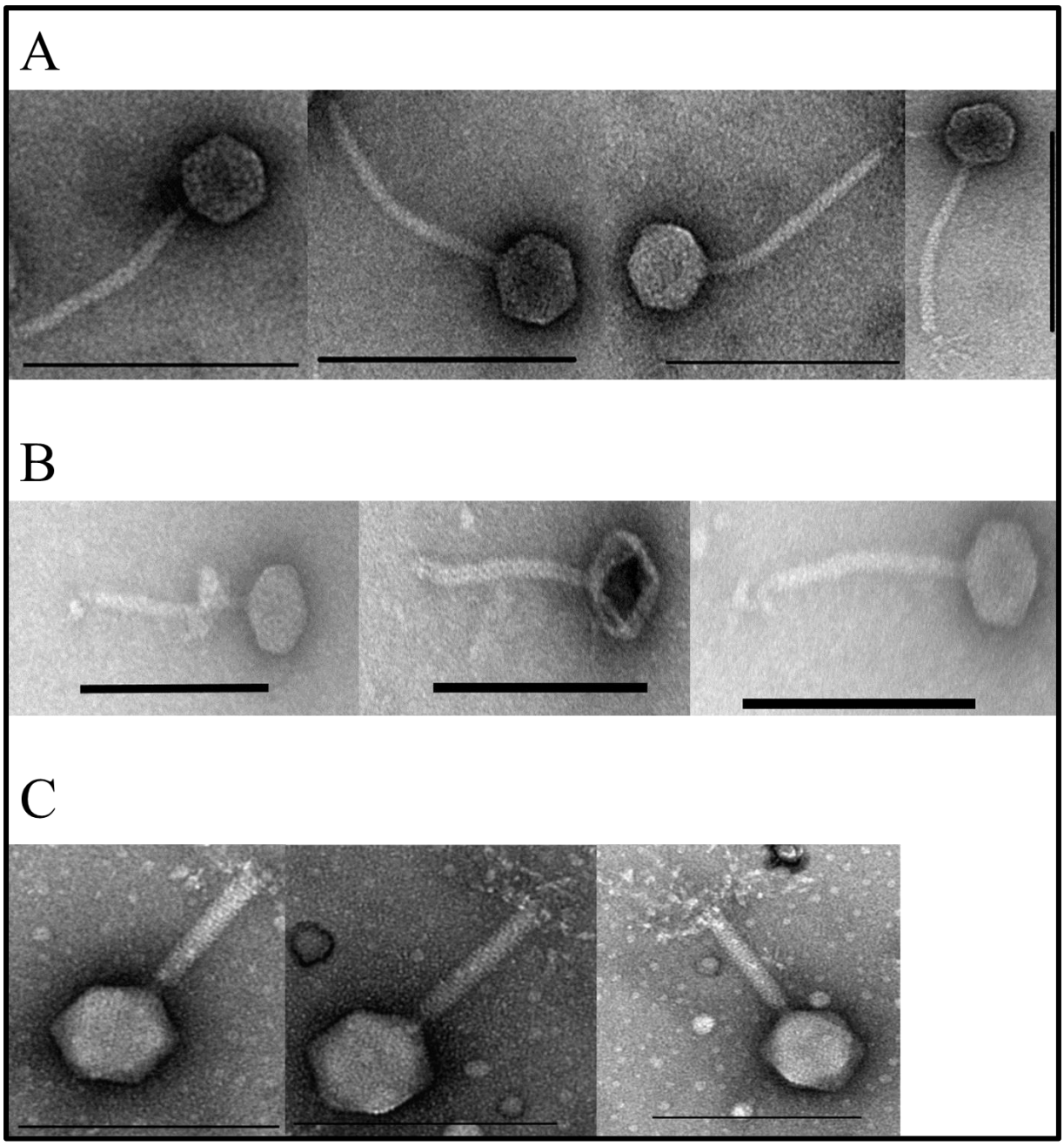



**Figure 3.3: Transmission electron microscopy analysis of the purified and propagated phages.**

Figure (A) shows the structure of the phage Kp2811 including the head, tail, and the tail fibres of the phage. Figure (B) shows the structural details of the phage Kp3153. Both phages as shown belong to the *Siphoviridae* family. Figure (C) shows the structural details of phage Kp2811M. The phage as shown belongs to the *Myoviridae* family (Scale bar = 200nm).

### 3.4.1.2 **Proteomic tree and Genome alignment**

The annotated genome of phage Kp2811 was compared with that of the phages most related to it. As a result, a proteomic tree was generated for phage Kp2811 using the VIPTree software (Figure 3.4A). These phages were aligned with phage Kp2811, and the homology was

confirmed between this phage and the related phages as shown in (Figure 3.5). In a similar manner, the annotated genome of phage Kp3153 was compared with the annotated genomes of the most related phages. A proteomic tree was generated as shown in Figure 3.4B using the VIPTree software and the phage genome was aligned with the most related phages to confirm the homology between phage Kp3153 and the related phages (Figure 3.6).

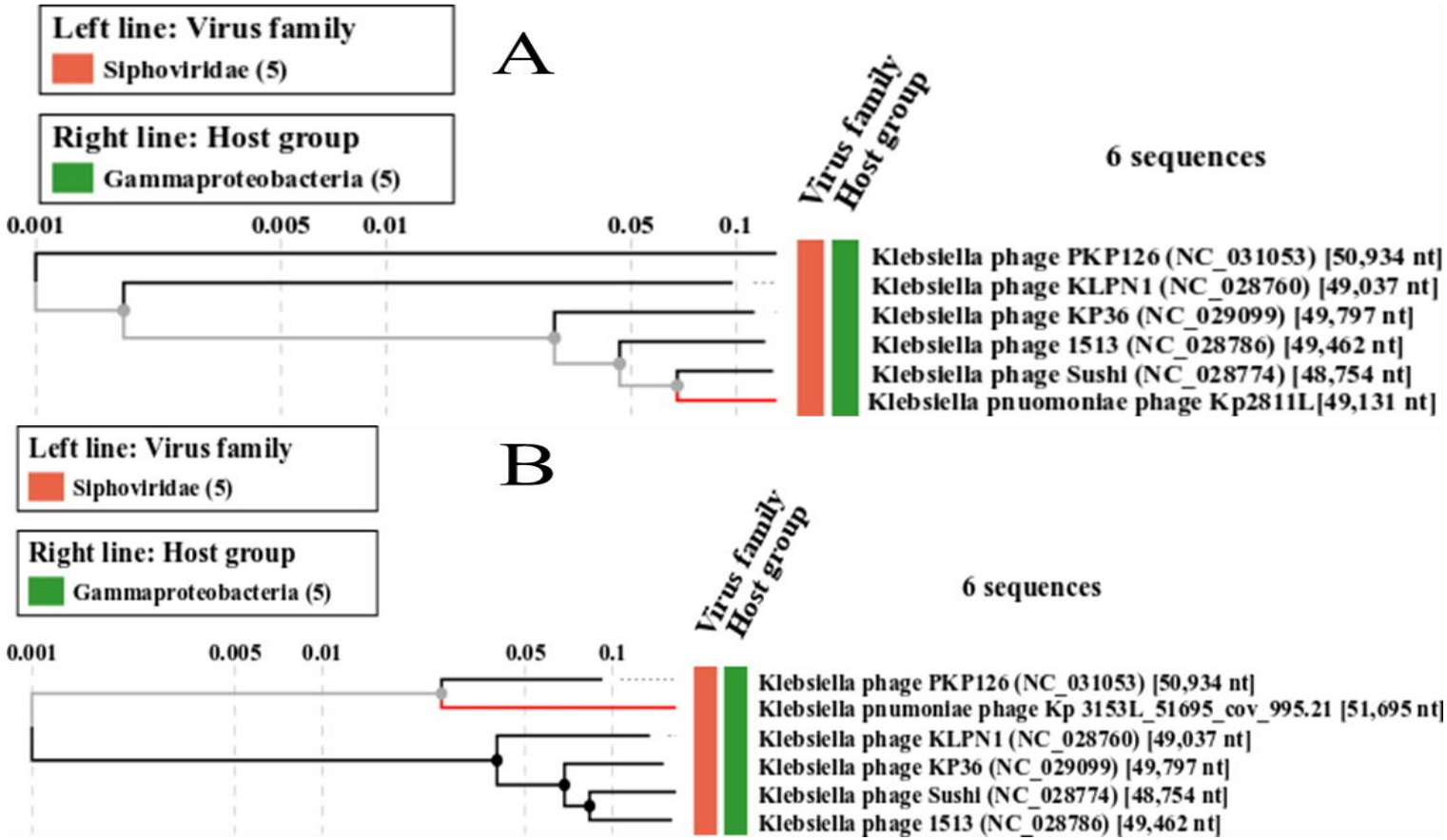



**Figure 3.4: Proteomic tree showing the most related phages to phage Kp2811 and phage Kp3153.**

Figure (A) shows all related phages that belong to the same family as Kp2811 and to the same host group, while figure (B) shows all related phages that belong to the same family as Kp3153 and to the same host group. Both trees were generated using the VipTree software.

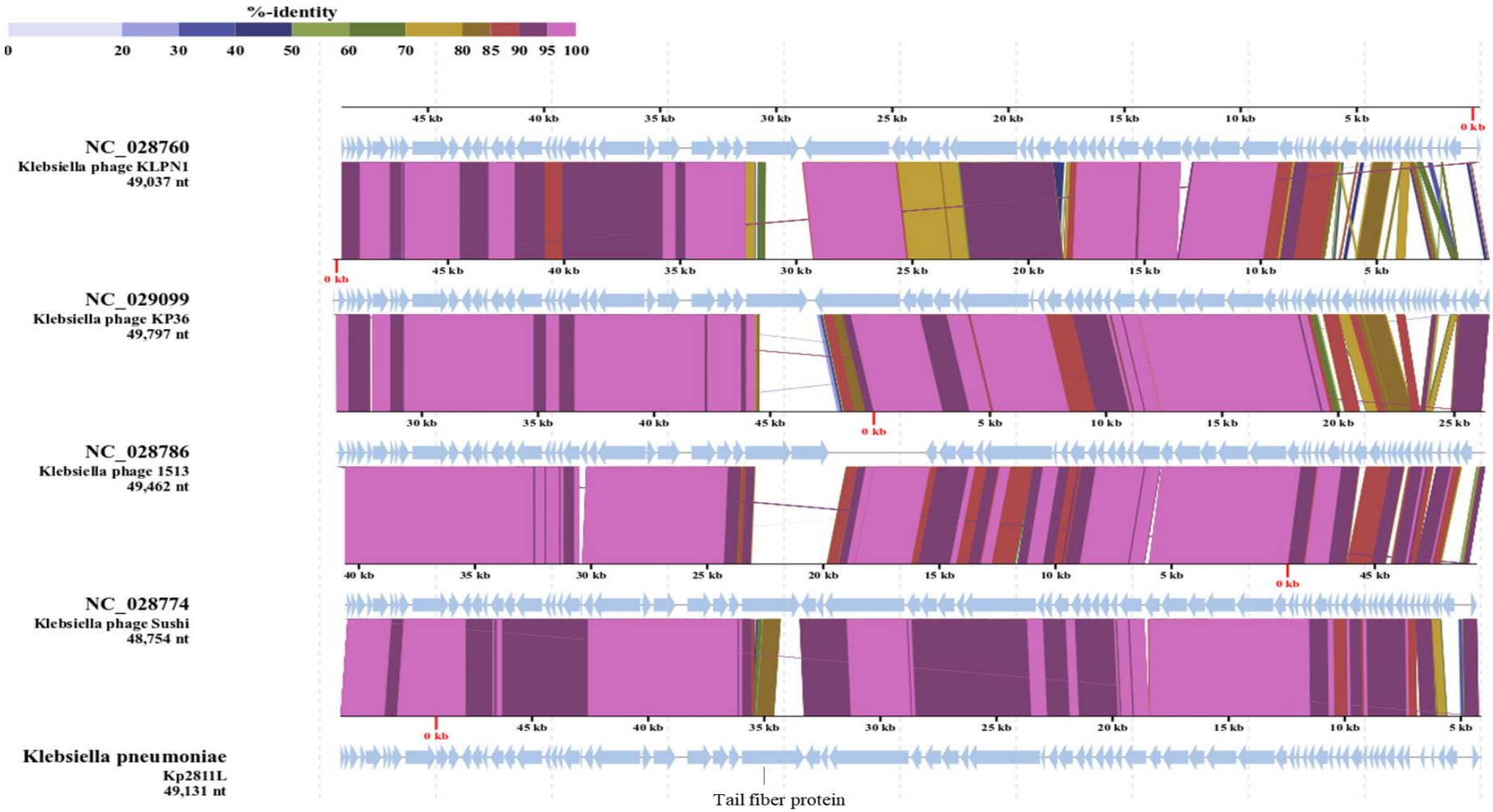



**Figure 3.5. Alignment of phage Kp2811 with other *K. pneumoniae* phages.**

The figure shows the homology of this phage with other similar phages from the same genus. The figure was generated by the VipTree software.

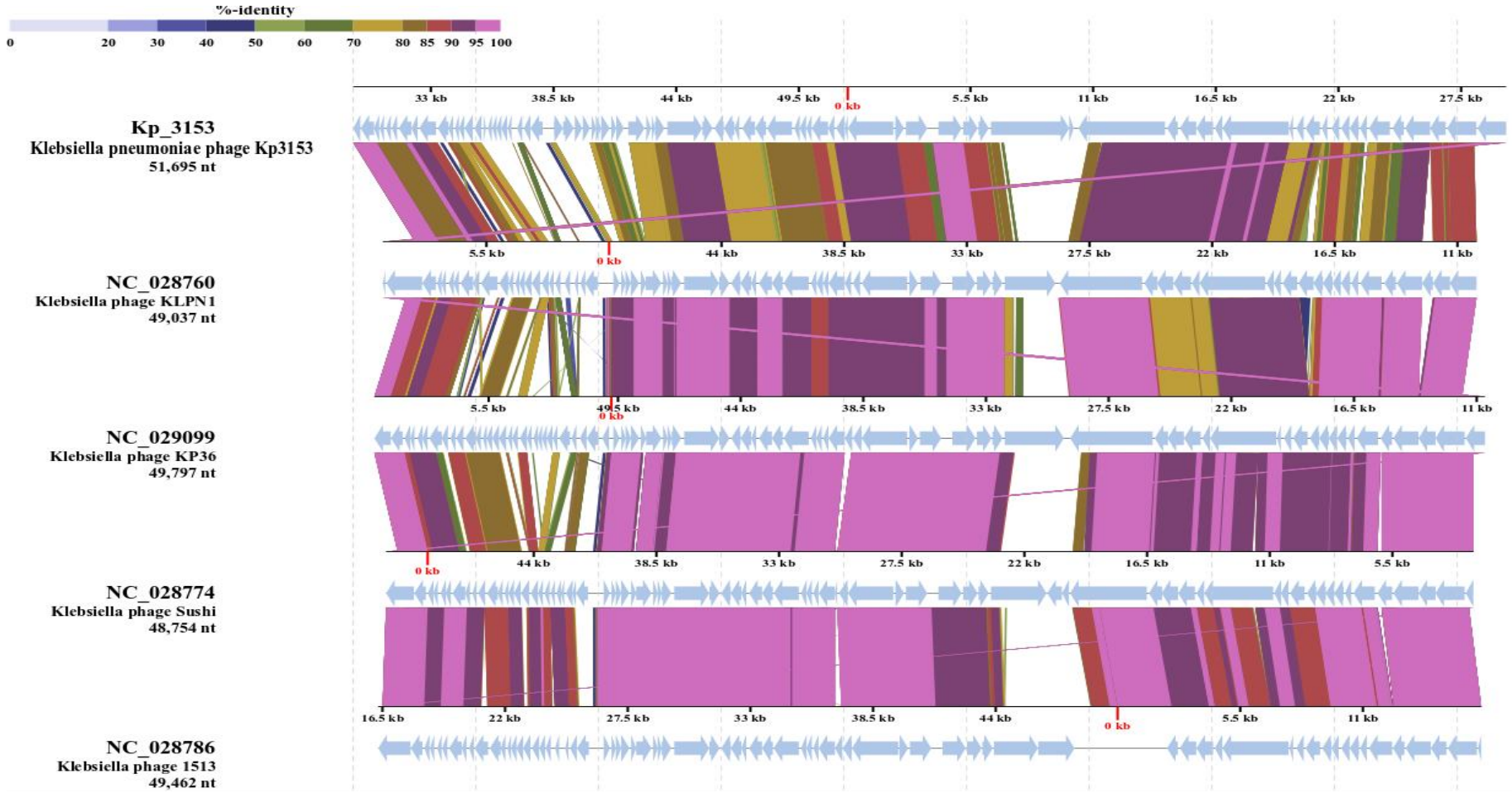



**Figure 3.6: Alignment of Kp3153 phage with other *K. pneumoniae* phages.**

The figure shows the homology of this phage with other similar phages of the same genus. The figure was generated by the VipTree server**.**

### 3.4.1.3 One-step curve experiments for phage Kp2811 and phage Kp3153

Phage growth rates were determined by the analysis of the growth cycle of the phage for one complete cycle; this was done by counting plaques formed at several time points in the one-step growth curve experiment. This experiment was performed for phage Kp2811 and phage Kp3153. As shown in (Figure 3.7A), the latent period of phage Kp2811 lasted for approximately 7 minutes, while the calculation of the burst size for one lytic cycle was found to be about 20 PFU per infected cell. On the other hand, the latent period of phage Kp3153 lasted for approximately 12 minutes (Figure 3.7B), while the burst size for one lytic cycle was found to be approximately 19 PFU per infected cell.

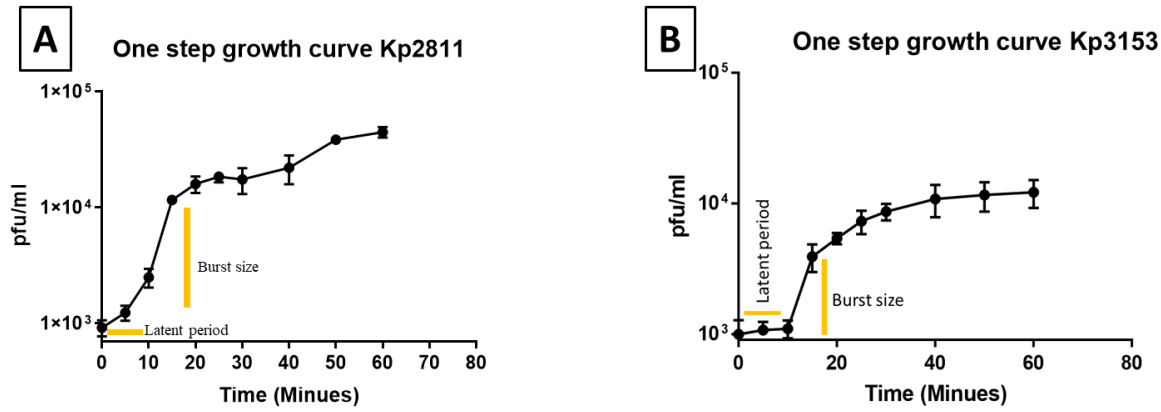



**Figure 3.7: One-step growth curve of phage Kp2811 and phage Kp3153.**

Graph (A) shows the latent period of phage Kp2811, which is around 7 minutes, and the average burst size of approximately 20 PFU per infected cell. Graph (B) shows the latent period of phage Kp3153, around 10 to 15 minutes, and the burst size, which is approximately 19 PFU per infected cell. The data was generated from three replicates for each phage.

### 3.4.1.4 Phage Kp2811 host range investigation.

The host range and the lytic activity of the isolated phages, phage Kp2811, phage Kp3153 and phage Kp2811M, were tested by plaque assays performed on several different lab strains of *K. pneumoniae* and *E. coli*. Table 3.1 shows the ability of phage Kp2811 to infect only 2 of the *K. pneumoniae* strains of the tested strains, one of which was the strain on which this phage was isolated. Additionally, the analysis shows the ability of phage Kp3153 to infect only 1 *K. pneumoniae* strain, and it shows the ability of phage Kp2811M to infect 3 *K. pneumoniae* strains. However, the tested phages show the inability to infect any of the *E. coli* lab strains which were used in the experiment.

**Table 3.1: A summary of the host range of phage Kp2811 against lab strains of *Klebsiella Pneumoniae* and *E. coli*.**

| Bacterial strain | Phage Kp2811 | Phage Kp3153 | Phage Kp2811M |
|---|---|---|---|
| ***K. pneumoniae KR2811*** | **Yes** | **No** | **Yes** |
| ***K. pneumoniae KR3153*** | **Yes** | **Yes** | **No** |
| ***K. pneumoniae KR2813*** | **No** | **No** | **Yes** |
| ***K. pneumoniae KR4010*** | **No** | **No** | **No** |
| ***K. pneumoniae KR640*** | **No** | **No** | **No** |
| ***K. pneumoniae KR4016*** | **No** | **No** | **No** |
| ***K. pneumoniae KR2044*** | **No** | **No** | **Yes** |
| ***Escherichia coli DH5α*** | **No** | **No** | **No** |
| ***Escherichia coli C600*** | **No** | **No** | **No** |

#### 3.4.1.5 Temperature and pH sensitivity of phage Kp2811

Investigating the stability of phages in various temperatures and different pH concentrations is essential information for the storage and downstream applications of phages. Various experiments were performed to investigate how isolated phages respond to different pH and temperatures. After a 1-hour incubation of phage Kp2811 at different temperatures, phage Kp2811 was found highly stable up to 40 ºC and 50 ºC (Figure 3.8A). There was a one log

reduction of the phage titre after incubating at 60 ºC. However, the phage was not able to survive temperatures of 70 ºC and above. The control used in this experiment was the main stock, which was stored kept at 4 ºC. Furthermore, phage Kp2811 was incubated in a buffer, modified to be in different pH conditions ranging from 1 to 10, for 2 hours. The phage showed high survival rate when exposed to pH values from 4 to 10 (Figure 3.8B). On the other hand, the phage was not able to survive at low pH values ranging from 1 to 3.

In a similar manner, the thermal and pH stability of phage Kp3153 was tested in order to find the optimum temperature for storage, and potential for different applications. After a one-hour incubation at different temperatures, and as shown in (Figure 3.8C), phage Kp3153 appeared to be highly stable at 40 ºC and 50 ºC. The phage titre reduced by almost one log when tested at 60 ºC. However, the phage was not able to survive in temperatures 70 ºC and above. The control of this experiment was to test the phage stock which is kept at 4 ºC. In addition, phage Kp3153 was incubated in a buffer ranging from pH 1 to 10 for 2 hours. The phage showed a high survival rate when tested against pH 3 to 10 (Figure 3.8D). On the other hand, the phage was not able to survive at pH 1 and 2.

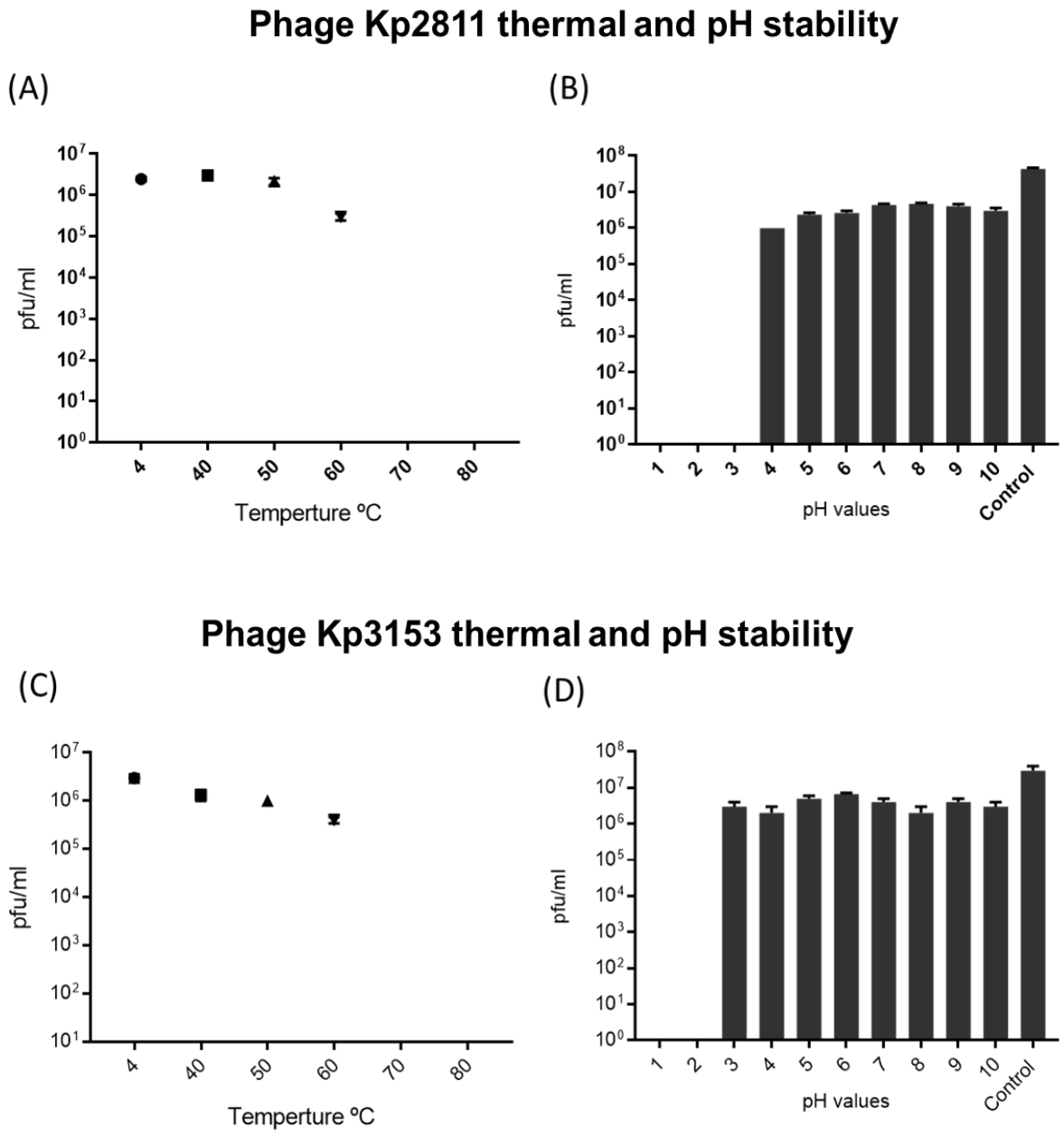



**Figure 3.8: Phage Kp2811L and phage Kp3153 thermal and PH stabilities.**

(A) Phage Kp2811L thermal stability, after 1 hour of incubation at different temperatures, the phage was not affected when incubated at 40 ºC and 50 ºC. At 60 ºC, the phage titre decreased by one-log. The phage could not survive when exposed to 70 ºC and above. A temperature of 4 ºC was used as the control for this experiment, as the phage is usually stored at this temperature. (B) Phage Kp2811L pH stability. After incubating and exposing phage Kp2811L to different pH solutions for 2 hours, the phages were able to survive pH values ranging from 4 to 10. On the other hand, no phage survival was observed from pH 1 to 3. (C) Phage Kp3153 thermal stability: After a one-hour incubation at different temperatures, the phage was not affected when incubated at 40 ºC, 50 ºC and 60 ºC. The phage was not able to survive at 70 ºC and 80 ºC. (B) Phage Kp3153 pH stability: After incubating Kp3153 phage in different pH solutions for 2 hours, the phages were able to survive in pH solutions ranging from 3 to 10. On the other hand, no phage survival was observed in pH 1 and 2. All the experiments were performed in three biological triplicates and the error bars represent SEM.

## 3.5 **Discussion**

The general aim of this project was to assess whether the interaction of bacteriophage with OMVs can reduce the immune response that is elicited by the OMVs alone upon interaction with immune cells. In other words, the OMVs, as discussed in (section 1.1.6), show an ability to stimulate pro-inflammatory cytokines upon interaction with immune cells. On the other hand, bacteriophages themselves, as discussed in (Section 1.2.6), do not often stimulate the immune cells to produce pro-inflammatory response. Additionally, OMVs can act as a first line of defence against environmental stressors for bacteria, and phage attack is one of such stressors. OMVs were shown to interact with bacteriophage, and this interaction resulted in the bacteriophage inactivation. In this approach, the plan is to assess whether the interaction of bacteriophage with OMVs will result in the disruption of the OMVs and eventually will spare the immune system the efforts of responding to OMVs. For that, the main elements of this projects are bacteriophages and OMVs. The main focus of this chapter is the isolation and characterisation of the phages with an ability to infect *K. pneumonia*e.

The first isolated and characterised phage was Kp2811. The sequence comparison of this phage was performed in order to define the most similar isolated phages to this phage. The Kp2811 sequence was found to be similar to previously isolated phages whose genomes are deposited in the NCBI database. The sequences of similar phages was extracted from the NCBI database and the average nucleotide identity (ANI) of phage Kp2811 was identified and compared with the other similar phages. The genomic sequence of Kp2811 has 91.2 % similarity with *Klebsiella* phage TAH8, 90.25 % with *Klebsiella* phage NJS3 and 85.2 % with *Klebsiella* phage Shelby. According to the species demarcation criterion, phages that are 95 % similar at the nucleic acid level can be considered of the same species (Adriaenssens and Brister 2017). Therefore, Kp2811 phage belongs to *Caudovirales* order, *Siphoviridae* family, *Tunavirinae* Genus and *Webervirus* species similar to other phages above. Nevertheless, it is a different and novel phage.

The second phage was Kp3153, which was found to be similar to previously isolated phages deposited in the NCBI database. However, the nucleic acid sequence of Kp3153 has 93 % ANI with *Klebsiella* phage Vb_KpnS_Penguinator and 94 % with both *Klebsiella* phage KOX1 and *Klebsiella* phage Vb_KpnS_Penguinator. According to the main species classification standards, phages that are less than 5 % different at the nucleic acid level can be considered to

be members of the same species (Adriaenssens and Brister 2017). This suggests that, despite the high similarity in their (ANI), Kp3153 phage belongs to the *Caudovirales* order, *Siphoviridae* family, *Tunavirinae* Genus and *Webervirus* species, and is a novel phage that is similar to the other phages listed above.

The host range testing of any isolated phage is important in order to define the ability of the phages to infect different bacterial strains. The host range testing of both phages, Kp3153 and Kp2811, revealed their ability to infect only a small number of bacterial strains from the lab collection. However, both phages were tested against a limited number of bacterial strains due to the limited access to other *K. pneumoniae* strains and lack of clinical strains to be tested. Nevertheless, Kp2811 and Kp3153 burst sizes were 19 and 20 PFU respectively, which revealed that both phages are able to perform strong lytic activity due to their ability to burst out of the bacterial cell relatively quickly. Because of that, each one of them can be a part of a phage cocktail fighting different bacterial strains. As future work, these isolated phages should take part in a host range study testing against wider, clinical and non-clinical bacterial strains collection.

The physical and chemical stabilities of both phages, Kp2811 and Kp3153, were determined to evaluate their features. Knowing whether the phage is stable when stored, transported or even when used in different applications such as spray-drying or encapsulation, is important in order to ensure that both phages are fit for use. A phage cannot be effective if it is not stored and prepared appropriately. The temperature stabilities of both phages were tested at different temperatures. Both phages were stable in temperatures ranging from 4 $^{o}$C to 60 $^{o}$C. On the other hand, none of the phages could withstand temperatures higher than 60 $^{o}$C; after one hour of incubation, they were inactivated. The stability of both phages was also tested at different pH. Phage Kp2811 shows an ability to survive in pH values ranging from 10 to 4, while phage Kp3153 was able to survive in different pH levels ranging from 10 to 3. None of the phages were able to endure pH levels of 1 and 2. Furthermore, phages need to be stable at a wider range of temperatures and different pH levels in order to be used in different phage applications, such as therapy, and be able to resist different environmental conditions.

The third isolated phage was Kp2811M. This phage showed a plaque morphology smaller than the plaque morphology of Kp2811 phage, due to which it was named Kp2811M (medium). Several attempts were made to identify the genomic sequence of this phage. Unfortunately,

none of the sequencing attempts were successful. The same extraction method (Section 3.3.2) that was performed to extract the genomes of other phages was performed to extract the genome of this phage. The extracted genome sequencing was performed by three different sequences facilities, two of the facilities were in University of Leicester and the third was in University of Birmingham. Genome sequencing is a vital step in phage characterisations because it identifies the novelty of the phage and whether this phage has been characterised previously.

The TEM images in (**Error! Reference source not found.**) show that this phage belongs to *Myoviridae* family; its size from head to tail is about 200 nm. Host range analysis was performed for this phage and it shows the ability of the phage to infect at least 3 different *K. pneumoniae* strains. Future work on Kp2811M phage, involves investigating the reasons behind the failed sequencing attempts and performing biological characterisation to study the ability of the phage in different environmental conditions. Additionally, future work should test the ability of the phage to infect different clinical and non-clinical *K. pneumoniae* strains.

# Chapter four

## 4. Extraction and purification of OMVs from *K. pneumoniae* and *E. coli*

## 4.1 **Introduction:**

Outer membrane vesicles (OMVs) are one of the virulence factors of Gram-negative bacteria. OMVs are spherical particles, ranging between 20-250 nm in diameter. They are naturally produced during bacterial growth (Hoekstra *et al.* 1976). OMVs are formed as small lumps on the outer membrane of the bacterium and eventually pinch off from the membrane, before being released. OMVs can scatter around the bacterial cells and can define certain biological functions (Kulp and Kuehn 2010).

OMVs have been studied thoroughly and it has been found that these vesicles play a role in the delivery of toxins, nutrients and other substances from the bacterial cell to other bacterial cells and to eukaryotic host cells. OMVs modulate the immune response of the host in addition to other functions (Ellis *et al.* 2010; MacDonald and Kuehn 2012b; Berleman and Auer 2013). The extraction and purification of OMVs in a sufficient amount and in a reproducible manner is challenging, and crucial for the downstream application of OMVs.

For this project, OMVs need to be extracted from two organisms of interest; one is *Klebsiella pneumoniae* (*K. pneumoniae*), a non-motile, capsulated Gram-negative bacterium, ubiquitous in many environments. It is classified as a member of *Enterobacteriaceae*. *K. pneumoniae* is one of the main causative agents of nosocomial infections. This bacterium is a major problem for the healthcare system, especially with its ability to adopt multidrug resistance genes. It is an opportunistic organism that can cause different human infections such as urinary tract infection, respiratory infection, bacteraemia, septic shock, as well as deep wound infections such as bed sores, especially for long-term hospital patients (Herridge *et al.* 2019).

The second organism is *Escherichia coli* (*E. coli*), a common commensal bacterium that is considered a part of the human microbiota. There are also numerous pathogenic strains of *E. coli*. *E. coli* is abundantly available in the environment and is frequently used as a model organism in research labs, due to its well-studied and defined genome and genomic modification (Fairbrother *et al.* 2019). *E. coli* has been used as a model to study and understand OMVs (Aguilera *et al.* 2014; Kulkarni *et al.* 2015). Although there are many pathogenic *E. coli* strains, the one used in this project was the non-pathogenic lab strain *E. coli* DH5α.

This chapter mainly focuses on extracting OMVs from *K. pneumoniae* and *E. coli* by the standard centrifugation method and attempts to optimize a method that is suitable and reproducible for extraction and purification of high-quality OMV preparations from Gram-negative organisms. For the purpose of confirming the extraction and to identify the presence of OMVs, the GroEL protein was targeted. GroEL is a conserved protein that belongs to the chaperone family and exists in many bacteria. It is important to the bacteria to mediate proper protein folding in order for these proteins to function properly (Hayer-Hartl *et al.* 2016; Washburn *et al.* 2019). GroEL is a membrane protein. Since the OMVs are made out of the bacterial OM, GroEL is part of OMVs.

These OMVs need to be extracted in sufficient quantity and as pure as possible for the purpose of interacting them with bacteriophages and to study the effect of this interaction on the host immune response.

In this chapter, we aimed to find the best possible method to isolate and purify OMVs from *K. pneumoniae* and *E. coli* and to validate the steps of this method to assure an excellent yield of OMVs and obtain the most purified sample. To achieve this, the following objectives were assigned:

- To extract *K. pneumoniae* OMVs by the centrifugation-based method.
- To test the quality, quantity and purity of this extraction.
- To optimize a protocol to extract OMVs from *E. coli*.
- To verify the quality of the optimized protocol extraction.

## 4.2 Methods

### 4.2.1 Bacterial strains identification confirmation by 16S RNA PCR

PCR confirmation for *K. pneumoniae* strains was performed by amplifying the 16S rRNA with the use of 16S rRNA universal primers, followed by sequencing. PCR was performed according to suggested protocol from manufacturer of BIOTAQ polymerase (bioline meridian bioscience, REF# BIO-21040). Briefly, the amplification reaction was carried out by using the following master mix reaction: 10 µM forward and reverse primers, (table 1), 2.5 mM dNTPs, 10x reaction buffer, 50 mM $MgCl_2$, 5 U/µl BIOTAQ polymerase and 10 ng/µl DNA template, which was prepared by boiling 50 µl of bacterial suspension at 100 °C for 10 minutes. After that, RNAse and DNAse-free water was added to reach a final reaction volume of 50 µL for each sample. Then, the master mix was aliquoted into small PCR tubes, 48 µl each, after which 2 µl of each sample were added to labelled tubes and loaded into a thermos-cycler (SensoQuest). The thermal cycling settings were as following: initial denaturation at 95 °C for 5 minutes followed by 30 cycles of denaturation step at 95 °C for 30 seconds, annealing step at 55 °C for 30 seconds, extension step at 72 °C for 45 seconds, and a final extension step at 72 °C for 5 minutes. After the completion of the 30 cycles, the samples were stored in 4 ºC until further sequencing.

**Table 4.1. Forward and reverse primers used for the 16S rRNA gene amplification**

| Primer | Sequence (5' to 3') | Purpose |
|---|---|---|
| 1391R | GACGGGCGGTGTGTRCA | To amplify 16S rRNA for bacterial identification |
| 8F | AGAGTTTGATCCTGGCTCAG | |

### 4.2.2 Gel electrophoresis

PCR products were loaded onto 1 % agarose gel, which is prepared by mixing 0.6 g agarose and 60 ml 1x TAE buffer (40 mM Tris base, 20 mM Acetic acid and 1 mM EDTA) and then microwaved for five minutes. Thereafter 0.5µg/ml GelRed was added to this, after which it was poured into a gel cast and left to solidify. The gel was run at 100 volts for 45 minutes. Bands were visualised by Chemodoc and then were excised using a scalpel while visualised under a UV trans-illuminator. PCR products were then extracted from excised gel fragments

using a gel extraction kit (QIAquick Spin Columns (100) Cat No. /ID: 28115) and following the manufacturer instructions.

Sequencing was carried out at GATC biotech, UK. Sequences were blasted against the NCBI non-redundant nucleotide collection using the Blast algorithm accessed online to assign bacterial species.

### 4.2.3 Outer membrane vesicles (OMVs) extraction and purification from *K. pneumoniae*:

#### 4.2.3.1 OMVs extraction-centrifugation based method:

OMVs were prepared from 30 ml overnight cultures as described previously (McBroom and Kuehn 2007; Manning and Kuehn 2011). Briefly, cells were pelleted (10,000 x*g*, 15 min, 4°C) and the resulting supernatants were filtered (0.45 µm, Millipore Durapore PVDF membrane). Filtrates were centrifuged (32,000 x*g*, 3 h, 4°C) and the OMV-containing pellets were re-suspended in phosphate buffered saline (PBS) (0.8 g KCl, 0.8 g $KH_2PO_4$, 46.8 g NaCl, 4.6 g $Na_2HPO_4$, 0.4 g $MgCl_2*6H_2O$, 0.4 g $CaCl_2$ in 4L $dH_2O$) supplemented with 0.2 M NaCl (DPBSS) and filter sterilized (0.45 µm Ultra-free spin filters, Millipore).

#### 4.2.3.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a technique used to separate proteins according to their electrophoretic mobility. To separate protein components of OMVs, 50 µl of the OMV lysate was boiled at 100°C for 10 minutes to lyse intact OMVs by heat and to denature their proteins. 20 µl of the boiled lysate was added to 10 µl loading dye, and the sample was then loaded into the wells of 12 % SDS-PAGE gels. Additionally, 3 µl of the protein marker was loaded along with the sample. The sample was ran at 200V for approximately 1 hour until the dye band reached the end of the gel. Finally, the gel was removed carefully from the supporting glass plates and placed in Coomassie brilliant blue solution to be stained, with gentle rocking overnight. The next day, the gel was washed with water to remove the excess stain and obtain a clear background.

#### 4.2.3.3 Western blotting

The Western blot was used to confirm the presence of a specific protein that is among the outer membrane proteins in order to verify and confirm the extraction and the purity of OMVs. This protein is known as GroEL, which is a chaperonin that can associate with model lipid membranes. The western blot is a technique widely used to identify proteins after SDS-PAGE

analysis (Mahmood and Yang 2012). After the separation of OMVs proteins via SDS-PAGE, the gel was placed on a nitrocellulose membrane to allow protein transfer under the influence of an electrical current. The gel and the attached membrane were placed between two sheets of Whatman filter paper (GE Healthcare Life Sciences). All of the components were equilibrated in Western buffer, and the transfer was performed at 30 mA overnight at 4 °C. After the blotting step, the membrane was blocked using 2 % milk for 30 minutes at room temperature, to block any nonspecific binding sites. Following this step, the membrane was washed with PBS and incubated overnight at 4 °C with the primary antibody, which was the rabbit polyclonal anti-GroEL antibody (1:1000 dilution, Enzo). The next day, the membrane was washed 3 times with a solution of PBS containing 0.1 % Tween 20 (PBST) to remove any nonspecific binding. Then, the secondary antibody, the goat anti-rabbit IgG alkaline phosphatase conjugate (1:3000 dilution, Enzo) was added, and this was incubated for 1 hour at room temperature. Finally, the membrane was washed 3 times with PBST, and the BCIP/NBT (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate) substrate (Sigma) was added to detect the presence of the protein as a dark band on the membrane. The reaction was stopped by washing with $dH_2O$.

#### 4.2.3.4 **Identifying OMV-associated proteins by mass spectrometry**

This experiment was performed to identify the main membrane-associated proteins that were present in the OMV lysate. Briefly, 25 µl of the OMVs lysate and 25 µl of a boiled OMVs lysate were sent to the mass spectrometry facility at the University of Leicester to perform trypsin digestion and mass spectrometry analysis for proteins recognition. The obtained results were analyzed by the Scaffold Software (version 4.8.9).

#### 4.2.3.5 **NanoSight particles tracking analysis (NTA)**

OMVs quantification is a crucial step to determining the yields of the OMVs at different stages of the extraction process; however, it is challenging due to their small size. Moreover, depending on the protein contents of the OMVs, counting the number of particles can be misleading because the OMVs can vary in size and protein composition, depending on the parent bacterial strain and the growth conditions. Using the commonly performed SDS-PAGE gel, Western blot analysis or Bradford protein assay to measure OMV particles can reveal incorrect result because of the possible presence of bacterial capsular fragments, broken flagella or other secreted proteins (Wieser *et al.* 2014).

OMVs can be measured using indirect methods that depend on the integration of the hydrophobic dye into the OMVs and then measuring the OMVs by their absorbance of the dye (McBroom *et al.* 2006). However, such quantification techniques can be easily influenced by impurities in the OMV lysate, which can lead to incorrect counting of the vesicles (Gerritzen *et al.* 2017).

The method of choice in this project to quantify the OMVs is the Nanoparticles tracking analysis (NTA), which is performed using the Nanosight LM10 instrument (Malvern Panalytical) equipped with NTA 2.2 analytical software. This was chosen to do its ability to measure the concentration of the particles in the lysate and determine the average size of the particles. The machine is equipped with a software that is capable of calculating the refractive index of individual particles. NanoSight analysis was performed as described in section (2.11).

#### 4.2.3.6 **Transmission electron microscope (TEM)**

TEM is the method which was chosen to demonstrate the presence of OMVs, identify their size, shape and overall appearance, as well as to inspect their purity according to the presence of contaminating non-OMV materials such as flagella and large protein aggregates.
The protocol used was explained in (2.10). Briefly, 4 µl of sample was applied onto a clean, negatively charged copper grid and incubated for 2 minutes. Then, the grid was stained twice with 5 µl of 1 % uranyl acetate, after which it was washed with 2 drops of distilled water. The grid was left to dry before being inserted into the electron microscope.

## 4.3 Results:

### 4.3.1 *K. pneumoniae* strain identification confirmation

In this experiment, a PCR targeting the 16s ribosomal RNA was used to confirm the identity of the *K. pneumoniae* strain which was used in this project. The 16S rRNA genes from the bacterial isolates were amplified using the 8f and 1391r primers. Analysis of the PCR amplicons on an agarose gel showed prominent DNA bands with approximate sizes of 1,400 bp. This indicates the specific amplification of the 16S rRNA gene. The amplified DNA fragments were excised and purified from the gel, and then subjected to sequence analysis using the same forward and reverse primers used in the PCR amplifications. The amplicons were sent to GATC Biotech Ltd. (Cambridge, United Kingdom) for Sanger sequencing. The sequence results confirmed that all chosen isolates were *K. pneumoniae* strains.
This was carried out on 6 isolates, as shown in Figure 4.1 below.

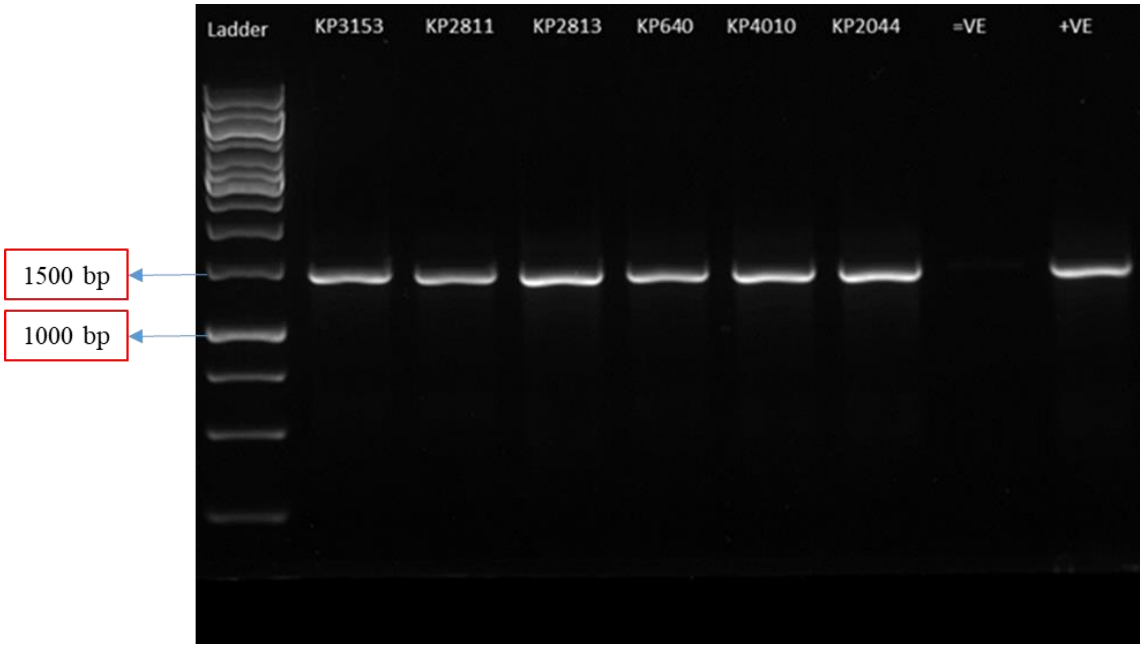



**Figure 4.1. Agarose gel electrophoresis analysis of the 16S rRNA genes amplified from six bacterial strains:**

Agarose gel electrophoresis analysis of 16S rRNA genes amplified from six bacterial strains. PCR amplified products were run on 1 % agarose gel. The DNA ladder used in Lane 1 was the GeneRuler DNA Ladder Mix 1 kb (ThermoFisher). Lanes 2 to 7 indicate the PCR-amplified 16S rRNA gene of the respective bacterial isolates. Lane 8 is the negative control and lane 9 is a known *E. coli* lab strain used as a positive control.

### 4.3.2 **Outer membrane extraction and purification**

In order to verify the purity of the extracted OMVs, the following schematic diagram (Figure 4.2) was followed to confirm the isolation of OMVs.

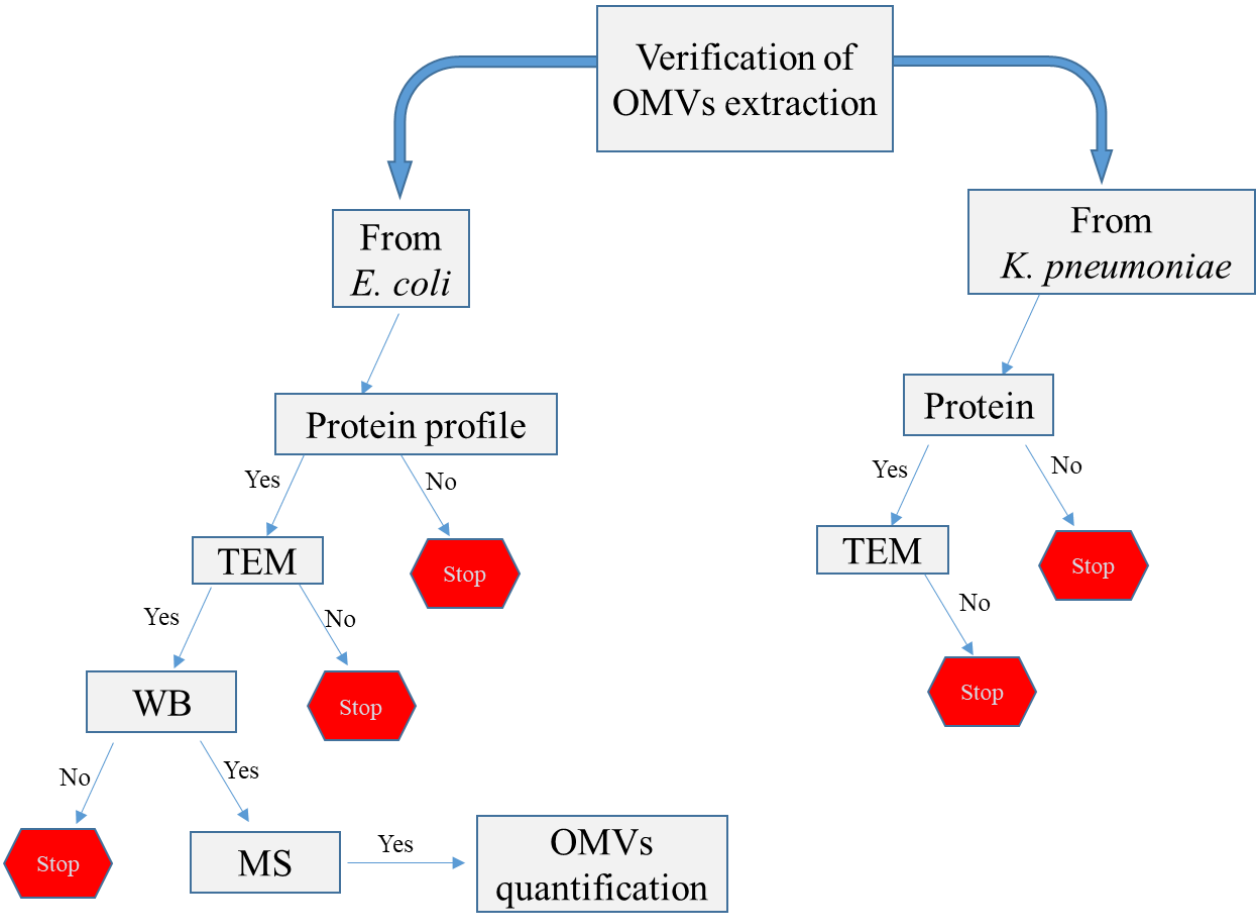



**Figure 4.2: A schematic diagram illustrating the steps that were followed to verify the quality of the OMV lysate.**

### 4.3.3 Assessment of the OMVs isolation from *K. pneumoniae* strains

#### 4.3.3.1 *K. pneumoniae* OMVs protein profile comparisons

At the beginning of this project, OMVs were extracted from two strains of *K. pneumoniae* (KP2811 and KP3153) using the centrifugation-based method (Section 4.2.3.1). 25 µl of the extracted OMV lysate and 25 µl of the bacterial cultures were boiled at 100 °C to lyse the intact OMVs and the bacterial cells and to release the proteins. The protein profiles for both of the extracted OMVs were checked and compared with the parent *K. pneumoniae* strains from which they were extracted. Figure 4.3 shows that both of the OMVs preparations from the two *K. pneumoniae* strains, *KP3153 and KP2811*, stained with Coomassie blue, showed almost no protein present in comparison to the parent cells. This indicates that the OMVs were isolated at a very low concentration, or not isolated at all.

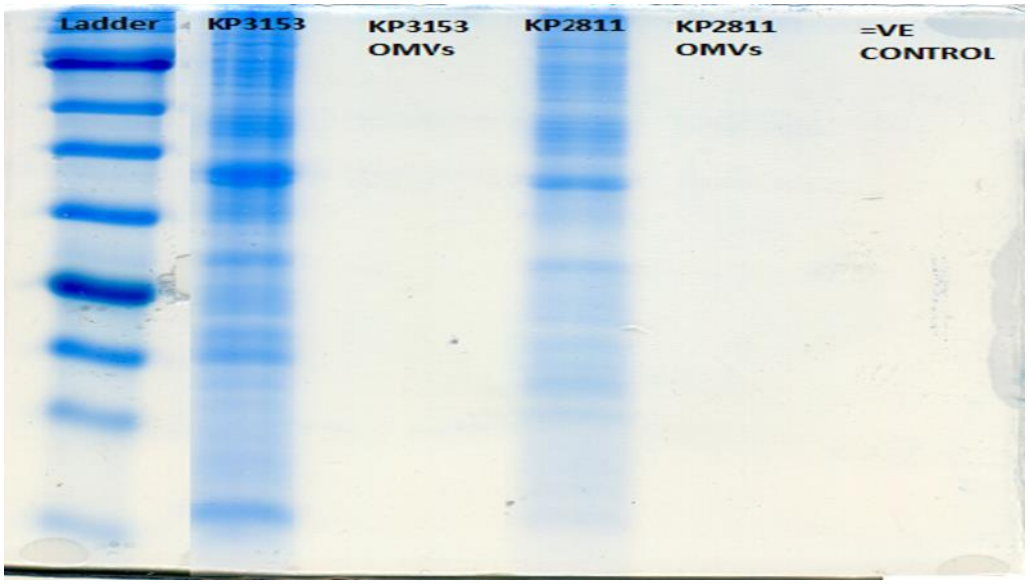



**Figure 4.3: Protein profiles of OMVs extracted from different *K. pneumoniae* strains:**

Two different extracts of OMVs were compared against the parent *K. pneumoniae* strain from which they were isolated. Lane 1 represents the protein ladder (Page-Ruler™ Plus Pre-stained Protein Ladder, 10 to 250 kDa Catalogue number: 26619). The sample in lane 2 represents the *K. pneumoniae* KP2811 protein profile, lane 3 represents the OMVs extracted from KP2811 strain, lane 4 represents the *K. pneumoniae* KP3153 protein profile, lane 5 represents OMVs extracted from KP3153 bacterial strain and lane 6 represents the negative control ($dH_2O$). No obvious protein bands are visible from the OMVs profiles, indicating that not enough OMVs were isolated.

#### 4.3.3.2 **Transmission electron microscope (TEM) analysis**

The OMV lysates that were extracted from the *K. pneumoniae* strains, KP 2811 and KP3153, were checked by TEM. The TEM analysis was used to check for the presence of OMVs and to test whether the use of the centrifugation-based method can lead to a successful extraction of the OMVs. Unfortunately, the TEM analysis demonstrated the unsuccessful extraction of OMVs from *K. pneumonia* strains. This is in agreement with the protein profile comparison and demonstrate that the centrifugation-based method failed. TEM images of this experiment were not shown due to the absence of OMVs.

At this point in the project, and after making a few attempts to extract OMVs from the *K. pnuemoniae* strains, KP2811 and KP3153, using the centrifugation-based method, it must be clearly stated that it is important to optimize a protocol for OMV extraction that is efficient, reproducible and able to produce pure OMV lysates at a high concentration. The bacterial strain that was used in this protocol optimization was *E. coli* DH5α.

### 4.3.4 Optimized protocol for extraction of OMVs from *E. coli* DH5α

#### 4.3.4.1 Overnight culture:

For the starter culture, *E. coli* DH5α was inoculated in 20 ml LB broth and incubated at 37 °C in a shaking incubator overnight. The following day, the 20 ml of overnight culture was divided into 2 flasks. Each flask contained 490 ml of LB, the total culture making up 1 L. It was then incubated overnight at 37°C in a shaking incubator.

#### 4.3.4.2 High speed centrifugation

After the overnight incubation, the *E. coli* DH5α culture was spun in the high speed centrifuge (Hitachi Koki Himac, CR22N, High-Speed Refrigerated Centrifuge from NuAire) at 6,000 x*g* for 25 minutes to eliminate the presence of bacterial cells. The supernatant was retained, and the sediment was discarded.

#### 4.3.4.3 Filtration and ultrafiltration:

In order to eliminate any remnant bacterial cells and to retain a high concentration of OMVs of different sizes, the supernatant was collected after the centrifugation step (Section 4.3.4.2) and was filtered twice through 0.45 µm bottle filters (Bottle top filter 500ml 0.45µm PES by SARSTEDT, Inc., Ref#: 83.1823.100). The filtered supernatant was then filter sterilized by ultra-filtration using Vivaspin ultrafiltration tubes (Vivaspin 20, Sartorios, REF# Vs2032) with a molecular weight cut-off of 50 kDa.

#### 4.3.4.4 Purification of the OMVs preparation:

Following filtration and ultrafiltration, the supernatant was further purified using the OMVs isolation kit (ExoBacteria™ OMV isolation kit for *E. coli* and other Gram-negative bacteria, System Biosciences, Cat # EXOBAC100A-1) by following the manufacturer's protocol. The kit purifies by capturing OMVs using a precipitation-free ion-exchange chromatography system that includes a capture resin and gravity column.

Briefly, the column was prepared by adding 1 ml of the resin, and equilibrating with 10 ml of the binding buffer, after which, up to 30 ml of the ultra-filtrated supernatant was added into the column. This was then incubated at 4°C with rotation for 30 minutes. Then, the mixture was allowed to flow through the column, and the resin was washed three times with 10 ml binding buffer. OMVs were then eluted using 1.5 ml elution buffer.

The optimized method of OMV extraction and purification is illustrated in the diagram below (Figure 4.4).

It must be stated that various other variations and methods were investigated before using the one outlined above (Section 4.3.4). To start with, changes were made in the volume of overnight culture from which the OMVs were extracted. To clarify, in previous published protocols, the volume of the overnight culture ranged from 30 ml to 100 ml. However, the overnight culture in the optimized protocol was increased to 1000 ml. Moreover, in previous protocols, centrifugation of the culture was performed in an ultra-centrifuge at 32,000 x*g* for 3 hours to pellet the OMVs, whereas the centrifugation step in this protocol was performed by a high speed centrifuge at 6000 x*g* for only 25 minutes, just to eliminate bacterial cells from the culture. Moreover, ultra-filtration Vivaspin tubes were used in this protocol with 50 kDa cut-off because it was noticed during the trials that using higher cut-off Vivaspin tubes, with 100 kDa, leaks unwanted non-OMVs materials such as flagella and large protein aggregates into the filtrate, which could affect the purity of the OMVs lysate and eventually interfere with downstream experiments, which seek to test the immune stimulation of the OMVs. Lastly, the OMVs isolation kit was added to the protocol in order to purify the OMVs from other unwanted proteins and bacterial fragments; this was a crucial and effective step that led to the development of the best OMVs extraction and purification protocol.

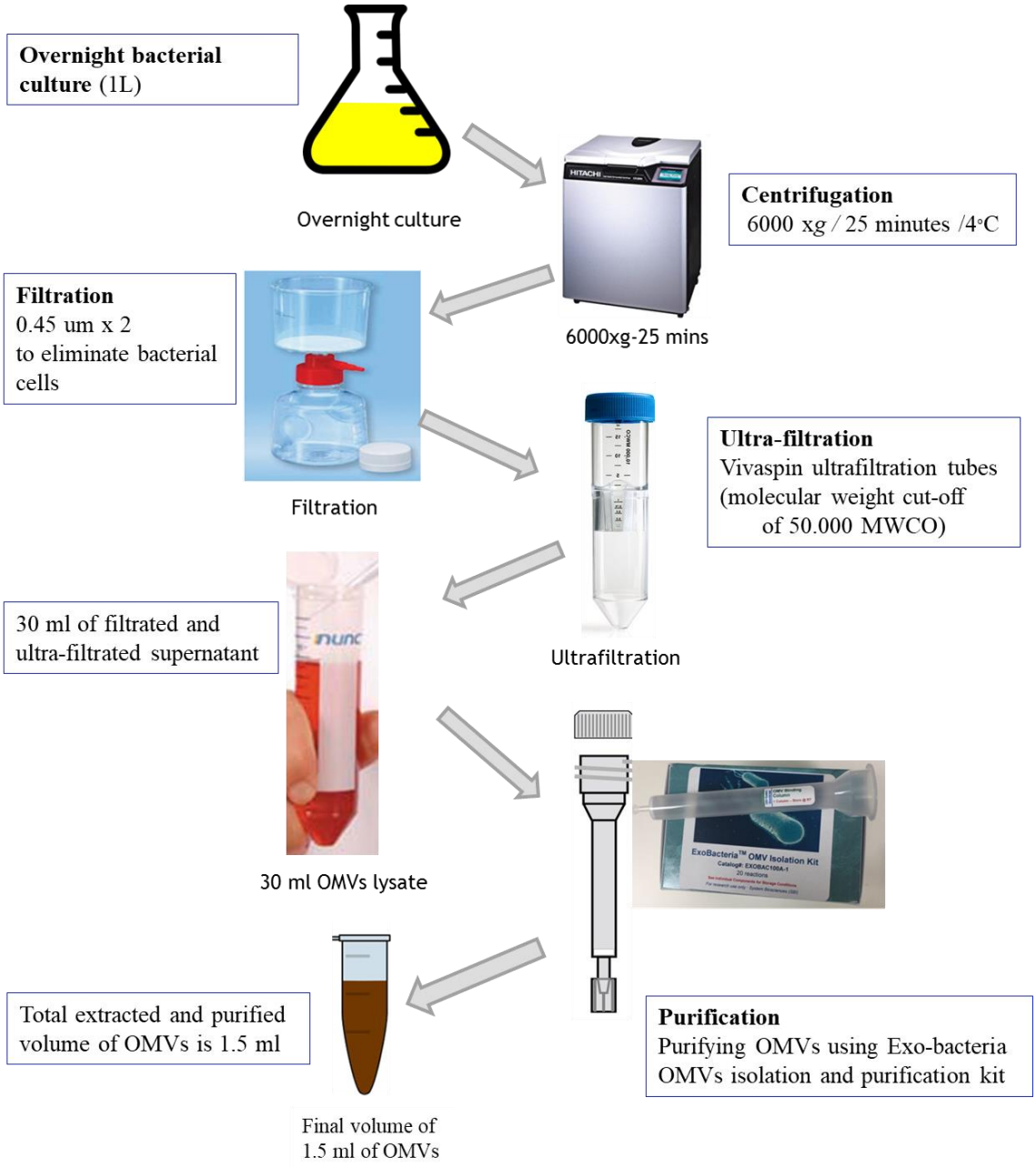



**Figure 4.4: A diagram illustrating the optimised protocol for the extraction and purification of OMVs.**
The steps protocol begins with 1 L of an overnight culture and ends with 1.5 ml of purified extracted OMVs.

### 4.3.5 Assessment of the OMVs isolation from *E. coli* strain.

#### 4.3.5.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting to confirm the presence of GroEL:

This is the first experiment to confirm the successful extraction of OMVs from *E. coli* DH5α. As described in (Section 4.2.3.2), the SDS-PAGE gel was performed for the boiled

OMVs extracted from *E. coli* DH5α. It has been previously shown (Section 4.1) that OMVs are associated with the outer membrane protein GroEL. This experiment was conducted to verify the presence of GroEL as a way to confirm the successful extraction of OMVs from *E. coli*. As shown in (Figure 4.5A), the boiled OMV lysate was analysed on a 12 % SDS-PAGE and compared with the GroEL positive control. The results firstly showed a protein profile that might be that of OMVs extracted from *E. coli* DH5α. The result of the SDS-PAGE gel revealed the presence of a band similar in size (~ 60 kDa) to the GroEL known positive control. This band was further investigated to confirm the presence of GroEL by performing Western blot using the primary antibody, the rabbit polyclonal anti-GroEL antibody, and the secondary antibody, the goat anti-rabbit IgG alkaline phosphatase. The Western blot analysis (Figure 4.5B) shows the presence of the GroEL protein with the expected molecular weight (~ 60 kDa) in a similar manner as the positive control.

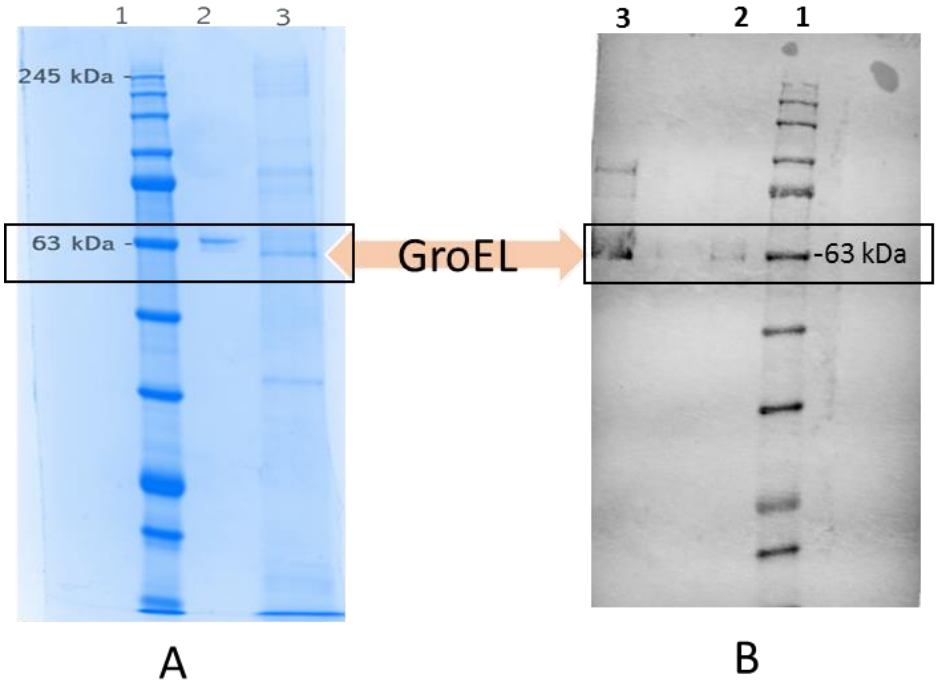



**Figure 4.5: Confirmation of the presence of outer membrane protein GroE at the OMVs extracted from *E. coli* DH5α.**

(A) 12 % SDS-PAGE gel showing the protein profile of boiled OMV lysate extracted from *E. coli* DH5α. Lane 1. Protein marker. Lane 2. GroEL recombinant protein as a positive control. Lane 3. Shows the OMVs protein profile. (B) Western blot proteins using polyclonal antibody anti-GroEL as a first antibody and Goat anti-rabbit IgG polyclonal antibody conjugated with alkaline phosphatase (AP). Lane 1. Protein marker. Lane 2 GroEL recombinant protein as positive control. Lane 3. OMVs protein profile.

#### 4.3.5.2 **Protein analysis by mass spectrometry:**

After visualizing the protein bands of the OMVs extracted from *E. coli* DH5α by SDS-PAGE gel, and after the confirmation of the presence of one of the main outer membrane proteins, GroEL, using the Western blot, 25 µl of the OMVs lysate was sent to the mass spectrometry facility at the University of Leicester to perform trypsin digestion, and for protein recognition. Mass spectrometry was performed to analyse the protein contents of the OMVs extracted from *E. coli* DH5α and to identify the main associated proteins that reflect the OMVs' composition. ( Figure 4.6) shows that the GroEL was the top scoring match for the peptides, furthermore, the top five proteins were found to be outer membrane proteins. Collectively, the mass spectrometry analysis shows that the most recognised proteins in the OMV lysate were outer membrane proteins. This may suggest that the contents of this lysate were generated from the bacterial outer membrane, and confirms that the contents of this lysate are mainly composed of OMV particles that were extracted from the outer membrane of *E. coli* DH5α.

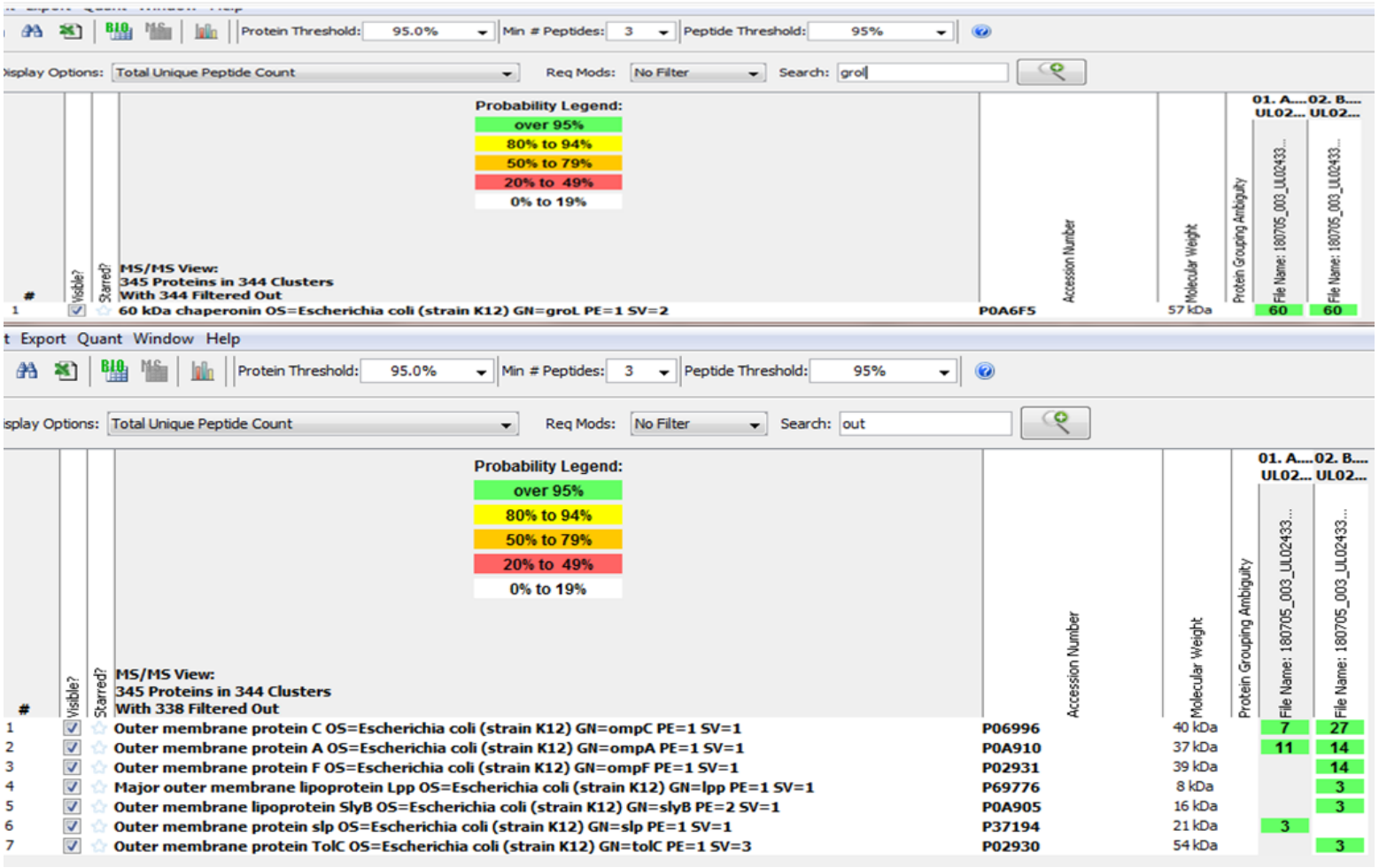



**Figure 4.6: OMVs protein profile in mass spectrometry.**
The top six proteins were outer membrane-related proteins and the most abundant protein was GroEL.

### 4.3.5.3 **Quantification of *E. coli* OMVs**

Nanoparticle tracking analysis (NanoSight) was used to determine the size and concentration of the extracted OMVs from *E. coli* DH5α. This analysis revealed that the size of the isolated OMVs from *E. coli* DH5α ranges between 120 to 250 nm. However, as shown in the concentration peak (Figure 4.7), the average size of most of the vesicles ranges between 153 to 218 nm. The concentration of the vesicles was approximately 6.0 x $10^6$ particles/ml.

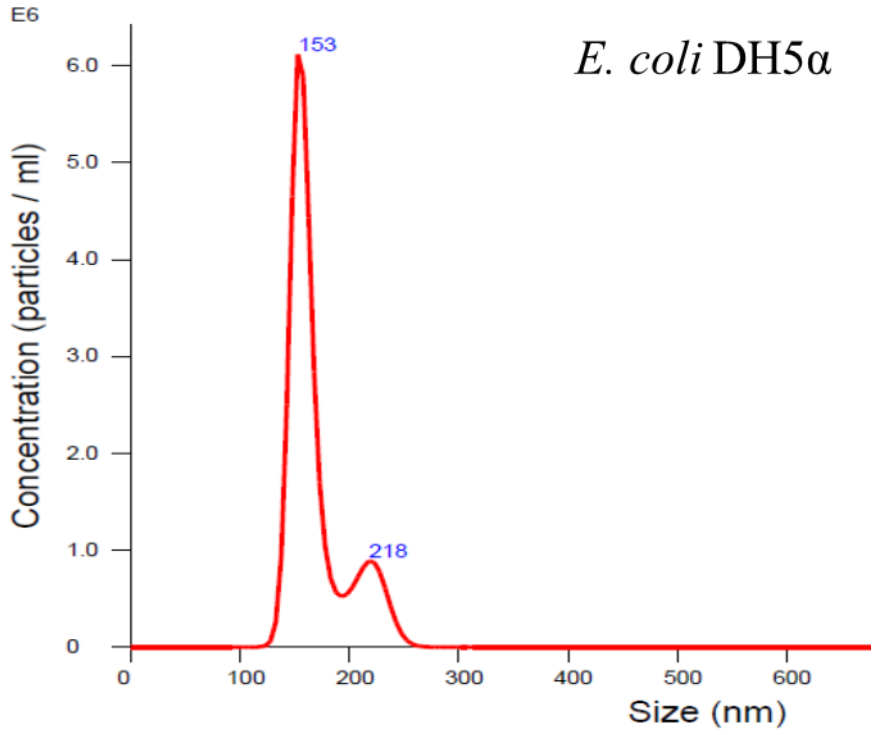



**Figure 4.7: Representative NanoSight size distribution and concentration of E. coli DH5α OMVs.**

The figure shows the average size of *E. coli* OMVs, which is between 120-250 nm, at a concentration of about 6.0 x $10^6$ particles/ml.

### 4.3.5.4 **Transmission electron microscopy (TEM) analysis**

TEM analysis was performed to assess and validate the extraction of OMVs after each step of the extraction process. The figures below show the comparison between different extraction protocols. (Figure 4.8) shows the presence of what are thought to be flagella/fimbriae that were isolated alongside the OMVs. These particular OMVs were extracted using the centrifugation-based method. (Figure 4.9) shows TEM analysis of the

OMVs' extraction using the optimized control before using the OMV isolation and purification kit. Various impurities can be seen in the sample, and the association of OMVs with what are thought to be flagella and/or fimbriae from the parent bacterial cell. On the other hand, (Figure 4.10) shows the successful extraction and purification of the OMVs extracted from *E. coli* DH5α using the optimized extraction method illustrated in (Figure 4.4). It was considered successful as the presence of both individual and clusters of OMVs can clearly be seen. Moreover, the images do not show any presence of unwanted non-OMVs, like materials such as flagella.

At this point, it can be concluded that the optimized OMV extraction method was successful in isolating pure OMVs that can be used in the phage-OMV interaction experiment.

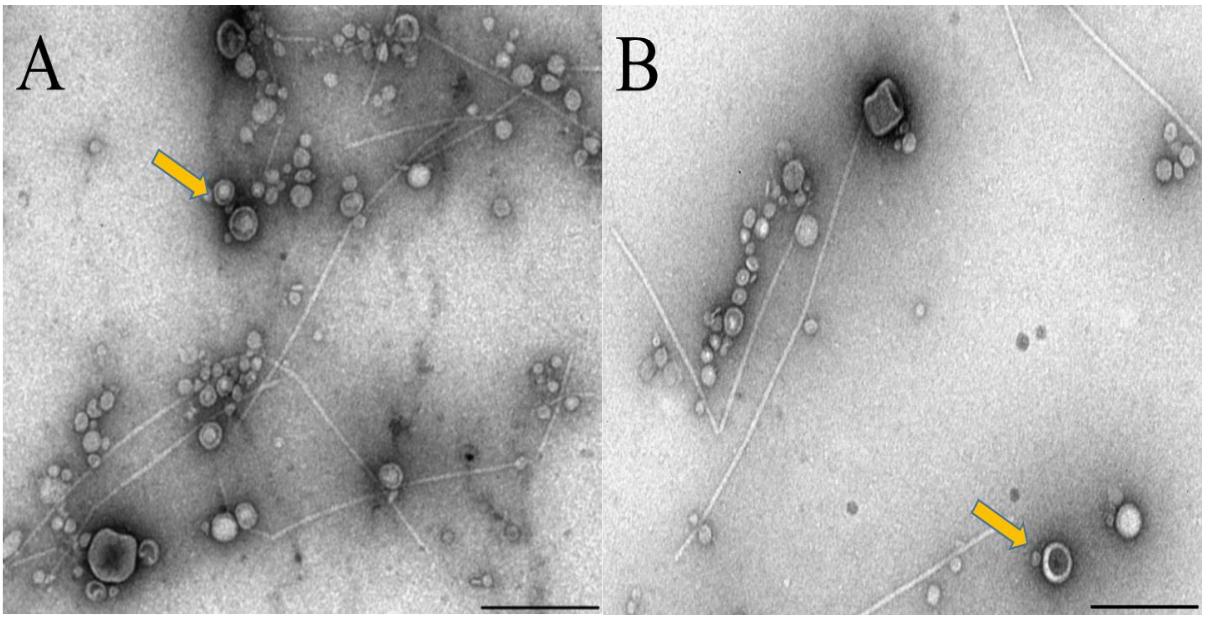



**Figure 4.8: TEM assessments of *E. coli* OMVs using the centrifugation-based method.**

Shown are the OMVs isolated (gold arrows) using centrifugation, filtration, ultrafiltration and ultracentrifugation. This figure shows the presence of suspected bacterial flagella or fimbriae alongside the OMVs in the OMV lysate.

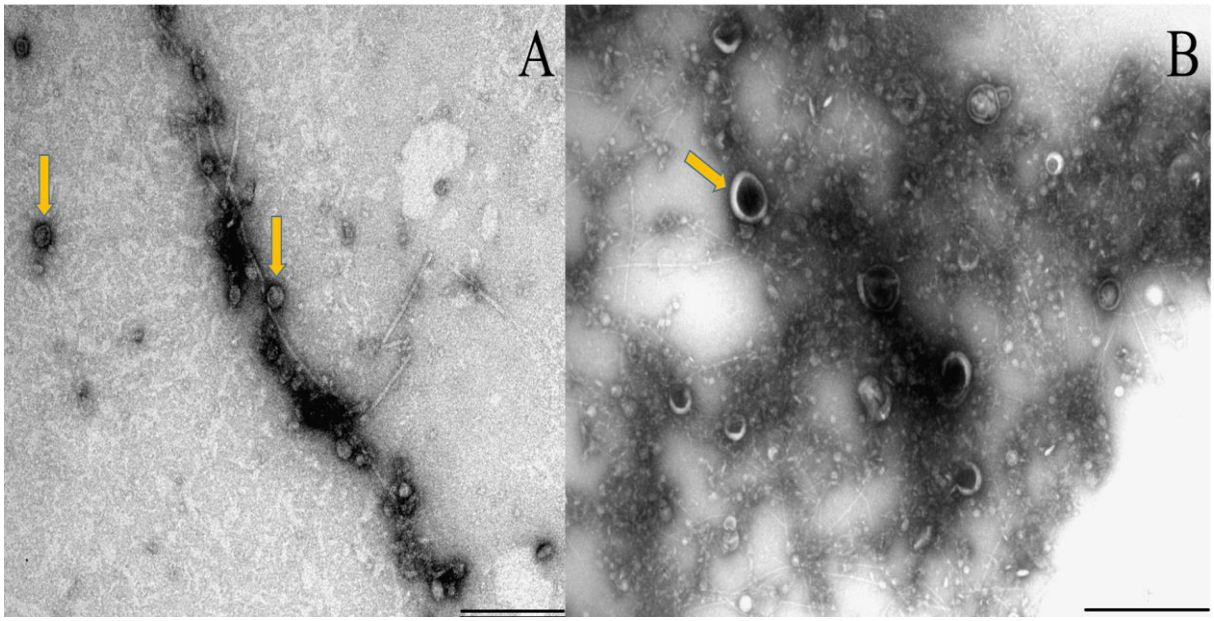



**Figure 4.9: TEM assessments of crude *E. coli* OMVs.**

The gold arrows indicate the OMVs that were extracted using the optimised extraction methods without the use of the purification kit. This figure shows the association of OMVs with non-OMVs like materials. Additionally, the presence of impurities can be seen before purification. A) Scale bar 200 nm B) scale bar 500 nm.

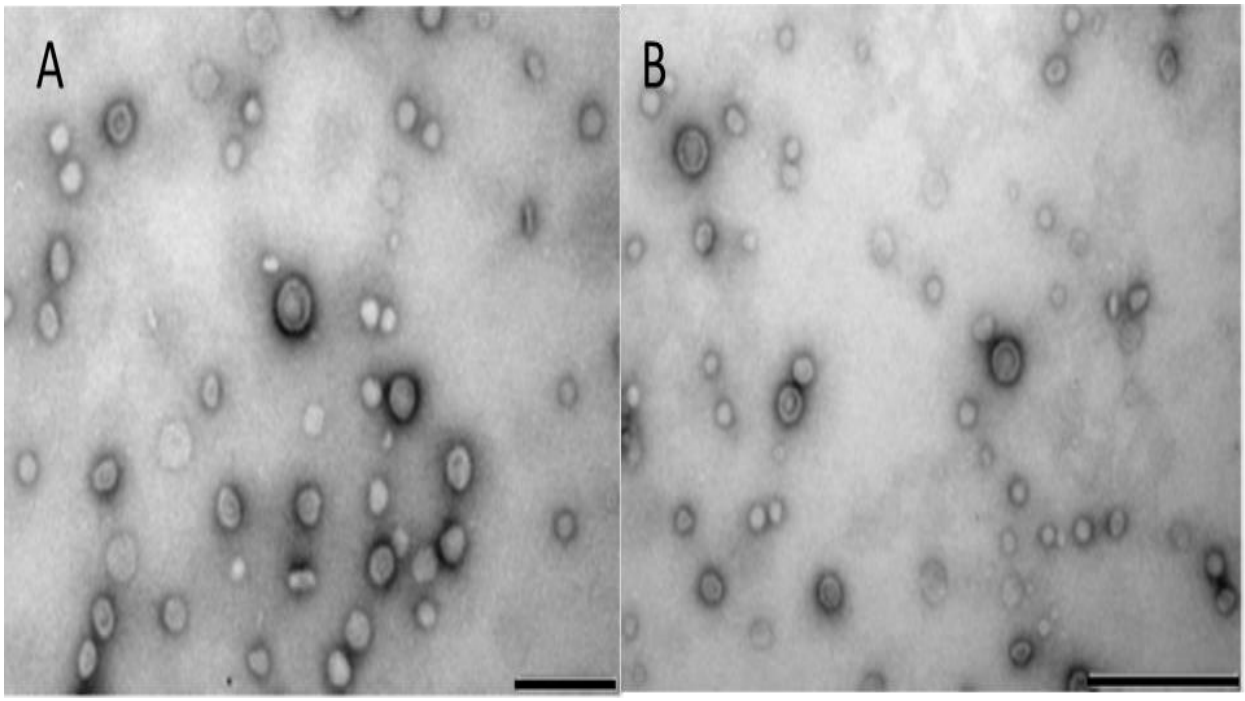



**Figure 4.10: TEM assessment of purified *E. coli* OMVs.**

Shown above are the OMVs isolated via centrifugation, filtration, and ultrafiltration with the use of the OMV purification kit. A) Scale bar 100 nm B) Scale bar 200 nm. The average size of the OMVs is approximately 20 – 45 nm.

## 4.4 **Discussion:**

*K. pneumoniae* OMVs were chosen to be studied in this project because they are known to be non-motile, lacking flagella which are highly immunogenic agents (Lauté-Caly *et al.* 2019). At the beginning of this project, the idea of extracting and purifying OMVs from *K. pneumoniae* was thought to be a secondary protocol. However, when the extraction process was first attempted following published protocols such as (Klimentová and Stulík 2015), it was found that the yields of OMVs was not high in terms of OMV concentration, nor were the extracts found to be pure (Section 4.3.3). As a result, it was necessary to optimize the process of extracting OMVs arose. The optimization focused on firstly extracting a high yield of OMVs, and secondly, purifying the OMVs extracts.

In this chapter, the trial experiments performed for the extraction and purification of OMVs from *K. pneumoniae* are shown. At the beginning of this project, it was attempted to extract OMVs from *K. pneumoniae* following the reported centrifugation-based protocol.

The purity and quantity of the OMVs extracted from K. pneumoniae were tested using several techniques, the first being the comparison between the protein profile of the boiled OMVs and the protein profile of the boiled parent bacterial cell. As shown in (Figure 4.3), the protein profile of the OMVs showed no bands in comparison with the protein profile of the parent cell. This was the first indicator that OMVs were not extracted successfully from *K. pneumoniae*. In addition to that, TEM was performed to visualise the OMVs. Unfortunately, visualising the OMV lysate under TEM revealed a very low concentration of *K. pneumoniae* OMVs.

At this point in the project and after making a few attempts to extract OMVs from the *K. pnuemoniae* strains, KP2811 and KP3153, using the centrifugation-based method, it was concluded that it would be necessary to optimize a protocol for OMV extraction that is efficient, reproducible and able to produce pure OMV lysates at a high concentration.

Based on these findings, *E. coli* was introduced to the project in order to test whether the low yield of OMVs was due to the protocol or the organism used in this protocol, *K. pnuemoniae*. OMVs were extracted from *E. coli* using the same protocol. The vesicles from *E. coli* were extracted at a higher concentration when compared to *K. pneumoniae*. However, along with the extracted OMVs from *E. coli*, a large quantity of flagella were also found when performing a TEM analysis of the sample (Figure 4.8). As it is known that flagella antigens are highly immunogenic agents for the host immune cells (Hajam *et al.* 2017), this was undesirable. Although the extraction protocol was successful in extracting the OMVs from *E. coli* at a high concentration, the purity of the OMV lysate was not optimal. For this reason, further optimizations were required for the protocol in order to exclude the bacterial flagella from the OMV lysate preparation. Therefore, an ultrafiltration step was introduced into the protocol. The vivaspin ultrafiltration tubes (Vivaspin 20, Sartorios, REF# Vs2042) with a molecular weight cut-off of 100.000 kDa, was used. However, the flagella were still found to be present when analysed by TEM. Therefore, ultrafiltration tubes with a smaller cut-off of 50.000 kDa were introduced. The TEM analysis after this step revealed a good concentration of OMVs with a low concentration of flagella, although some flagella were still present. At this point, one last step was added to the protocol, this being the OMV isolation and extraction kit. The use of this kit resulted in the purest and highest yield of OMVs; significantly, the bacterial flagella and other impurities were not found to be present, as shown in (Figure 4.10).

After the optimization and validation of this protocol, the extraction of OMVs from *K. pneumoniae* using the optimized protocol was attempted. Unexpectedly, the OMVs from *K. pneumoniae* were not extracted at a high concentration, despite the use of this newer protocol. It has been reported that flagella synthesis influences the production of OMVs (Manabe *et al.* 2013), suggesting that the quantity of OMVs produced by non-motile bacteria may be less than those produced from motile bacteria. Therefore, and for the main purpose of this project, the idea of extracting OMVs from *K. pneumoniae* was replaced with the idea of extracting OMVs from *E. coli* DH5α.

## 4.5 **Conclusion:**

Despite following the OMV extraction and purification methods, OMVs from *K. pneumoniae* could not be extracted at a high concentration. This led to the validation and the use of published protocols for OMV extraction using *E. coli* DH5α as a model organism. The same validated method was applied to extract *K. pneumoniae* OMVs. Unfortunately, the OMV yield from *K. pneumoniae* was not sufficient. Eventually, the OMVs extracted from *E. coli* DH5α were used for the main purpose of this project. Finally, the extraction and purification of OMVs using *E. coli* DH5α was optimized. The extracted OMVs were checked by NanoSight, protein analysis and visualized under TEM.

# Chapter five

## 5. Interaction of phages with bacterial outer membrane vesicles: immunological aspects.

## 5.1 Introduction

OMVs are round proteoliposomed nanoparticles that are produced from the outer membrane of Gram-negative bacteria. They are secreted naturally during normal bacterial growth (Kulp and Kuehn 2010). OMVs play different roles in microbial virulence and in the modulation of the host immune responses. They also play a role in the communication between the bacterial cells and host cells and between different bacterial cells (Kuehn and Kesty 2005).

As part of the normal growth of Gram-negative bacteria, OMVs are produced by both pathogenic and commensal bacteria (Kulp and Kuehn 2010). OMVs that are produced by commensal bacteria have been found to be involved in the maturation of the host immune system (Shen *et al.* 2012). On the other hand, OMVs that are produced from pathogenic Gram-negative bacteria have been found to facilitate the establishment of infection in hosts (Goes *et al.* 2020). Thus, for both symbiotic and infectious situations, OMVs are important surfaces and carriers which need to be studied.

OMVs from different Gram-negative bacterial species have been found to elicit the pro-inflammatory response. For example, OMVs that were extracted from *Porphyromonas gingivalis* were found to stimulate the immune response. This stimulation which occurred in the gingival tissues was caused by dysregulating inflammation that is involved in chronic periodontitis (Cecil *et al.* 2016). In another study, after 24 hours of incubation, a low dosage of OMVs from *H. pylori* were found to increase the proliferation of gastric epithelial cells. However, at higher doses, it was found that OMVs from *H. pylori* arrested the growth of the epithelial cells, increased toxicity and stimulated the production of IL-8 (Ismail *et al.* 2003). In a similar scenario, OMVs from *K. pneumoniae* were able to significantly increase the mRNA expression of cytokines such as IL-8, IL-6, IL-1β and TNFα (Martora *et al.* 2019; You *et al.* 2019). OMVs isolated from *P. aeruginosa* were found to increase the expression of IL-8 after interaction with epithelial cells for 24 hours (Bauman and Kuehn 2006). Similarly, OMVs that were isolated from *Legionella pneumophila* and *Acinetobacter baumannii* were found to stimulate the host immune response by upregulating the expression of the chemokines genes (Galka *et al.* 2008; Jun *et al.* 2013). In a similar study, OMVs isolated from *Salmonella typhimurium* were found to not only enhance the production of pro-inflammatory cytokines, such as TNFα and IL-

12, but also to stimulate antigens presenting cells (APCs) in a similar manner as their parent bacterial cells. It was found that these vesicles contain antigens (AGs) recognized by *Salmonella* specific B and T cells, which resulted in the generation of antibodies (Abs), thus immunizing the host from bacterial infection caused by the parent bacteria (Alaniz *et al.* 2007). Furthermore, *Vibrio anguillarum* OMVs were found to stimulate the production of TNF-α, IL-1β and IL-6 (Hong *et al.* 2009). Other cytokines were elicited by OMVs of *Neisseria meningitides* such as IL-10, type I interferon-dependent and IFNϒ (Durand *et al.* 2009). Thus, all these isolated OMVs from different bacteria were found to elicit an inflammatory response from the host immune cells.

In a different approach, bacteriophage have been recognized as an alternative potential treatment option to cure patients with infection caused by multidrug resistance bacteria (Kutter *et al.* 2015), mainly because the lytic action of a phage is not affected by the bacterial antibiotic resistance mechanism. Moreover, bacteriophages are considered a safe option to treat bacterial infections (Speck and Smithyman 2016). In another study using a well-known T4 phage that infects *E. coli*, it has been shown that the phage itself along with the head proteins did not stimulate the production of pro-inflammatory cytokines or inflammatory mediators of the host (Miernikiewicz *et al.* 2013).

Bacteriophages are known to interact with OMVs. This interaction can lead to the inactivation of phages. This has been illustrated in a study conducted by (Manning and Kuehn 2011) which showed that the interaction of *E. coli* OMVs with phage T4 resulted in a reduction of T4 phages titre and infectivity. In another study, *Vibrio cholera* OMVs showed an ability to inhibit virulent phages via a receptor-dependant mechanism (Reyes-Robles *et al.* 2018). This shows that OMVs may interfere with phages in bacterial infection treatment and impact their efficacy. What is unknown, however, is the impact of such interaction on the OMVs and their contribution to the modulation of the host immune system.

## 5.2 Aim and objectives

The aim of this set of experiments was to interact purified OMVs from *E. coli* with a phage that is able to infect the parent *E. coli*, and to study the effect of such interaction on the aspects of the production of pro-inflammatory cytokines, namely TNFα by macrophages.

### 5.2.1 Objectives

- Purification of *E. coli* phage EL
    - To measure the level of endotoxin present in the phage lysate.
    - To minimise the presence of the endotoxin.
    - To purify the phage lysate to minimise the presence of immunogenic stimuli in the lysate.
- Interaction between *E. coli* OMVs and EL phage
    - Confirmation of the interaction between phage EL and the E. coli OMVs.
    - Incubation of the OMVs-phage complex, OMVs alone and phage alone with J774 macrophage cell line.
- Measure the level of TNFα produced, which is released from the macrophages cell after incubation under different conditions.

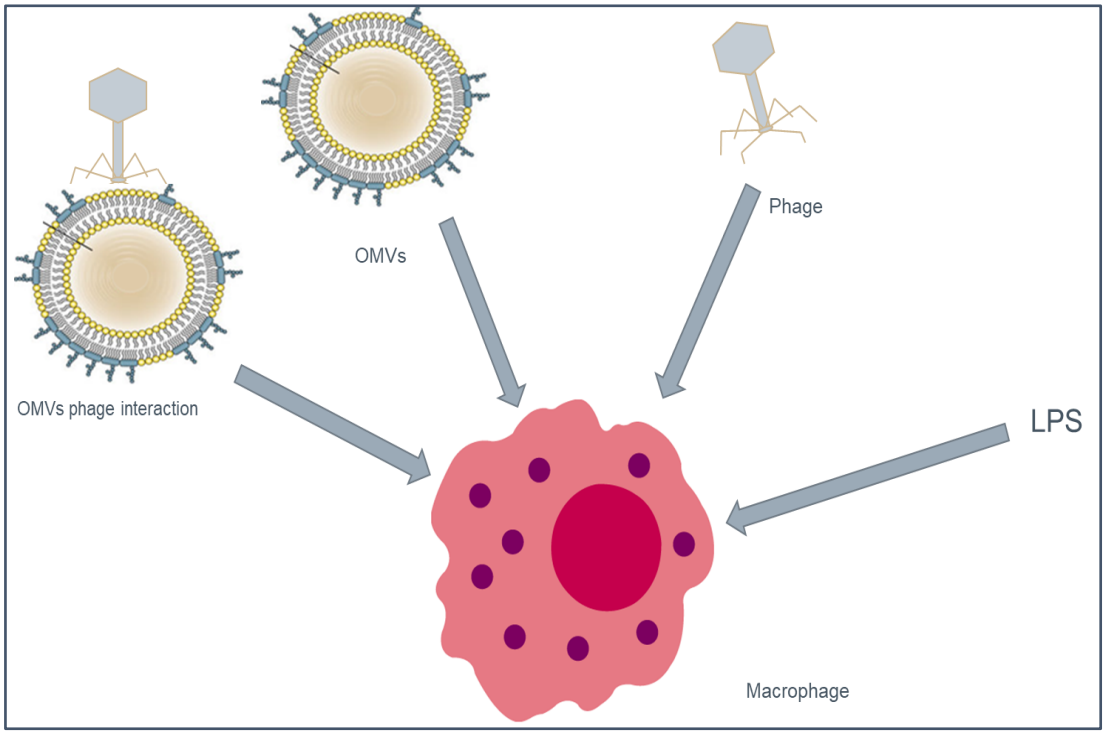



**Figure 5.1: A diagram illustrating the experimental design.**

The first condition is the interaction of macrophages with OMVs, which are known to elicit host immune responses. Second condition is to assess the interaction of phages with OMVs, and whether this will result in a reduction of the immune response compared to the OMVs alone. The third condition is to assess whether the phage alone can induce the host immune response. The fourth condition is the use of a positive control, LPS, which is known to stimulate the host immune response.

## 5.3 Methods

### 5.3.1 Endotoxin removal kit

To test the level of endotoxins in the phage lysate sample, the Pierce LAL (*Limulus* Amebocyte Lysate) Chromogenic Endotoxin Quantitation Kit (Ref# 88282, Thermo Scientific, UK) was used following the manufacturer recommendations. The kit is designed to measure Endotoxin concentrations by a chromogenic signal generated in the presence of endotoxins. A standard curve was generated using 5 points (1, 0.5, 0.25, 0.1 and zero EU/ml) of *E. coli* endotoxin standard included in the kit (Figure 5.2). The samples were prepared in microplates and measured using an absorbance reader with a wavelength of 405 nm.

### 5.3.2 Endotoxin removal

To minimize the presence of endotoxin to the minimum level, the Pierce™ High Capacity Endotoxin Removal kit (Thermo Scientific™, UK, and REF # 88274) was used following the manufacturer protocol. This kit comprises 5 spin columns that contain porous cellulose beads that have been surface modified and covalently attached with modified ε-poly-L-lysine, which has a high affinity for endotoxins.

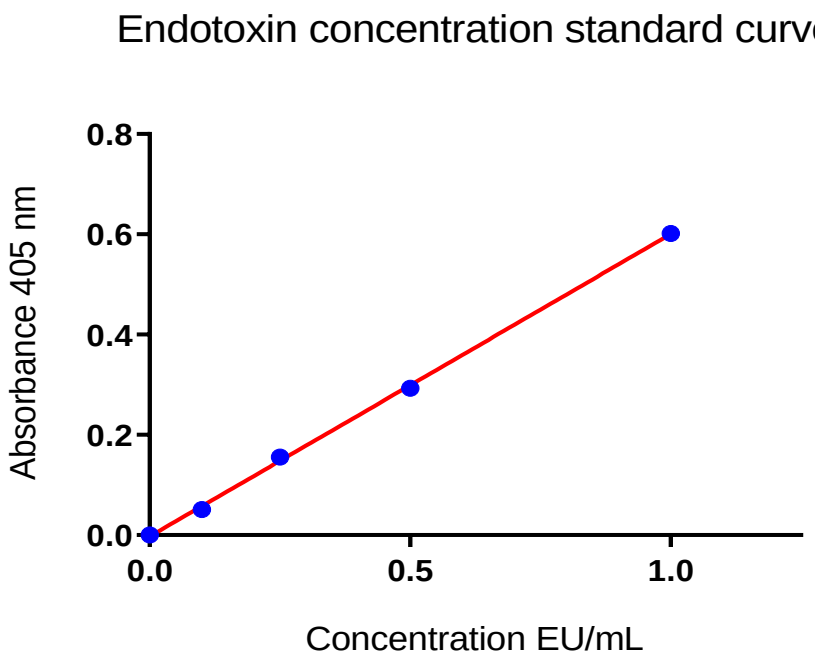



**Figure 5.2. Endotoxin concentration standard curve.**

5 points standards curve was generated from known concentration of LPS (endotoxin) standard. The standards concentrations used to generate this curve are 0, 0.1, 0.25, 0.5 and 1 EU/ml. The plate was then read by a plate reader at 405 nm ($R^2$ = 0.9994).

### 5.3.3 Phage purification using anion-exchange chromatography and CIM® monoliths:

Phage EL was purified in order to minimise the presence of any immunogenic stimuli in the lysate. This purification was performed using anion-exchange chromatography with Convective Interactive Media monolithic column (CIMs). 5 ml of phage lysate (1.2 x $10^{11}$ PFU/ml) was dialysed at 4 ºC overnight against binding buffer (20 ml of 50 mM Tris 7.5 and 8 mM $MgSO_4$. 7 $H_2O$). The column used in this assay was 1 ml quaternary amine (QA), which is a monolithic column developed by BIA Separations (Slovenia). The column was attached to the AKTA™ FPLC™ system (General Electric Healthcare), and then washed with the binding buffer. The sample was then injected into the machine and eluted using the elution buffer (50 mM Tris 7.5 and 8 mM MgSO4. 7 $H_2O$ and 2M NaCl) (Adriaenssens *et al.* 2012; Clokie 2018). The fractions were monitored by observing the real-time UV absorbance. The data that was collected while the fractions were eluted, and was analysed using the UNICORN™ version 7.0 software and GraphPad Prism version 7.04 software. The phage titre in each fraction was determined by spot tests (**Error! Reference source not found.**).

### 5.3.4 **Phage and OMVs interaction and assessment**

This interaction was achieved by the mixing of phage stock in SM buffer and OMVs at a ratio of 1:1 and incubated for 15 minutes at 37 ºC. This interaction was assessed by TEM. Briefly, 4 µl of the mixture was transferred onto a clean copper grid, left for 2 minutes, then stained twice with 5 µl of 1 % (w/v) uranyl acetate and dried by filter paper. The grid was then washed twice with one drop of distilled water each time and left to dry, before being inserted into the electron microscope. To assess the specificity of the interaction, OMVs extracted from *P. aeruginosa* PAO1 were mixed with phage EL. Similarly, this interaction was assessed by TEM.

### 5.3.5 **Cell line**

The cell line used in this study was J774 mouse macrophage-like cell line (provided by Dr Cordula Stover, Department of Infection and Immunity, University of Leicester. Another batch was provided by Anas Malas (Department of Infection and Immunity, University of Leicester). The cell line was maintained in DMEM (Dulbecco's Modified Eagle's Medium) media with high glucose (Thermo Fisher scientific, UK, and Ref # 11965092) supplemented with L-glutamine, 10 % (v/v) fetal bovine serum (FBS), penicillin ($10^2$ IU.ml$^{-1}$), and streptomycin (100 µg.ml$^{-1}$).

### 5.3.6 **Thawing cells**

The cryovials containing the frozen cells were taken from –150 °C and defrosted within one minute by slight swirling in a water bath at 37 °C. Once completely thawed, they were centrifuged at 200 x*g* for 5 minutes (Allegra X-22 centrifuge, Beckman Coulter, US). The supernatant was removed, and the pelleted cells were washed once by adding growth media to neutralize the toxic effect induced by the remainder of the freezing media. Following centrifugation, once the supernatant was discarded, the cells were re-suspended in 1 ml of media and placed into a tissue culture flask containing growth media. The next day, the media was changed. The cells were checked for their attachment and viability using an inverted microscope on a daily basis, with the media being changed every 3 days until confluence was reached.

### 5.3.7 **Cell line subculture (passage)**

To avoid an over-confluent culture, the cell cultures were split when they reached 70 % confluence. To split the cell line, the culture medium was pipetted into a waste container. The flask was then washed with 5 ml PBS to remove any unattached dead cells. After that, 2 ml of trypsin was added to the flask and then incubated for 10 minutes at 37 ºC to allow the cells to detach from their monolayer and culture surface. During this step, they were closely monitored using the inverted microscope, until most of the cells observed floated off. After that, the detached cells suspension was transferred to a culture tube and diluted with 10 ml of culture media containing serum to terminate the proteolytic reaction of trypsin and centrifuged at 200 x*g* for 5 minutes. The pellet was then re-suspended in 5 ml of the supplemented media and seeded into the culture flask.

### 5.3.8 **Cell counting**

The counting chamber was prepared by placing the cover slip on the chamber properly. Then, approximately 10 µl of the mixed cell suspension was added to the front of the gap between the chamber and the cover slip, to allow the sample to be drawn inwards via capillary force. Under microscopy, all cells defined within all four squares were counted and divided by 4 to obtain the average number of cells per ml (expressed as $x10^4$ /ml). The common formula used in counting is: No. of cells (4 squares) / 4 = Average of cell number ($x10^4$ /ml).

### 5.3.9 **Freezing J774 cells**

For long-term storage, the cells were frozen at -150 ºC. To do this, the cell medium was aspirated from the flask in which they were grown. Thereafter, the cells were washed with 5 ml of PBS; afterwards, the PBS was decanted and replaced with 1 ml of warm trypsin to detach the cells from the flask and then incubated for 10 minutes at 37 ºC. To terminate trypsin activity, 1 ml of supplemented medium was added to the flask. The mixture was then aspirated into a Falcon tube and centrifuged at 200 x*g* for 5 minutes. The supernatant was then discarded, and the pellet was re-suspended in 1 ml of 5 % (v/v) DMSO (Dimethyl Sulfoxide) in FBS. The re-suspended pellet was then transferred to labelled cryovials tubes. These vials were placed in a tube rack (Mr frosty) filled with isopropanol and placed at -80 ºC overnight to allow the cells to freeze slowly at a rate of ~1 ºC /

minutes. Finally, the next day, the frozen vial was transferred to the ultra-low temperature tissue culture freezer at -150 ºC.

### 5.3.10 Tissue culture plate layout and the interaction of J774 cell lines with the conditions

Six-well plates were used to introduce the conditions and the controls (Figure 5.3) to the macrophage cells in the cell line. The cells were passaged and incubated until approximately 70 % to 80 % confluence was obtained. Thereafter, 100 µl of OMV lysate, OMV-phage complex, phage lysate, positive control (1000 ng/ml LPS) and negative control (PBS) were added to well 1, 2, 3, 4, 5 respectively. Nothing was added to the sixth well to assure that there was no contamination in the cell lines in the experiment incubation period. After the addition of the samples, the plate was incubated for 4 hours, and then again for 24 hours. After each incubation period, a 1 ml aliquot was collected from each well and centrifuged for 1 minute at 13,000 x*g* to eliminate the presence of the macrophage cells. The supernatants were then collected into 1.5 ml Eppendorf tubes and were either tested by ELISA or frozen at -20 ºC to be tested later.

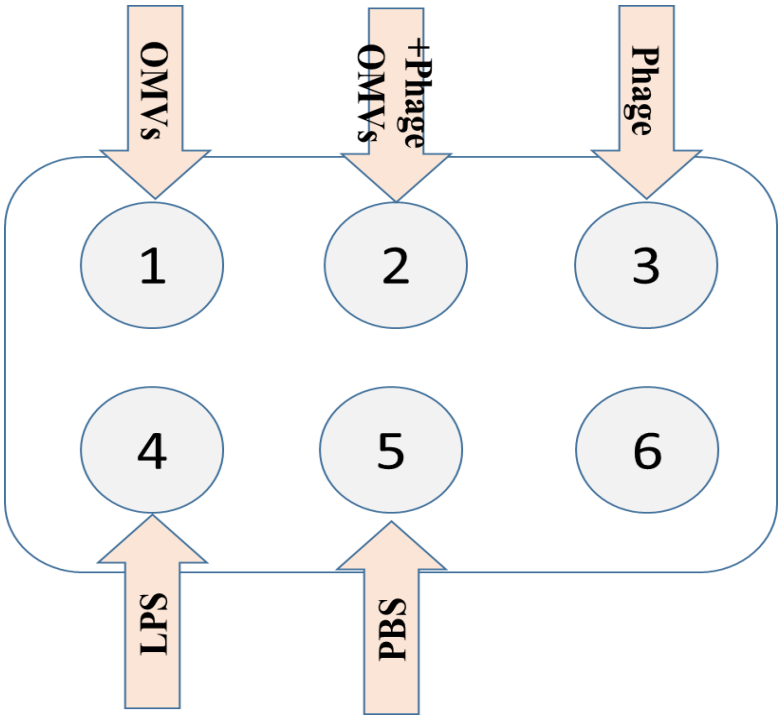



**Figure 5.3: Tissue culture plate layout.**

Shown is the layout of the samples added to each well. (1) 100 µl of OMV lysate, (2) 100 µl of phage-OMV complex, (3) 100 µl of the phage lysate, (4) 2 µl of 1000 ng/ml LPS and (5) 100 µl of PBS.

### 5.3.11 **Murine TNF alpha ELISA**

ELISA experiments were performed to test the level of TNFα that was released from macrophage cell line J774 upon exposure to the following samples: purified OMVs, phage-OMV complex, purified phage alone, positive control (1000 ng/ml LPS) and negative control (PBS). Following the manufacturer's instruction, 96-well microplates were coated with the TNFα capture antibody diluted in carbonate buffer (15 mM $Na_2CO_3$ and 35 mM $NaHCO_3$) to a concentration of 1 µg/ml. 100 µl of this diluted antibody was added to each well. The microplate was then sealed and incubated overnight at room temperature. The next day, the excess antibodies were removed, and the wells were washed four times with 300 µl washing buffer (0.05 % (v/v) Tween 20 prepared in sterile filtered PBS). The plate was then inverted and blotted on paper towels to drain the remaining buffer. Thereafter, the wells were blocked with 300 µl of 1 % (w/v) BSA and incubated for 2 hours at room temperature. The plate was then washed and dried again, as before. From this step and beyond, the wash buffer was the diluent used. 8 points of 2-fold dilutions of the murine TNFα standard was prepared, starting from a concentration of 2500 pg/ml to zero (Figure **5.4**). 100 µl of each standard and 100 µl of each sample were immediately added in triplicate to each well and then incubated at room temperature for 2 hours. The plate was then washed and blotted as before. After this, 100 µl of detection antibody, which was diluted to 0.5 µg/ml, was added to each well and incubated for 1 hour at 37 ºC. The plate was then washed and dried again. 100 µl of 0.025 µg/ml Streptavidin HPR conjugate antibodies were added to each well and then incubated for 40 minutes at room temperature. The streptavidin conjugate antibody was diluted to 1:4000 in diluent for a final volume of 10 ml. The plate was then washed for the last time. 100 µl of substrate solution TMB 3, 3', 5, 5'-Tetramethylbenzidine (Sigma) was added to each well, and the colour development was monitored for up to 20 minutes at room temperature. After this, the reaction was stopped with the addition of 50 µl of the 25 % (v/v) sulphuric acid (stop solution). The plate was then analysed using the ELISA microplate reader (LT4500, Labtech serving scientists) with a wavelength of 450 nm.

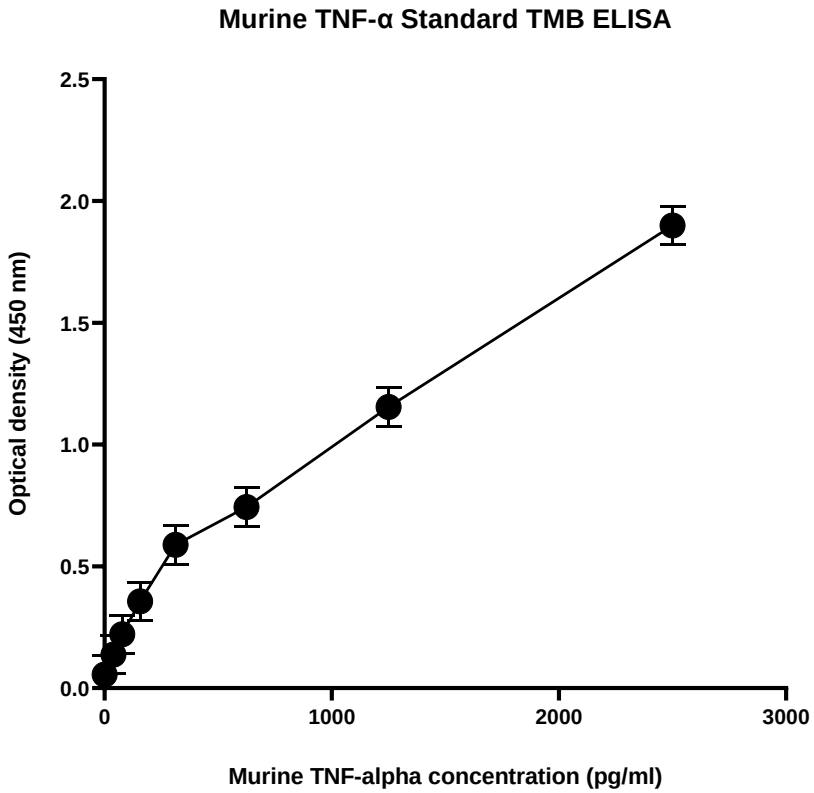



**Figure 5.4: Murine TNF α standard curve.**

8 points standard curve was generated from the known concentration of the murine TNF α standard, which was prepared by double serial dilution according to manufacturer instructions. The standards start from 2500 pg/ml to 12.5 pg/ml. The absorbance was then read via a microplate reader at 450 nm. Data is presented as mean ± standard error of mean (SEM) with linear regression ($R^2$) = 0.9.

## 5.4 Results

### 5.4.1 Phage purification using CIM® monolithic column

Phage EL was purified using anion-exchange chromatography column in an attempt to remove possible immunogenic stimuli from the lysate. The purified particles were eluted in a rate of 1 ml /minute and an absorbance peak was detected, representing the quality of the purification method (Figure 5.5). Eluted fragments under the phage peak were collected separately, after which the phage titre was determined via spot test (Table 5.1). The fragments with the highest concentration were then pooled together. There was no significant reduction in phage titre as the purification was performed for a phage lysate of 1.2 x $10^{11}$, and the retained pooled fragments of the purified phage was 3.0 x $10^{10}$.

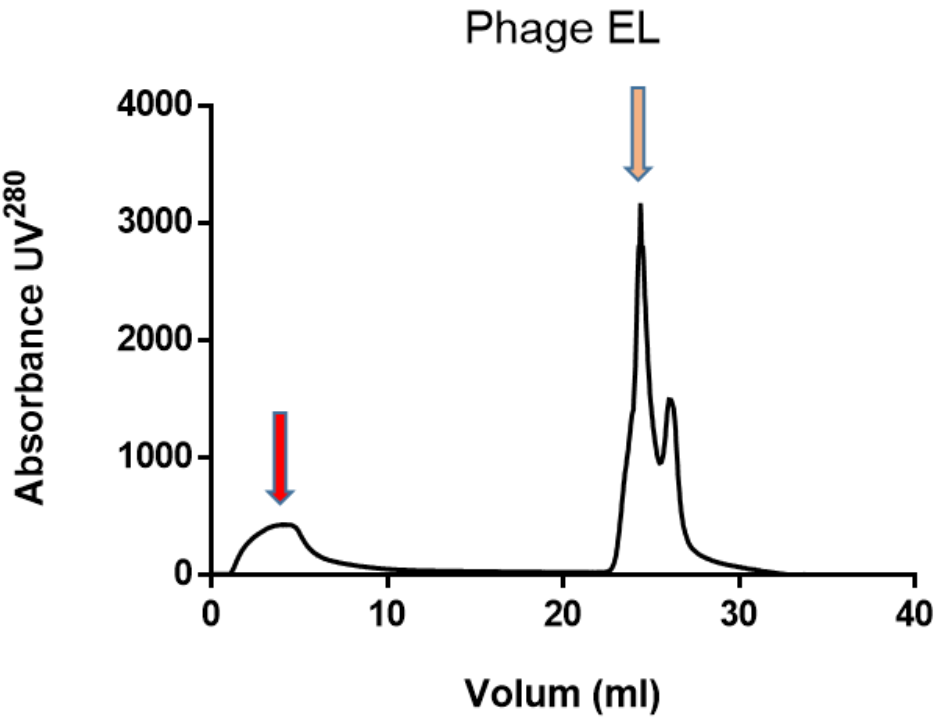



**Figure 5.5: Linear gradient purification of EL phage.**

The red arrow indicates the flow-through, while the orange arrow indicates the peak which corresponds to the presence of phage EL. The phage elution started at 23 minutes, the peak at approximately 25 minutes and the end of elution at approximately 30 minutes.

### 5.4.2 **Phage titration after purification**

Purified and eluted fractions of phage EL were tested by spot tests in order to determine the phage titre. Table 5.1 below shows the titration of 9 fractions that were eluted every minute from 20 minutes to 28 minutes, corresponding to the elution of phage EL.

**Table 5.1: Titration of phage EL after purification via anion-exchange chromatography.**

| Minutes | Phage titre | Minutes | Phage titre | Minutes | Phage titre |
|---|---|---|---|---|---|
| 20 | 0 | 23 | $4 * 10^{10}$ | 26 | $1 * 10^{9}$ |
| 21 | $6 * 10^{3}$ | 24 | $2 * 10^{10}$ | 27 | $3 * 10^{10}$ |
| 22 | $2 * 10^{6}$ | 25 | $2.4 * 10^{9}$ | 28 | $2 * 10^{7}$ |

### 5.4.3 **Endotoxin level of purified and un-purified phage lysate**

The endotoxin removal kit was used on the phage lysate sample after purification via the CIM® column as an additional step to minimise the presence of endotoxin in the lysate. The phage lysate was tested for the presence of endotoxin by using the Peirce® LAL (Limus amebocyte lysate) endotoxin quantification kit (Thermo Scientific, UK). The lysate endotoxin was tested before purification, using both methods (anion-exchange chromatography and endotoxin removal kit), and after purification. (Figure **5.6**) shows the amount of endotoxin before purification via the CIM® column and using the removal kit, and after purification and using the removal kit. The figure shows a significant reduction in the endotoxin level after purification using the CIM® column and the endotoxin removal kit.

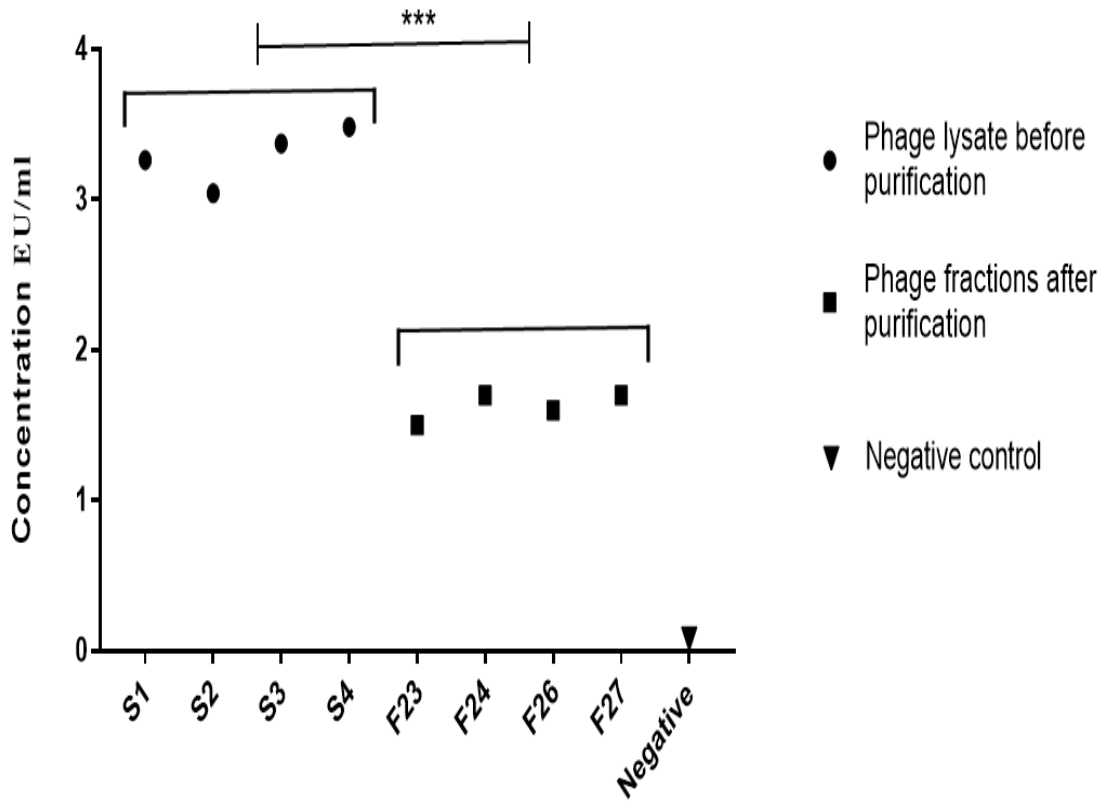



**Figure 5.6: Comparison of endotoxin level in phage lysate before and after purification.**

This figure shows the endotoxin level of 4 samples from the original phage lysate after propagation and before purification using both the CIM® column and endotoxin removal kit, in comparison with 4 samples that were purified using both the CIM® column and the endotoxin removal kit. The figure shows a significant reduction of endotoxin amounts in the samples before and after purification. Although this figure shows reduction of the endotoxin level after purification, it does not show a total elimination of the endotoxin. P value = 0.0023, significance is considered if p value is < 0.05.

### 5.4.4 Visualization of OMVs and OMV-phage complex

TEM was used to visualize the purity of the negatively stained *E. coli* OMV lysate before the interaction with phage EL. The isolated and purified OMVs show typical morphology of the extracted *E. coli* OMVs, where their size is between 70 to 100 nm (Figure 5.7). TEM was used to assess the interaction between *E. coli* OMVs and phage EL, which as previously mentioned, is capable of infecting *E. coli*. The images revealed that phage EL phage interacted successfully with *E. coli* OMVs in the mixture (Figure 5.8). Upon interaction, the phages attached to the OMVs and released their DNA. To assess the specificity of this interaction and whether other phages are able to interact with OMVs in the same manner, other phages and OMVs were tested. The *E. coli* phage, phage EL, was

mixed with OMVs extracted from *P. aeruginosa* PAO1. The TEM analysis of these samples showed no productive interaction between the OMVs of *P. aeruginosa* PAO1 and *E. coli* phage (Figure 5.9).

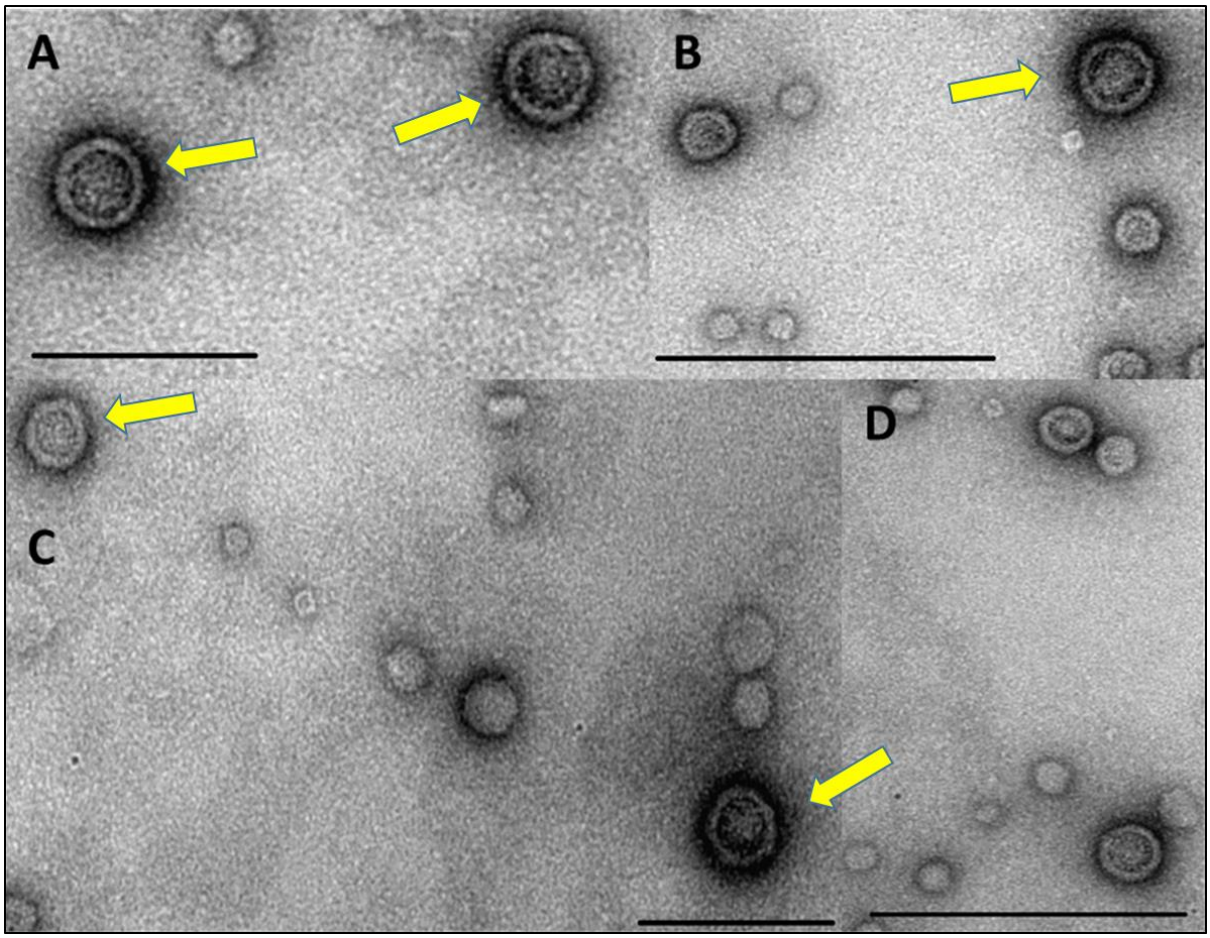



**Figure 5.7: TEM images showing *E. coli* OMVs before interaction with EL phages.**

This figure shows examples of negatively stained *E. coli* OMVs (Yellow arrows). (A) And (C) scale bars indicate 100 nm. (B) And (D) scale bars indicate 200 nm.

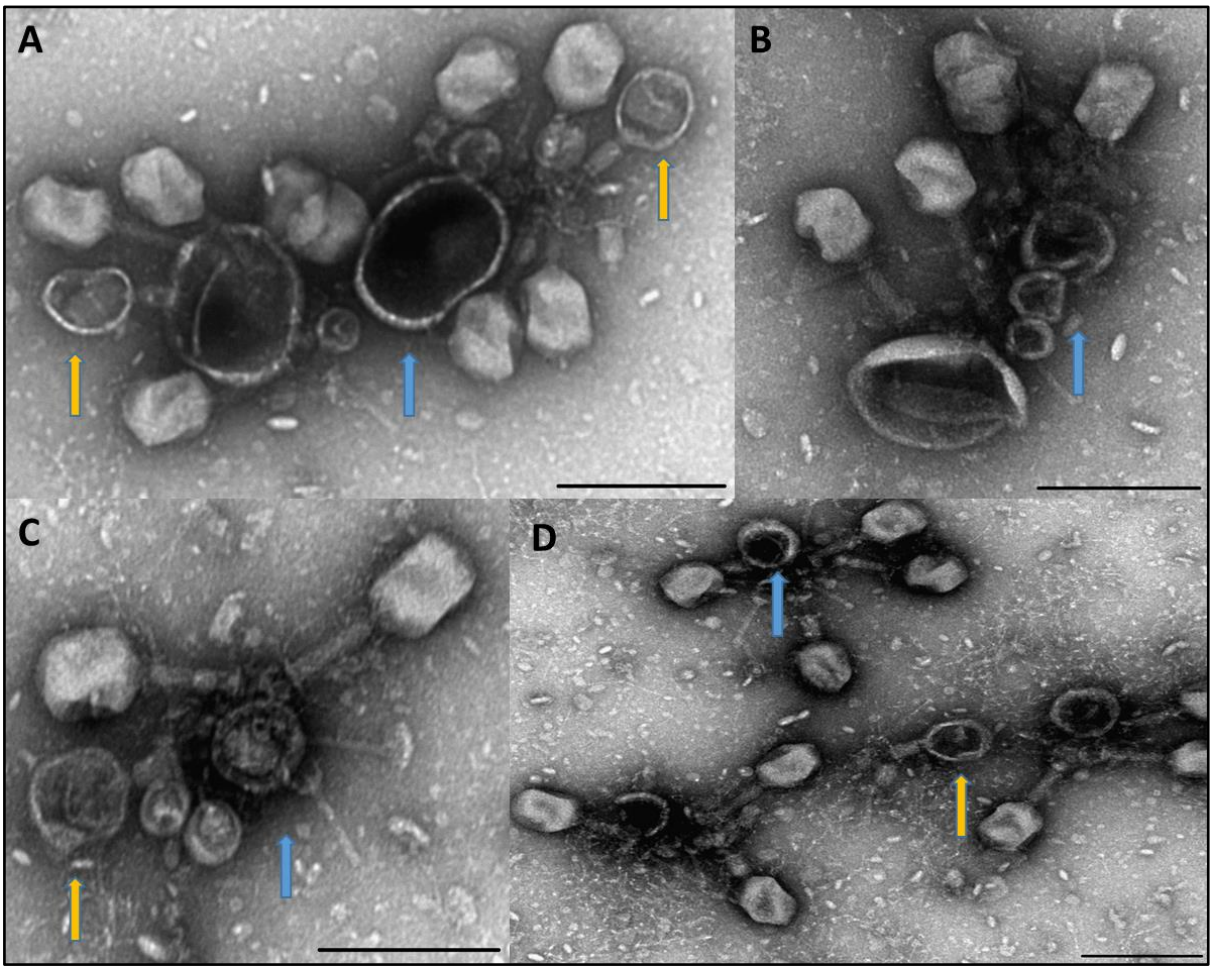



**Figure 5.8: TEM analysis of the interaction between phage EL and OMVs of *E. coli*.**

Multiple TEM images show the interaction between the E. coli OMVs and phage EL. The phage EL tail connects to the membrane of the OMVs and contracts. The blue arrows indicate the OMVs involved in the interaction. The gold arrows indicate empty phage capsids. Scale bar = 200 nm.

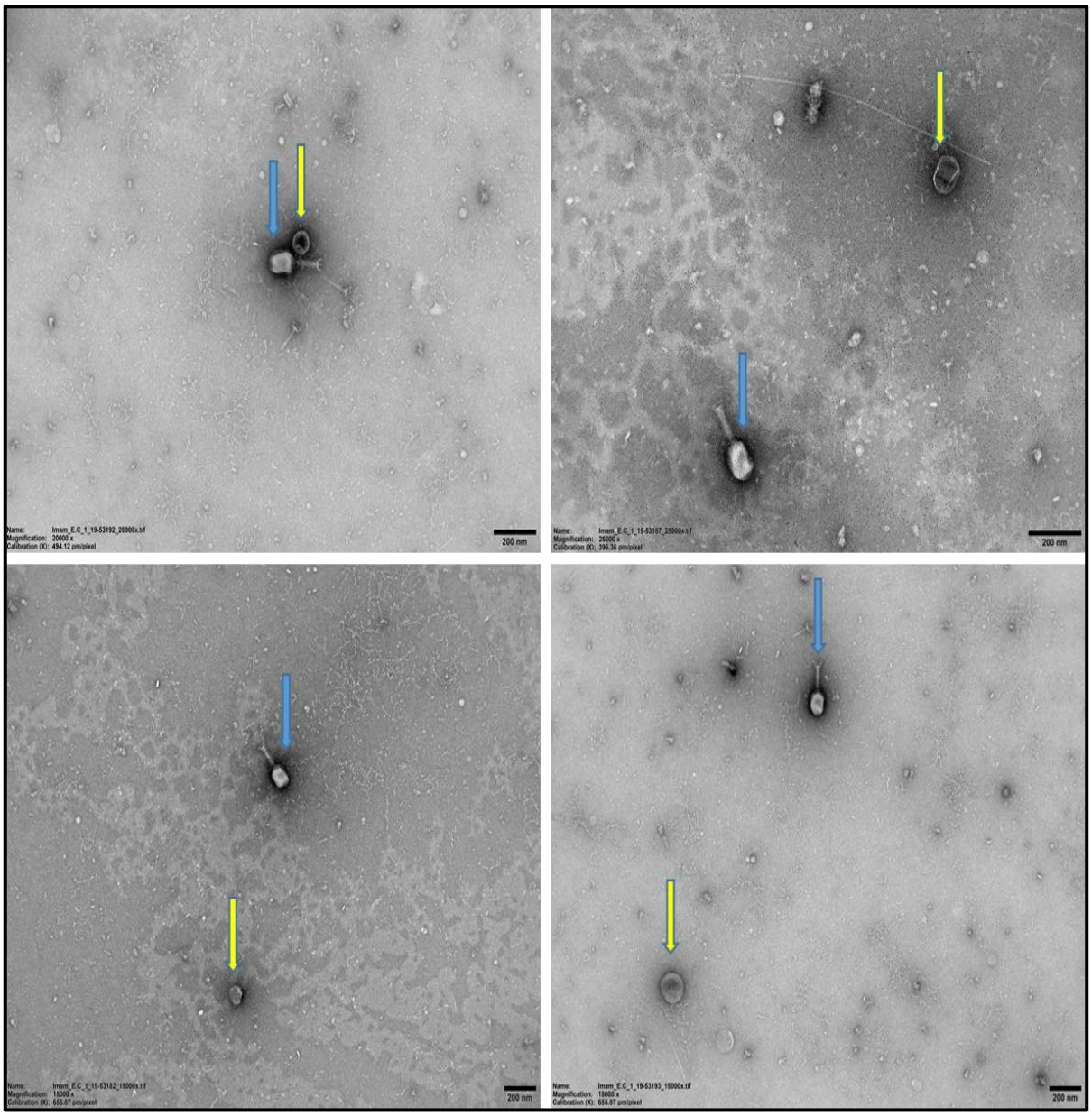


**Figure 5.9. TEM analysis of the *E. coli* phage EL interacting with OMVs from *P. aeruginosa*.**

These images show a lack of interaction between phage EL, which infects *E. coli* (blue arrow) and the OMVs of *P. aeruginosa* (yellow arrow). No sign of interaction can be observed, such as attachment, OMVs disruption, phages with contractile tails or empty capsids, highlighting the specificity of the interaction between OMVs and phages. Scale bar indicates 200 nm.

### 5.4.5 **Investigation of the effect of the interaction of OMVs with phage**

An investigation into the ability of the phage to alter the inflammatory response elicited by OMVs alone upon interaction with immune cells was critically required. This was assessed by investigating whether the interaction of phage with OMVs can affect the level of TNFα which are released by the macrophages in comparison with the immune response that is elicited by the OMVs alone. For that reason, three different samples, OMVs alone, OMV-phage complexes and phages alone, and the control for this experiment, which was 1000 ng/ml LPS, were incubated with the macrophages using cell line J774. Two time points were assessed: 4 hours and 24 hours. After each incubation period, the supernatant was collected from each sample and centrifuged for 1 minute at 13,000 x*g* to eliminate the presence of the macrophage cells. The supernatant was then collected and tested by ELISA to assess the level of TNFα present.

The results in (Figure 5.10) show the immune responses elicited by different samples at different time points. After four hours of incubation, there were no significant differences in the level of TNFα produced upon interaction of the cells with OMVs, phages, or phages interacting with OMVs. On the other hand, after 24 hours, the level of TNFα that is released by macrophages upon interaction with the phage-OMV complex was significantly reduced compared to that of OMVs alone. It is important to note that phages alone stimulated the release of TNFα, but at a level significantly lower than that stimulated by OMVs. This may be due to the residual presence of LPS in the phage samples which was not completely removed.

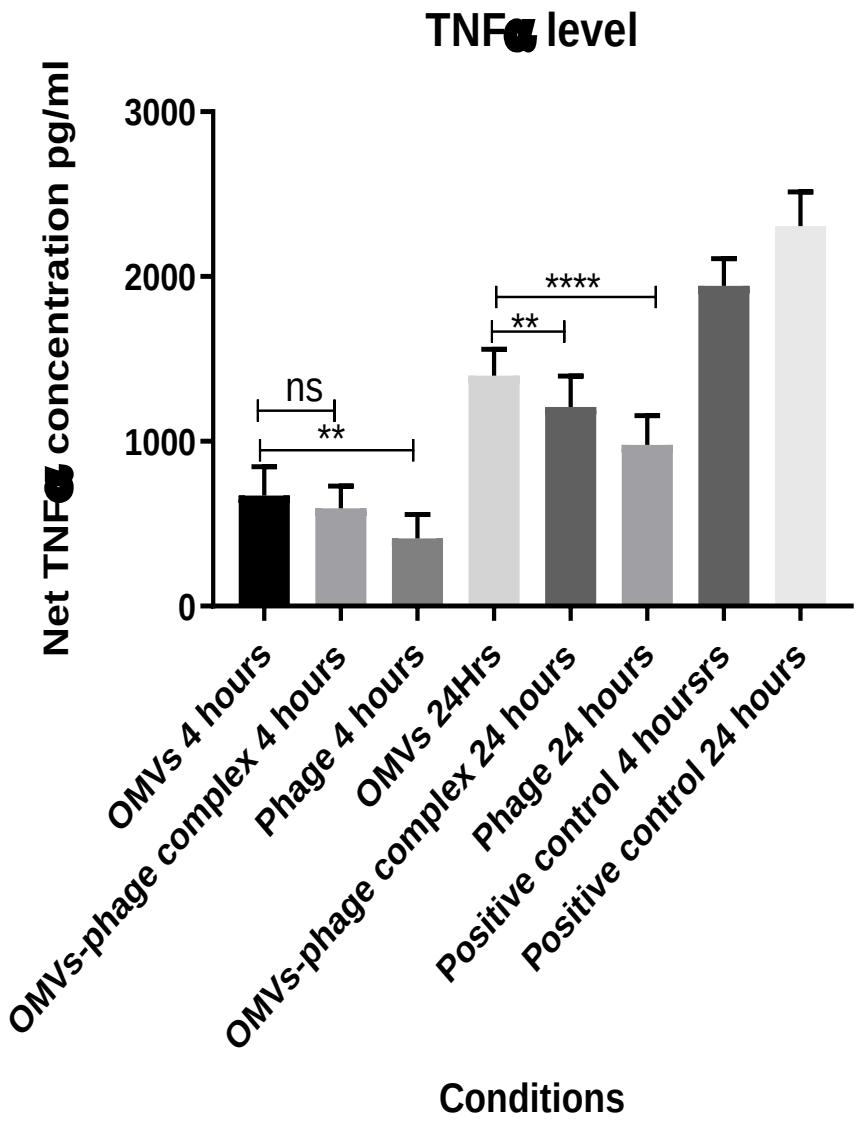



**Figure 5.10: Quantifying the amount of TNFα induced from macrophage cells J774 upon interacting with either phages, OMVs, or phage and OMV complexes.**

In a six-well plate, *E. coli* OMVs, *E. coli* OMVs interacted with phage EL, and EL phage alone were incubated with the cell line for 4 hours and 24 hours. The supernatant was then collected and spun down. TNFα was then measured via ELISA. This figure shows the comparisons between these different conditions and the positive control, which is 1000 ng/ml LPS. After 4 hours of incubation, the figure shows no significant reduction in the level of the induced TNFα upon the interaction of OMV-phage complex with macrophage cells in comparison with the level of TNFα that is induced upon the interaction of the macrophage cells with the OMVs alone. After 24 hours incubation, the figure shows a significant reduction in the induced TNFα upon interaction of OMV-phage complex with the macrophage cells in comparison with the level of the induced TNFα upon the interaction of macrophage cells with OMVs alone. Data was normalised by deduction of the negative control from each value. Data is presented as mean ± standard error of mean (SEM) of at least 4 independent experiments. Significance was assessed by analysis of variance (ANOVA) and Turkey's multiple comparisons test. Significance was considered if P value was $< 0.001$.

## 5.5 Discussion

This chapter primarily focuses on the work carried out to investigate interactions of OMVs with phages and to study the immune response stimulated from J774 macrophage cells upon interaction with either OMVs alone, phages alone or OMV-phage complexes. As discussed in (section 5.1), OMVs are known stimuli that modulate the host immune response, stimulating the production of pro-inflammatory cytokines such as TNFα and IL-8. This stimulation has been reported for different OMVs isolated from different Gram-negative organisms. On the other hand, phages have already been used for therapeutic purposes, meaning that they are considered safe to use on humans (Speck and Smithyman 2016). Phages do not usually stimulate the host immune response (Miernikiewicz *et al.* 2013). However, phage lysate might contain endotoxins, which are considered highly immunogenic and may stimulate the immune response (Szermer-Olearnik and Boratyński 2015). Endotoxins (LPS) are composed of Lipid A, which is the hydrophobic anchor of lipopolysaccharide that forms most of the outer layer of Gram-negative bacteria (Zhang *et al.* 2013). As mentioned, some phage lysates might contain endotoxins. Thus, as long as the endotoxin levels meet the legal limit of the British pharmacopeia, which is < 5 EU/kg of body weight, which equals <0.2 EU/ml, phages are still considered safe for use as therapeutic agents. However, even small amounts of endotoxin can stimulate an immune response (Fedele *et al.* 2020).

For that reason, the phages used in this study needed to be tested for the presence of endotoxins before they could interact with OMVs in order to test the effect of this interaction. The phage EL lysate was tested for the presence of endotoxin. As mentioned, the outcome of this experiment showed that endotoxin was present in the lysate. Various purification methods have been reported to successfully remove the endotoxin contamination in phage lysates. Three different purification methods were applied to reduce the presence of endotoxins in the phage lysate: ultra-filtration, purification via anion exchange chromatography, and the use of an endotoxin removal kit. These three methods have been reported to be 3 of the most efficient methods for the reduction of endotoxins in phage lysate (Hietala *et al.* 2019). Two of these methods were used in this work to reduce the level of endotoxin: purification via CIM® column and the endotoxins removal kit. The level of endotoxins was tested before and after the purification methods.

The results showed that although the endotoxin level decreased to the maximum possible level from the phage lysate, it was not totally eliminated.

After purifying OMVs and the phage, these were mixed in a ratio of 1:1; the OMV-phage interactions were verified and visualised via TEM.

Three conditions were involved in testing the immune response of the J774 macrophage cell line. The first condition was to verify the elicitation of the immune response by OMVs alone. The second condition was the OMV-phage mixture. This condition was to assess the effect of the OMV-phage interaction on the host immune response. The third condition was the phage alone, in order to assess the immune response against phages specifically. Other conditions were positive control (LPS) and negative control (PBS). These different preparations were incubated with confluent macrophages for 4 or 24 hours. After the incubation, the supernatants were tested for the presence of TNFα to assess the immune response elicited upon different stimuli to the immune cells (macrophages). The immune response cannot be investigated as a whole in this chapter, and TNFα was used in this study as an indicator and an example of the immune response stimulation. TNFα is a well-studied cell signalling protein (cytokine) which is involved in acute systemic inflammation (Patten *et al.* 2017). Thus, it was chosen as an appropriate marker of immune response. OMV-phage complex showed no significant reduction in the level of TNFα produced after 4 hours incubation in comparison with the elicitation of the immune response from OMVs alone. However, after further incubation, at the 24-hour point the effect of the phage on the immune response that was induced from OMVs was significantly lower than the immune response that was induced from the OMVs alone. As mentioned above, phage EL was purified using multiple purification methods. Nevertheless, endotoxin was not completely eliminated from the lysate, and may have induced the release of TNFα from immune cells. Thus, the interaction of OMVs and the phage could have resulted in a higher drop in immune response release if the phage used in this interaction did not stimulate the immune response at all (Figure 5.10).

Taken all together, these findings suggest that the phages can inactivate or "detoxify" the OMVs, possibly by disrupting them. As evidence for that, the elicited immune response against the OMVs was significantly higher than the elicited immune response against the OMV-phage mixture. This novel finding indicates the possible additional role that phages

can have in therapy and in the fight against bacterial infections: the use of phages for therapy will not only target bacteria, but also one of the bacterial virulence factors.

# Chapter six

## 6. Induction of prophages upon bacterial interactions with macrophages and preliminary investigation of phages in whole blood.

## 6.1 Introduction

Phages infect and propagate within bacteria; this infection occurs via two possible life cycles, these being lytic or lysogenic cycles. Briefly, in the lytic life cycle, the phage attaches to receptors on the bacterial cell, injecting its DNA and initiating the phage replication programme, taking over the host cell machinery. This leads to the production of progeny phages. On the other hand, while the lysogenic life cycles begins in the same manner as the lytic life cycle, the presence of the integrase results in the phage genomic material being integrated within the bacterial genome, thus becoming a prophage. Prophage replicates with the host for many generations. Eventually, the lysogenic phage can be induced, replicated and released under certain conditions. Phages that can undergo both cycles are referred to as temperate phages (Orlova 2012; Monteiro *et al.* 2019).

Prophages can contribute towards the survival chances of their hosts in the environment. They can provide their hosts with metabolic or virulence properties, resistance mechanisms and alter bacterial genomes, thereby providing a better chance of survival and thriving in the harsh environment (Menouni *et al.* 2015).

One of the most popular prophages in phage research is phage Lambda (λ phage). It was firstly discovered in 1951 by Esther Lederberg, who found the ability of *E. coli* K-12 to release this phage upon exposure to UV light. Since this discovery, and due to the ability of the Lambda phage to undergo two phage lifecycles as a temperate phage, it has been used as a model system to study and understand phage biology (Casjens and Hendrix 2015).

The lifecycle of the Lambda phage, like most phages, is initiated by the recognition of its receptor on the target bacterium. Thereafter, the chemieric J protein, which is located at the end of the phage tail, binds to the maltose pore receptor LamB, which is located on the OM of the bacterial cell. Afterwards, the phage DNA is injected via a long flexible tail ejection system into the bacterial cytoplasm. After this point, and with the help of ligase and gyrase enzymes, the viral DNA integrates with the bacterial host DNA (Chatterjee and Rothenberg 2012).

As a temperate phage, the Lambda phage can undergo two types of replication life cycles. The first one is the lysogenic pathway. The phage genome integrates with the host cell's chromosome in a very stable, silent state and exists as part of a symbiotic relationship (Semsey *et al.* 2015). The second cycle is the lytic lifecycle. This cycle is responsible for the production of phage progeny virions, killing the bacterial host cell and then infecting different bacterial hosts. The phage might undergo any of these lifecycles depending on the nutritional state of the bacterial host, the number of infecting phage particles and the exposure to one of many environmental stressors (Erez *et al.* 2017). The lytic-lysogenic decision depends on the presence of specific factors, such as the phage proteins; CI repressor and Cro. If the concentration of Cro proteins is more than the concentration of CI repressor proteins, the phage will undergo a lytic lifecycle. If the other way around, the phage undergoes a period of latency and enters the lysogenic lifecycle (Casjens and Hendrix 2015).

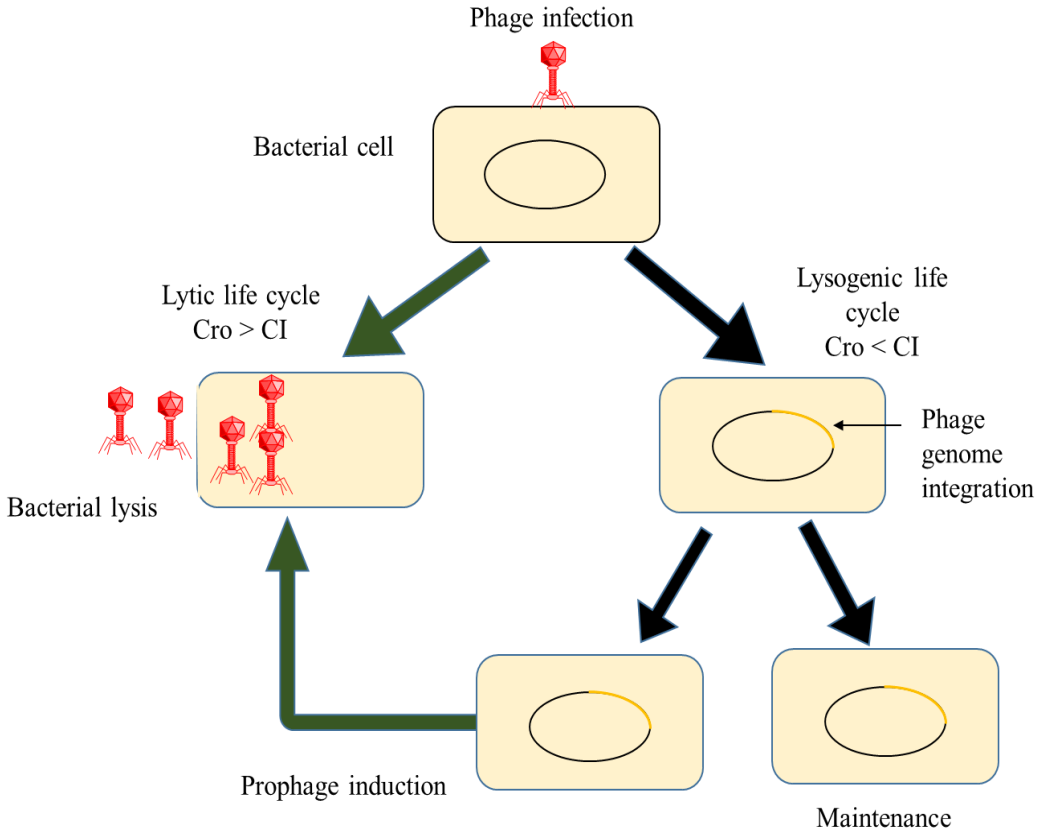



**Figure 6.1: Temperate phage lifecycle.**

The diagram illustrates the life cycle of a temperate phage such as phage Lambda. The phage can undergo either a lytic lifecycle (red arrows) and produce progeny, or it can undergo a lysogenic cycle (black arrow) by integrating within the bacterial chromosomal DNA and maintaining the period of latency.

Temperate phages can produce progeny by switching from a state of latency to a lytic lifecycle; this can occur via an induction event. Prophage induction can be initiated by applying stress on the bacterial host. These stressors can either be brought on by heat treatment of the temperature-sensitive repressors, chemotherapeutic effects and bacterial DNA-damage by damaging agents such as UV light or antibiotics (mitomycin C) (Janion 2008; Rokney *et al.* 2008). Moreover, prophages may be spontaneously induced without external triggers. This induction might be as a result of the bacterial replication that can cause DNA damage (Nanda *et al.* 2015). From an evolutionary standpoint, it makes sense that the prophage would prefer to be induced when the host cell is under stress, allowing for the prophage to escape its weak host and find a better host to infect and survive within.

Phages can attach to OMVs using OMV receptors such as siderophore receptors, and OMVs in this case act as a collector of phage particles such as tails, and as a defence mechanism that prevent the spread of phages (Kharina *et al.* 2015). Upon induction, lysogenic phages were found to be associated with OMVs, and as it is known that the production of OMVs is induced and upregulated during infection, this upregulation of OMVs production contributes to the survival of pathogenic organisms within the host (Bauwens *et al.* 2017). In this chapter, the main focus is to investigate whether the induction of prophages also happens under *in vivo* conditions, and whether immune cells can act as a trigger to initiate the induction of prophages.

## 6.2 **Aim and objectives**

This study aimed to run pilot experiments to investigate 2 different phenomena:

- First was to investigate whether prophages can be induced upon interaction with macrophages.
- Second was to investigate the ability of prophages to be induced upon interaction with whole blood.

## 6.3 Methods

### 6.3.1 Bacterial strain preparation

The bacterial strain *E. coli* NM522 n4, which contains the lysogenic phage (phage Lambda) integrated into the bacterial genome, was used to compare different induction conditions. This bacterial strain was prepared from an overnight liquid culture. The bacterial culture was diluted 1 in 100 in 5 ml LB, after which it was incubated at 37 °C on a shaker incubator till the $OD_{600}$ of the culture reached 0.4. Afterward, the culture was centrifuged at 2,000 x*g* for 10 minutes to form a pellet. The supernatant was removed and the pellet was then resuspended in 5 ml DMEM.

### 6.3.2 Prophage induction

Described below are three methods which were performed to induce phage Lambda. This was done in order to compare the methods and check whether the interaction of macrophages with bacterial strain *E. coli* NM522 n4 would result in the induction of its prophage.

### 6.3.3 Spontaneous induction

The bacterial strain *E. coli* NM522 n4 was streaked onto LB agar. A lawn was made using 6 ml of sloppy agar and 300 µl *E. coli* DH5α, which was poured on top of the LB agar and then left to solidify. The plate was then incubated for 6 hours at 37 °C. The next day, the plates were checked for the presence of plaques.

The prophage was induced spontaneously using DMEM (Dulbecco's Modified Eagle's Medium) media-high glucose (Thermo Fisher scientific, UK, and Ref # 11965092) supplemented with L-glutamine, 10 % (v/v) fetal bovine serum (FBS), penicillin ($10^2$ $IU.ml^{-1}$), and streptomycin (100 $\mu g.ml^{-1}$). 1 ml of this media was added to cover the surface of a well of a six-well plate; 100 µl of *E. coli* NM522 n4 washed cells were resuspended in the media, and the plate was incubated overnight at 37 °C. After this incubation time, 1 ml was collected from each well and centrifuged for 5 minutes at 2,000 x*g*. The supernatant was then collected, filtered using 0.22 µm filters and tested for the presence of the induced phage via spot test assays.

### 6.3.4 Mitomycin C induction

The lysogenic phage that is present within the bacterial genome of *E. coli* NM522 n4 was induced using the antibiotic mitomycin C. Using a six-well plate, 1 ml of supplemented DMEM was added into each well. Thereafter, 100 µl of a prepared bacterial suspension of *E. coli* NM522 n4 was added to the media. Mitomycin C was then added to a final concentration of 1 µg/ml, after which the plate was incubated for 6 hours at 37 °C. 1 ml of media was collected, centrifuged, filtered and tested via spot test assays in a similar manner as performed with the spontaneous induction experiment mentioned above in section 6.3.3.

### 6.3.5 Lambda phage induction by Macrophages

In a similar manner, Lambda phage was induced by macrophages in a six-well plate. The macrophages were grown to a confluence of 70 to 80 %, as described in (Section 5.3.7). The media was then removed and replaced with 1 ml of fresh supplemented DMEM. Thereafter, 100 µl prepared bacterial suspension of *E. coli* NM522 n4 was added to the media. The plate was then incubated for 6 hours at 37 °C. After incubation, 1 ml of media was collected from each well, centrifuged for 5 minutes at 2,000 x*g* and filtered using 0.22 µm filters. The titre of the induced phage was determined via spot test assays.

### 6.3.6 Ethical Statement

Whole blood samples were collected from healthy volunteers by a phlebotomist in accordance with the principles of ethical standards. All participants provided written consent prior to their enrolment in the study, (Supplementary 1). All protocols were reviewed and approved by Dr Chris Bayliss, University of Leicester.

### 6.3.7 Spot test

The concentrations of the phages tested in the aforementioned experiments were determined via spot test. As described in (2.7), 6 ml of sloppy agar was mixed with 300 µl of the bacterial host was poured on top of LB agar and left to solidify. The phage lysate was serially diluted by transferring 10 µl of the phage into 90 µl of SM buffer. This dilution took place in a microplate from well 1 to well 12. 10 µl of each dilution was spotted on top of the solidified sloppy agar. The agar plates were then left on the bench until the spot was almost dry. Thereafter, the plates were incubated at 37 °C overnight.

The next day, the titre was determined by counting the plaques visible at the highest dilution.

## 6.4 Result

### 6.4.1 Comparison of induction methods of phage Lambda from *E. coli* NM522 n4

This experiment was performed to test whether phage Lambda is inducible upon interaction with a macrophage. This potential induction was compared with the natural spontaneous induction that occurs with phage Lambda, and induction using 1 µg/ml mitomycin C. After inducing the phage with different methods, the induced phages were quantified using spot tests. As shown in (Figure 6.2), the induction of phage Lambda using mitomycin C was, as expected, the most efficient method when compared with the spontaneous and macrophage inductions. This is because mitomycin C targets and causes damage to the bacterial DNA. The crucial part of this experiment was to determine whether the induction of phage Lambda with the use of macrophages is different when compared with natural spontaneous induction. As shown in (Figure 6.2) below, the induction of phage Lambda using macrophages was significantly higher than the spontaneous induction, and lower than the induction caused mitomycin C.

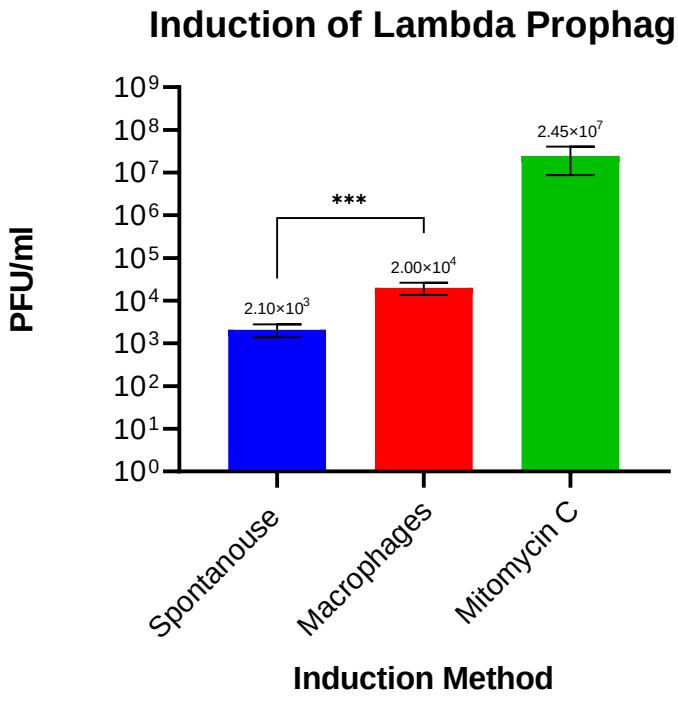



**Figure 6.2: Comparisons of Lambda phage induction from *E. coli* NM522 n4.**
Three different methods of prophage induction were tested to induce phage Lambda. As expected, induction with mitomycin C was the most efficient method for induction. Phage Lambda induction using macrophages was significantly higher than spontaneous induction. Data is presented as mean ± standard error of mean (SEM). Differences were considered significant if P value was < 0.001. (n = 3).

### 6.4.2 **Survival and recovery of Lambda phage from whole blood**

Phages are potential therapeutic agents which can combat bacterial infections. However, this experiment was performed in order to check whether phage Lambda is induced when mixed with whole blood in a similar manner as with macrophages. Furthermore, it was important to test whether the induced phage is able to survive in whole blood after different incubation periods, as it would not be possible to detect any possible induction if the induced phage was rapidly inactivated. Blood samples in this experiment were collected from healthy volunteers in accordance with the ethical requirements.

This experiment started with the addition of *E. coli* NM522 n4 ($OD_{600}$ = 0.4) to whole blood samples. Afterwards, this mixture was incubated at 37 °C for specific durations; 0 minutes (control), 30 minutes, 2 hours and 4 hours. Then, 300 µl of the mixture was collected for each time point, and was spun at 2,000 x*g* for 15 minutes; the sera were collected, and spot test assays were performed to assess the titre of the induced and recovered phage after incubation with whole blood. As shown in Figure 6.3, the initial phage titre, which would have been the result of spontaneous induction in the bacterial inoculum, was 7.5 x $10^3$ PFU/ml. After 30 minutes of incubation, the phage titre had increased to 1.0 x $10^5$. This is almost a 14-fold increase in phage titre in comparison with the initial titre. After 2 hours of incubation, the phage titre was maintained at a similar level as that measured at the 30 minutes time point. After 4 hours of incubation, a 6-fold increase was found in the phage titre in comparison to the initial sample titre, but it was 8-fold lower than that measured after 2 hours of incubation. This reduction in titre might be the result of phage depletion caused by binding to bacteria or their remains, or clearance of the phage by the immune cells. However, it must be clearly stated that this experiment is a pilot experiment, and the results obtained are only preliminary and need to be investigated further. To summarise, this experiment shows that phage Lambda can be induced upon interaction with whole blood, as well as be recovered after being incubated with whole blood.

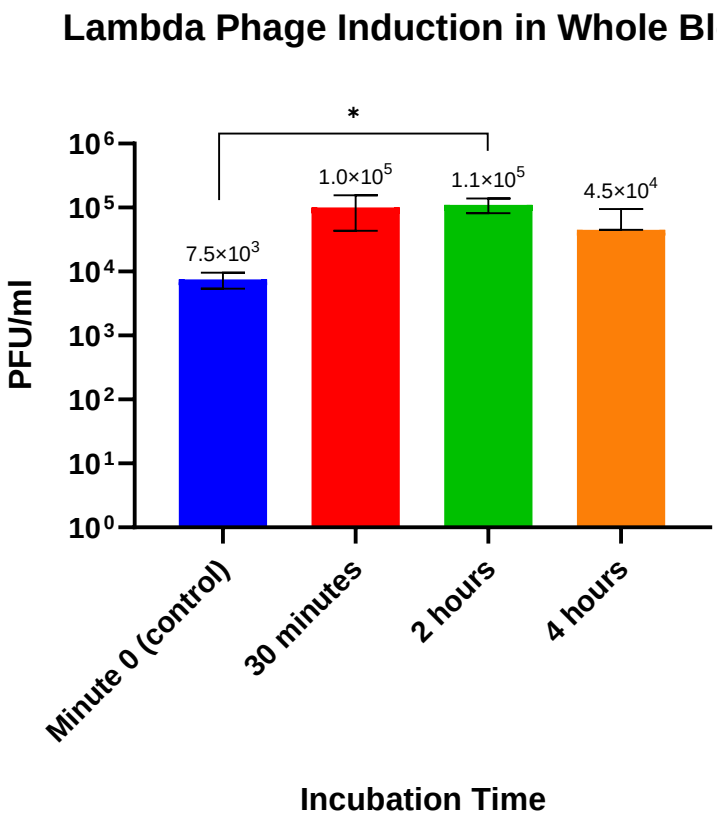



**Figure 6.3: Induction and survival of Lambda phage from whole blood sample.**

The bacterial strain *E. coli* NM522 n4 at an OD600 = 0.4, was added to whole blood samples. Four time points, 0 minutes (control), 30 minutes, 2 hours and 4 hours. At these points, samples were collected, centrifuged and the supernatant was used to quantify the amount of phage present. As shown, there was a 14-fold increase in the phage titre after 30 minutes and 2 hours, in comparison with the initial titre of the control at time point 0. Additionally, a 6-fold increase was observed after 4 hours of incubation in comparison with the initial titre. A t test was performed to compare each time point and the control time point of minute 0. Comparing between 0 and 30 minutes resulted in a significant difference, where a p value of $< 0.05$ was obtained. In almost the same manner, a significant difference was found between minute 0 and 2 hours. On the other hand, no significant difference was found between minute 0 and 4 hours (p value of 0.3965).

## 6.5 Discussion

Most of the bacterial species that are part of the human microbiota are lysogenic. Although the biological role of these biomes is not clear, prophages are induced from these lysogens in the human biomes, such as the gut. It is estimated that the production of prophages in the intestine is almost 200-fold higher than *in vitro* (Duerkop *et al.* 2012).

It has been reported that hydrogen peroxide influences the induction of prophages (Łoś *et al.* 2010). Additionally, as is known, one of the main elements of the antimicrobial mechanisms of macrophages is the production of hydrogen peroxide. Therefore, it can be suggested that prophages can be induced upon interaction with macrophages due to hydrogen peroxide activity.

The ability of the Lambda prophage to be induced from the lysogenic strain *E. coli* NM522 n4 has been investigated in this chapter. The lysogenic *E. coli* NM522 n4 was incubated for 6 hours with macrophages, which resulted in a 6-fold increase in the amount of prophages induced when compared to the spontaneous induced prophages. This experiment confirms the capability of macrophages to induce prophages from lysogenic strains. Although the amount of phages induced by macrophages was not as high as observed with mitomycin C, this can be justified by the fact that mitomycin C directly targets the lysogen DNA and causes damage to most of the bacterial cells, eventually leading to prophage induction. However, in our experiment, only a fraction of bacteria may be in direct contact with macrophages, and even fewer could be taken up and thus exposed to oxidative stress. Furthermore, it has been described previously in the work of (Łoś *et al.* 2010; Górski *et al.* 2018) that induction of prophages using mitomycin C was 10 times more efficient than hydrogen peroxide.

In addition, this chapter outlines the work carried out to investigate the ability of the Lambda prophage to be induced from the lysogenic *E. coli* nm522 n4 upon incubation with whole blood samples. The results revealed that the components of the whole blood samples were also able to induce phage Lambda, and that the phage was capable of surviving in whole blood even after 4 hours of incubation. This was concluded after comparisons were made between multiple incubation time points, which were also compared with a 0 minute incubated control. This 0 minute presumably represents the

amount of spontaneous induced phages likely to be present in the original bacterial inoculum. However, this experiment showed an increase in the titre of phages when incubated for 2 hours in the blood. After further incubation, the phage titre decreased at the 4-hour time point. These results do not conclusively point to why this occurred. However, it can be said that the reduction in the titre is normal and possibly due to the lack of host cells that can help in the propagation of the phage in the whole blood, or due to the clearance of the phage itself. Furthermore, this experiment provided preliminary results that must be investigated thoroughly. As a future plan, the bacterial cells shall be quantified alongside the phage titre.

This approach can add to published literature that suggests the use of induced phages as biomarkers to diagnose, indicate and confirm the causative agent of an infection (Brown-Jaque *et al.* 2016). Using prophages as a diagnostic tool can lead to an accurate and early diagnosis of infections, and therefore allow early disease management and better recovery rates.

Phage therapy is a great candidate for the treatment of sepsis. Due to the ability of phages to eliminate bacteria, phage administration during the early stages of sepsis could be useful (Górski *et al.* 2018). By proving the ability of the phages, both lytic and lysogenic, to survive in whole blood, the findings from this work will support the study of using phage therapy in the treatment of infections, particularly septicaemia.

# Chapter seven

## 7. General discussion

## General discussion

Bacteriophages have garnered global interest in recent years, particularly due to the increased emergence of pathogens displaying antimicrobial resistance. Additionally, many bacteria possess resistance to multiple antimicrobials, through different resistance mechanisms (Azam and Tanji 2019). When antibiotics become inefficient, alternative treatment options are required, and bacteriophages can be an excellent candidate for combating and treating infections, especially MDRs infections. There are several reports suggesting that phages can be used successfully to treat infections caused by multidrug resistant organisms that could not be treated with antibiotics. The more is known about phages, their biology and the mechanisms they use to kill bacteria, the more support will be provided in favour of phage therapy to be considered as a viable treatment option.

Many studies have focused on the ability of lytic phages to treat bacterial infections (Hung *et al.* 2011; Górski *et al.* 2017b; Kortright *et al.* 2019). Several studies reported the successful use of such bacteriophages to treat infections which could not be treated with antibiotics (Dedrick *et al.* 2019). Moreover, most of the bacterial species forming human microbiota are lysogenic (Duerkop *et al.* 2012). Although the biological role of these biomes is not fully understood, it is clear that many prophages are induced from the lysogens inside human bodies, particularly in the gut. It is estimated that the induction of prophages in the intestine is almost 200-fold higher than under *in vitro* conditions (Duerkop *et al.* 2012).

Despite the evidence that we are constantly exposed to phages, and that such exposure could be highly beneficial for our health, the role of phages, both lytic and lysogenic, in supporting the immune system to combat bacterial infections *in vivo* is not well studied. The research in this project focused on investigating aspects of the poorly understood, yet commonly occurring *in vivo* interaction between three factors: innate immune system, bacteria and phages. The main part focused on the phage interactions with the first line of bacterial defence against stressors, which are bacterial outer membrane vesicles (OMVs), with the aim of investigating the outcome of such interactions and their impact on subsequent interactions with the immune cells, specifically with macrophage cells.

OMVs produced by different bacteria are considered to be bacterial virulence factors. They have been found to stimulate an immune response upon interaction with immune cells (Martora *et al.* 2019; You *et al.* 2019). Additionally, it was proposed that OMVs may serve as "bacterial innate immunity", as bacteriophages have been found to interact with OMVs (Manning and Kuehn 2011) and lose their infectivity as a result. What happens when such an interaction occurs *in vivo*, during the bacterial infection of a human host? To begin addressing this question, this project was designed to investigate whether OMV-phage interactions may alter the immune response.

The OMVs from *E. coli* were isolated and mixed with phage EL, and their interaction was assessed using TEM. The analysis of TEM images revealed actual physical attachments between phages and OMVs. It also showed the presence of phages with contracted tails and empty heads, indicating the event of irreversible binding, which leads to phage DNA ejection. Similar electron microscopic images have been obtained and reported previously in different publications for different phages (Manning and Kuehn 2011; Parent *et al.* 2014). Once the productive interaction of OMVs from *E. coli* and phage EL were confirmed, the main components needed to be purified before they could be used to assess whether their interactions have immunological consequences.

The ability of the OMV-phage mixture to stimulate an immune response was investigated by assessing the interaction of this mixture with the J774 macrophage cell line. The outcome of this experiment was compared with the outcome of the interaction of macrophages with OMVs alone, and phages alone. As already mentioned, OMVs are a known stimuli that modulate host immune responses, stimulating the production of pro-inflammatory cytokines such as TNFα and IL-8. This stimulation has been reported for different OMVs isolated from different Gram-negative organisms (Ismail *et al.* 2003; Alaniz *et al.* 2007; Martora *et al.* 2019; You *et al.* 2019). On the other hand, phages used for therapeutic purposes (Altamirano and Barr 2019) do not usually stimulate the host immune response (Miernikiewicz *et al.* 2013). TNFα was chosen as an appropriate marker for the immune response due to its involvement in acute systemic inflammations (Moledina *et al.* 2019; Müller *et al.* 2019). The level of TNFα that was induced from macrophages cells upon interaction with OMV-phage mixture, OMVs alone and phages alone, was measured using the ELISA technique. A significant reduction in the level of TNFα produced by macrophages after a 24-hour incubation with OMV-phage mixtures

was observed, in comparison with the elicitation of the immune response from OMVs alone. This finding might suggest that the phages can inactivate or "detoxify" the OMVs, possibly by disrupting them. This novel finding indicates the possible additional role that phages can have in therapy and in the fight against bacterial infections; the use of phages for therapy will not only target bacteria, but also one of the bacterial virulence factors.

Before mixing OMVs with phages, in this project, a period of time was spent on optimizing a method to extract and isolate OMVs from Gram-negative bacteria in a pure form. Several attempts were made to isolate OMVs from *K. pneumoniae* using the centrifugation-based method. However, these attempts were not successful. Upon further investigation, it was found that OMV production from Gram-negative bacteria is associated with the ability to synthesise flagella, including the synthesis of flagella proteins (Manabe *et al.* 2013). Therefore, the experimental difficulties with regards to the isolation of OMVs from *K. pneumoniae* encountered in this project suggest that bacteria that do not form flagella, in other words immotile bacteria such as *K. pneumoniae*, would not produce large quantities of OMVs, as motile bacteria would. Due to the difficulty faced with the extraction of OMVs from *K. pneumoniae* and in the interest of time management, the project shifted its focus to extracting OMVs from a motile bacterium, *E. coli*. The project was diverted to optimize a reproducible and efficient method to isolate pure OMVs at a high concentration using *E. coli* cells.

In this project, a new method was optimized successfully to extract OMVs from *E. coli*. The efficacy of this novel method was proven to extract OMVs from *E. coli* in a reproducible matter. The extracted OMVs were pure when visualised by transmission electron microscope (TEM) in comparison to the extracted OMVs from *E. coli* using the centrifugation-based method. This optimized protocol was used to extract OMVs from both *E. coli* and *K. pneumoniae*. The protocol resulted in reproducible results every time it was used to extract OMVs from *E. coli*. However, OMVs from *K. pneumoniae* were not extracted to a high concentration and purity using the optimized method. In order to draw a final conclusion about the efficacy of the optimised method and its potential ability to extract OMVS, this protocol needs to be used to extract OMVs from different Gram-negative bacteria, and motile and immotile bacterial strains, including different strains of *K. pneumoniae*.

As OMVs from *K. pneumoniae* were not successfully extracted in this project despite using two different methods, and the main purpose of this project was to investigate the immunological outcome of interacting OMV-phage mixtures with immune cells, phage EL, which is a phage capable of infecting *E. coli*, was introduced and used to interact with OMVs from *E. coli*.

According to the initial main aim of this project, which was to test the effect of *K. pneumoniae* phage on OMVs from *K. pneumoniae*, the isolation and characterisation of phages infecting *K. pneumonia* was an important part. The main reason for isolating these phages was to use them for research with OMVs and macrophage cells. However, the isolation and characterisation of new phages capable of infecting *Klebsiella* are important in their own right, as they are a useful addition to the already isolated sets that may be used to fight these important bacterial pathogens.

For these purposes, three different phages were successfully isolated from sewage samples: Kp2811, Kp3153 and Kp2811M. These phages were named after the bacterial strains that were used to isolate them and according to their plaque morphology. Each phage underwent 5 rounds of purification before they were propagated in order to increase their concentrations. Phage Kp2811 and phage Kp3153 were successfully characterised by sequencing their genomes, along with a few other experiments to biologically characterize them. The sequence analysis for both revealed the similarity of these phages to previously isolated phages whose genomes were deposited in the NCBI database. However, despite this similarity, phages Kp2811 and Kp3153 can be considered as novel phages, due to the differences in their sequences and host range. Phage Kp2811 and Kp3153 are infecting only a limited number of *K. pneumoniae* strains in our small collection. However, the host range analysis for phages Kp2811 and Kp3153 should be determined against a broader range of bacterial strains. It should also be noted that to date, most Klebsiella- specific phages which have been isolated have rather narrow host specificities (Kęsik-Szeloch *et al.* 2013; Ciacci *et al.* 2018; Tabassum *et al.* 2018). One of the important findings in this project was the isolation of phage Kp2811 and phage Kp3153, which can be considered good additions to already described phages to fight infections caused by *K. pneumoniae* strains.

Despite the several attempts to sequence phage Kp2811M, unfortunately, the phage genome was not sequenced successfully. The reasons that led to the unsuccessful sequencing of this phage are not fully known, since the method used to extract phage Kp2811M genome was the same at that used for phage Kp2811 and Kp3153.

All the three phages are able to infect different strains of *K. pneumoniae*. Therefore, they can be used for therapeutic purposes as such, or by harnessing their enzymes to be used as antimicrobial products. Many researchers focus on using phage enzymes instead of using intact phages because of the legislative hurdles that are associated with phage therapy (De Smet *et al.* 2017). One of the enzymes that can be used to degrade the membrane of bacterial cells is endolysin, which is produced by phages. The main function of this enzyme is to degrade the peptidoglycan layer within the bacterial cell wall; it plays a role in the exit of phages and lysis of the host cell (Briers *et al.* 2007; Briers *et al.* 2014). Further work could be to investigate the host range of these phages on a wider collection of bacterial strains to support their potential use for therapy on their own, or as part of a phage cocktail against *K. pneumoniae* infections.

A pilot experiment was conducted at the end of this work to investigate the induction of lysogenic phages upon interaction with macrophage cells and with whole blood samples. This experiment has provided preliminary results of the possible outcome of the interaction between lysogenic bacteria and blood cells. This work has shown that macrophage cells may be able to induce lysogenic phages. The induction using macrophages was compared with the level of mitomycin C induction and spontaneous induction; the result revealed that the use of macrophages successfully induced prophages to a level that was lower than mitomycin C induction, but higher than the spontaneous induction. From an evolutionary standpoint, this makes sense for the lysogenic phage; as the bacterial cell is encountering stress and possibly going to die, the lysogen will selfishly escape to better its own chance of survival. Similarly, this work demonstrated that prophages can be induced upon interaction with the components of whole blood samples, and the capability of the phages to survive in the whole blood sample even after 4 hours of incubation. These results support the use of induced phages as diagnostic tools (Brown-Jaque *et al.* 2016) that can lead to an accurate and early diagnosis of infection, and eventually early disease management and better recovery rates.

In summary, this study demonstrated the ability of phages to interact with OMVs, and as a result of this interaction, the OMVs become inactivated and possibly disrupted. This can support the usage of phages as a treatment to fight bacterial infections alongside antibiotics, which can then lower the excessive use of antibiotics which is promoting the generation of antibiotics resistance mechanisms. Additionally, three novel phages that can infect *K. pneumoniae* have been isolated, two of which were successfully characterised. The next step is to continue the characterisation of these phages by studying their potentials in treating a wide range of *K. pneumoniae* strains, and see how effectively they work as a cocktail with other phages. Moreover, an important methodological development from this project was the validation of the OMV extraction and purification protocol, which can be followed to isolate highly pure OMV preparations that can be used in various OMVs applications, such as vaccination. In addition to that, this project has preliminarily assessed a novel phenomenon, which is the induction of lysogenic phages upon interaction with macrophages cells and components of whole blood samples. This can allow for further understanding of the behaviour of lysogenic phages inside host cells and highlights the ability of prophages to be induced upon interaction with immune cells. This approach can provide an opportunity to research into phage behaviour within the host.

## 7.1 Future work

The results and outcomes of this project could lead to various avenues of further study in the future. The following ideas are suggested for future work:

1. To expand the host range specificity of the isolated phages in this project, by testing the phages against a wide range of *K. pneumoniae* strains.

2. The use of phages Kp2811, Kp3153, and Kp2811M with other phages to produce phage cocktails with a wider host range that can be used in animal model experiments.

3. Assessing the ability of phage-derived molecules, such as the endolysin enzyme from phages Kp2811 and Kp3153, to inhibit bacterial infections.

4. Validating the optimised methods for the extraction of OMVs and testing whether this method could be used to extract OMVs from different Gram-negative organisms.

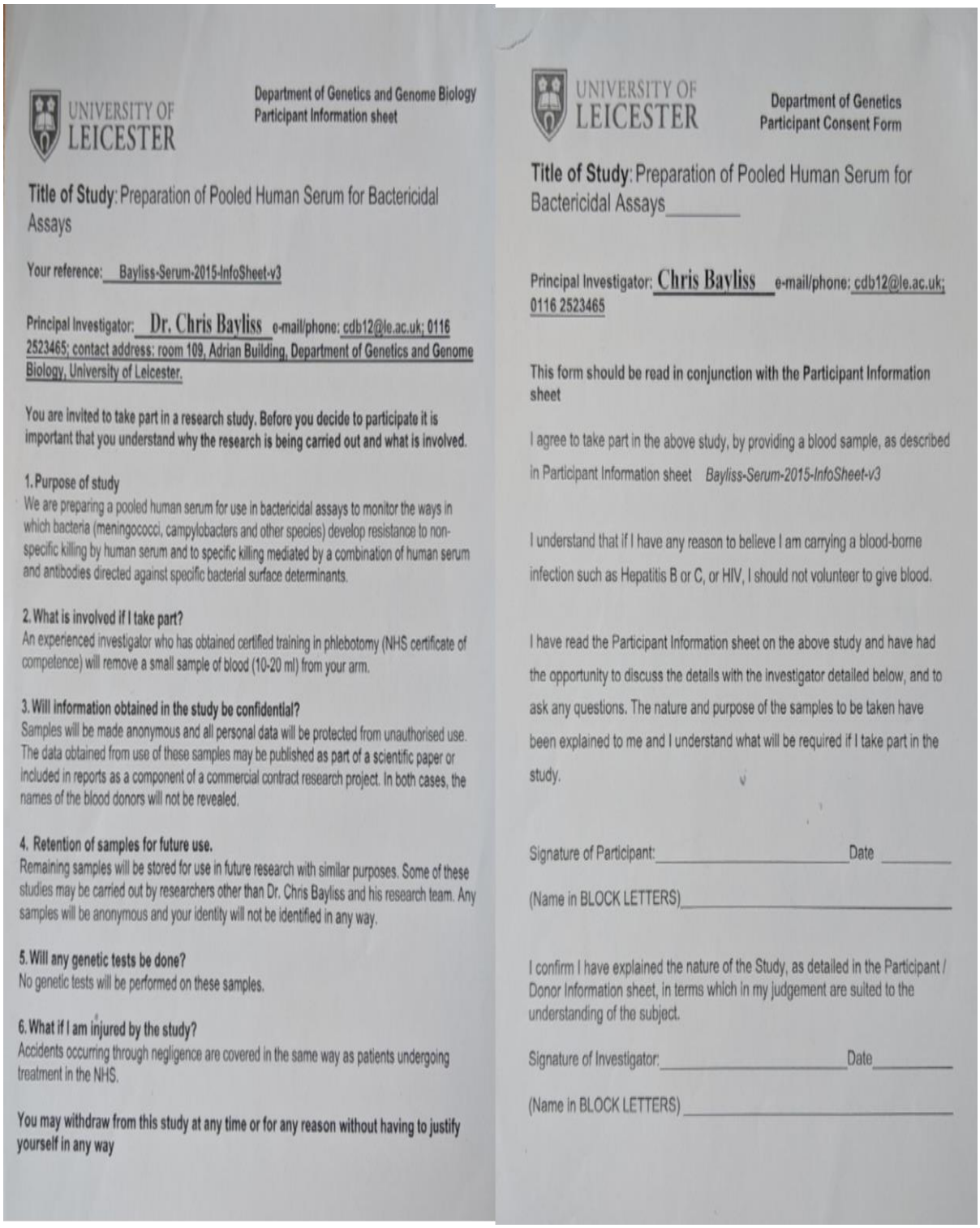
5. Assessing the interaction of phages with different types of OMVs.

6. Assessing the immunological aspect of phage-OMV interactions by using different phages and OMVs and targeting different cytokines, such as IL-8 and IL-10.

7. Studying the mechanisms that lead to the induction of prophages upon interaction with immune cells and apply the same approach with other prophages.

# Supplementary

Supplementary 1. Example of ethic approval that was required to be signed by volunteers of the study.



UNIVERSITY OF LEICESTER

Department of Genetics and Genome Biology
Participant Information sheet

**Title of Study**: Preparation of Pooled Human Serum for Bactericidal Assays

Your reference: Bayliss-Serum-2015-InfoSheet-v3

Principal Investigator: Dr. Chris Bayliss e-mail/phone: cdb12@le.ac.uk; 0116 2523465; contact address: room 109, Adrian Building, Department of Genetics and Genome Biology, University of Leicester.

**You are invited to take part in a research study. Before you decide to participate it is important that you understand why the research is being carried out and what is involved.**

**1. Purpose of study**
We are preparing a pooled human serum for use in bactericidal assays to monitor the ways in which bacteria (meningococci, campylobacters and other species) develop resistance to non-specific killing by human serum and to specific killing mediated by a combination of human serum and antibodies directed against specific bacterial surface determinants.

**2. What is involved if I take part?**
An experienced investigator who has obtained certified training in phlebotomy (NHS certificate of competence) will remove a small sample of blood (10-20 ml) from your arm.

**3. Will information obtained in the study be confidential?**
Samples will be made anonymous and all personal data will be protected from unauthorised use. The data obtained from use of these samples may be published as part of a scientific paper or included in reports as a component of a commercial contract research project. In both cases, the names of the blood donors will not be revealed.

**4. Retention of samples for future use.**
Remaining samples will be stored for use in future research with similar purposes. Some of these studies may be carried out by researchers other than Dr. Chris Bayliss and his research team. Any samples will be anonymous and your identity will not be identified in any way.

**5. Will any genetic tests be done?**
No genetic tests will be performed on these samples.

**6. What if I am injured by the study?**
Accidents occurring through negligence are covered in the same way as patients undergoing treatment in the NHS.

**You may withdraw from this study at any time or for any reason without having to justify yourself in any way**

UNIVERSITY OF LEICESTER

Department of Genetics
Participant Consent Form

**Title of Study**: Preparation of Pooled Human Serum for Bactericidal Assays

Principal Investigator: Chris Bayliss e-mail/phone: cdb12@le.ac.uk; 0116 2523465

**This form should be read in conjunction with the Participant Information sheet**

I agree to take part in the above study, by providing a blood sample, as described in Participant Information sheet *Bayliss-Serum-2015-InfoSheet-v3*

I understand that if I have any reason to believe I am carrying a blood-borne infection such as Hepatitis B or C, or HIV, I should not volunteer to give blood.

I have read the Participant Information sheet on the above study and have had the opportunity to discuss the details with the investigator detailed below, and to ask any questions. The nature and purpose of the samples to be taken have been explained to me and I understand what will be required if I take part in the study.

Signature of Participant: _____ Date _____

(Name in BLOCK LETTERS) _____

I confirm I have explained the nature of the Study, as detailed in the Participant / Donor Information sheet, in terms which in my judgement are suited to the understanding of the subject.

Signature of Investigator: _____ Date _____

(Name in BLOCK LETTERS) _____

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