Significant Differences Characterise the Correlation Coefficients between Biocide and Antibiotic Susceptibility Profiles in *Staphylococcus aureus*

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Abstract: There is a growing concern by regulatory authorities on the selection of antibiotic resistance due to the use of biocidal products. We aimed to fill the gap of detailed information on large surveys by investigating the relationship between biocide and antibiotic susceptibility profiles of a large number of *Staphylococcus aureus* isolates using four biocides and antibiotics commonly used in clinical practice. The minimal inhibitory concentration (MIC) for the most clinically-relevant antibiotics was determined according to standardized methodology for over 1600 clinical *S. aureus* isolates and compared to susceptibility profiles of benzalkonium chloride, chlorhexidine, triclosan, and sodium hypochlorite. Evaluation of the relationship between antibiotic and biocide susceptibility profiles was performed using non-linear correlations.

The main outcome shows an absence of any strong or moderate statistically significant correlation when comparing either triclosan or sodium hypochlorite susceptibility to susceptibility for any of the tested antibiotics. On the other hand, correlation coefficients for MICs of benzalkonium chloride and chlorhexidine are above 0.4 for susceptibility to quinolones, beta-lactams, and also macrolides.

Our data do not support any selective pressure for the association between biocide and antibiotic resistance and furthermore do not allow for a defined risk evaluation for some of the compounds. Importantly, our data clearly indicate the absence of risk of selection for antibiotic resistance for the compounds triclosan and sodium hypochlorite. These data, hence infer that biocide selection for antibiotic resistance has had so far a less significant impact than feared.

Keywords: *Staphylococcus aureus;* antibiotic resistance; biocides; benzalkonium chloride; chlorhexidine; triclosan; sodium hypochlorite

INTRODUCTION

Antimicrobial substances comprise, in addition to pharmacological products (antibiotics), a series of antimicrobial chemical compounds marketed as biocides, disinfectants, antiseptics, or preservatives. Given that both antibiotics and biocides are characterized primarily by their antimicrobial action it is obvious that the selective pressure excreted by these compounds on bacteria may have analogies, and thus might converge in similar mechanisms of resistance or co-existence of resistance. In such a way, selective pressure exerted by any of these types of antimicrobial agents on bacterial populations translates to the selection of those cells which are resistant, with the literature being full of examples for both co- and cross-resistance [1, 2].

Taking into consideration these studies, in recent years national and international agencies and consumer associations have raised awareness on a risk of widespread biocide use in health care settings, in the environment, and in cosmetic and food industry towards selection of antibiotic-resistant bacteria. This is witnessed by the 2009 report of the Scientific Committee on Emerging and Newly Identified Health Risks on biocides in general, the report of the Scientific Committee on Consumer Safety in 2010, and the most recent report (December 2013) of the Food and Drug Administration (FDA) [3-5].

All these reports pose significant questions which highlight as one of the main issues that, despite the information obtained *in vitro* on laboratory strains, there is a lack of coherent and relevant epidemiological data linking utilization of compounds to toxicity, resistance and aspects of co- and cross-resistance to antibiotics [6]. We have recently reported epidemiological cut-off values of biocide susceptibility profiles for over 3300 strains of different species [7]. Here we report the correlation of the susceptibility profiles between antibiotics and biocides in the largest group of these strains in order to provide a clear dataset onto which to base future risk evaluations. The minimum bactericidal concentrations (MBC) of all biocides were also evaluated. This is particularly relevant because biocides are usually utilized at bactericidal concentrations, and hence changes on MBC can reflect changes on the actual susceptibility to these antimicrobial agents.

MATERIALS AND METHODS

To investigate the relationship between susceptibility profiles to biocides and antibiotics we determined the susceptibility profiles to the most commonly used antibiotics in 1632 clinical *S. aureus* isolates with known susceptibility profiles to the biocides chlorhexidine, benzalkonium chloride, sodium hypochlorite and triclosan [8-10].

The evaluation of the relationship between biocide and antibiotic susceptibility profiles were computed using non-linear correlations. Bivariate correlations were calculated using Matlab® 7.10.0.499 (R2010a). Spearman's correlation coefficient was computed for each bivariate combination of these variables in order to find non-linear associations between the biological variables. For each computed Spearman's correlation coefficient, a hypothesis test was performed in order to test for statistical association between each pair of variables and p-value was calculated followed by a Bonferroni correction to control the family-wise error rate due to multiple testing.

RESULTS AND DISCUSSION

Using the non-linear correlation approach, there was no strong relationship between any biocide and antibiotic phenotypes. Indeed, the data analysed showed weak to moderate bivariate correlations. This was a result that matches with a previous study of a smaller group of antibiotics where only the profile of both benzalkonium chloride and chlorhexidine were associated with multi-drug resistance [10, 11]. In this dataset we found, after performing the correction for family-wise error rate, a series of pairs of variables with a statistically significant Spearman’s correlation coefficient. The highest correlation coefficient characterized compounds of the same class (i.e. resistance to one beta-lactam is correlated to resistance to another beta-lactam). High correlation coefficients were found for macrolides (>0.86), quinolones (>0.77) and beta-lactams (>0.67). The shading in Table 1 also indicates clearly the pairwise relationship between macrolides, beta-lactams, quinolones, and also quinupristin/dalfopristin. Such association is reflected by the well-known occurrence of multi-drug resistance in methicillin-resistant staphylococcal clones [12]. The deep

analysis of recent literature produced on the resistance to biocides and the results obtained in the present work lead to the following observations: (i) the MICs to chlorhexidine and benzalkonium chloride have a statistically significant coefficient of 0.5 in accordance with the fact that both compounds are effluxed by the NorA and QacABCGHJ efflux pumps [8]; on the contrary, absence of any correlation between MICs and MBCs for both chlorhexidine and benzalkonium chloride in accordance of absence of correlation of any known death-preventing and MBC-increasing resistance mechanisms [8], and (iii) a correlation coefficient of 0.6 between the MICs and MBCs for triclosan which are in accordance with the molecular characterisation of phenotypes conferred by *fabI*-related resistance mechanisms, influencing both inhibition and cell death [9].

The main outcome relative to the scope of this work was the characterisation of bivariate correlations between biocide and antibiotic susceptibility profiles of a collection of staphylococcal bacteria, where we compared both the MICs and MBCs of biocides to the MICs of antibiotics (Table 1). A previous study was done in this direction but with a smaller group of antibiotics [10]. The analysis of the same set of strains using machine learning techniques, had shown only a link between simultaneous reduction in susceptibility to chlorhexidine and benzalkonium chloride and resistance to antibiotics [10]. However, using the same approach we were unable to find specific biocide/antibiotic associations [10]. In the current article, we expand our analysis by studying the independent correlations on the susceptibilities of a large group of antibiotics and a set of the most frequently used biocides: chlorhexidine, triclosan hypochloride and benzalkonium chloride.

The data obtained here showed absence of any strong or moderate statistically significant correlation when comparing either triclosan or sodium hypochlorite's susceptibility with to susceptibility for any of the tested antibiotics. On the other hand, correlation coefficients for MICs of benzalkonium chloride are above 0.4 associated with the susceptibility to quinolones, beta-lactams and also macrolides. This correlation might be due to the well-described association of *qac* determinants and the beta-lactamase transposon Tn*552* which are located on psK41-like plasmids, widespread among clinical multi -rug resistant *S. aureus* clones since early '50s and also among

127 animal isolates of different staphylococcal species [8]. Similar data can be seen for chlorhexidine.
128 The somewhat weaker data for chlorhexidine are due to the less evident phenotype conferred by the
129 QacA efflux pump [8]. Our present data-set included only methicillin-resistant *S. aureus*
130 (MRSA)/methicillin-sensitive *S. aureus* (MSSA) breakpoints and as such could not be included in
131 the multivariate analysis, but methicillin resistance was clustered essentially as data for Cefaclor
132 (Table 1) [10].

133 A large survey on over 3300 isolates of *S. aureus, Salmonella* spp., *Escherichia coli, Candida*
134 *albicans, Klebsiella pneumoniae, Enterobacter* spp., *Enterococcus faecium*, and *Enterococcus*
135 *faecalis* has recently shown that biocide resistant subpopulations are uncommon in natural
136 populations of clinically-relevant microorganisms [7]. In that study both MICs and MBCs for
137 biocides followed a normal distribution, in contrast to antibiotics where selection clearly defines
138 bimodal distribution and resistance phenotypes. Upon the tested species *Enterobacter* spp., *E. coli*
139 and *S. aureus* exhibited for selected biocides discrete subpopulations. Interestingly in enterobacteria
140 subpopulations with susceptibility to benzalkonium chloride were not linked to the presence of qac
141 genes [7]. On the contrary, the defined genetic mechanisms of *S. aureus* for reduced susceptibility
142 to triclosan, benzalkonium chloride and chlorhexidine could be identified in subpopulations with
143 reduced susceptibility [7-9]. This definition of genetically distinguishable subpopulations of *S.*
144 *aureus* with decreased biocide resistance are at the basis of this work. We here perform a
145 correlation analysis of the biocide susceptibility profiles to antimicrobial drug resistance in general
146 by testing over 1600 clinical *S. aureus* isolates. Data clearly show that there is no correlation of
147 susceptibility profiles for the biocides triclosan or sodium hypochlorite and any clinically-relevant
148 antibiotic. On the contrary, our data show a significant relationship with a moderate correlation
149 between susceptibility profiles to chlorhexidine and benzalkonium chloride. Although a relationship
150 between multiple drug resistance (MDR) efflux pumps and quinolone resistance is known, it should
151 be noted that while *in vitro* mutants in the promoter of the *norA* efflux pump gene confer resistance
152 to norfloxacin and fluoroquinolones, this is not observed for all clinical isolates with *norA* promoter

mutations [8]. More importantly, resistance to benzalkonium chloride has been attributed to the activity of *qac* efflux pumps in the vast majority of clinical isolates and none of the QacABCG pumps was shown to confer fluoroquinolone resistance [8], still not ruling out that gyrase or topoisomerase mutation may be facilitated in strains carrying *qac* efflux pumps. These observations would thus favour the hypothesis that cross-resistance between benzalkonium chloride and chlorhexidine on one side and fluoroquinolones on the other could be involved in co-selection of strains. In the case of beta-lactams, the fact that the *blaZ* gene is carried by most if not all *qacA* plasmids would indicate that co-resistance could be the driver of co-selection of resistant strains. In this context, it should be noted that the recently identified IS*256* associate *sh-fabI* gene which confers reduced susceptibility to triclosan was found in few occasions on *qacA* carrying plasmids and plasmids which also harbour cadmium resistance pumps [9]. These data indicate that there are numerous distinct mechanisms potentially responsible for the association of the *qacA* carrying psK41-like plasmids to MDR staphylococcal clones and that this association cannot be pinpointed to a single mechanism.

CONCLUSION

The data presented here have shown that in *S. aureus* there is no correlation between of susceptibility profiles to triclosan or sodium hypochlorite and any clinically-relevant antibiotic. On the contrary, the data have further show that there is a significant relationship with a moderate correlation between susceptibility profiles to the bisbiguanide chlorhexidine and the quaternary ammonium compound benzalkonium chloride and some classes of antibiotics. In the light of the recently published observations that most clinically-relevant bacterial species do not show evidence for presence of subpopulations with decreased biocide susceptibility, our data suggest that global biocide use appears not to have resulted in a clinically-relevant impact on antibiotic resistance. While our data do not allow for inference as to the direction of selective pressure in the case of the association between susceptibility profiles to some biocides and antimicrobial resistance, they clearly rule out the possibility that such evidence exists at present for other compounds. While not addressing toxicity of the biocides, this report would answer some of the other questions relating to the risk for human health raised by the recent FDA report on the Safety and Effectiveness of Consumer Antiseptics [5].

CONFLICT OF INTEREST

The authors confirm that this article has no conflicts of interest.

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Table 1. Correlation coefficient between antimicrobial susceptibility profiles of over 1600 clinical *S. aureus* isolates

	CLX	BZC	SHC	TRI	CLX*	BZC*	SHC*	TRI*	P	AMC	CXM	CEC	CPD	SXT	CC	E	CLR	AZM	TEL	SYN	TEC	VA	CIP	LVX	GAT	MFX	TE	LZD
CLX	1.00																											
BZC	0.53	1.00																										
SHC	0.01	0.05	1.00																									
TRI	0.07	0.01	0.21	1.00																								
CLX*	0.07	0.02	0.09	0.04	1.00																							
BZC*	0.04	0.02	0.17	0.03	0.38	1.00																						
SHC*	0.15	0.14	0.42	0.19	0.31	0.33	1.00																					
TRI*	0.13	0.10	0.21	0.61	0.11	0.08	0.37	1.00																				
P	0.15	0.19	0.07	0.01	0.03	0.02	0.05	0.03	1.00																			
AMC	0.25	0.32	0.06	0.04	0.06	0.03	0.03	0.05	0.75	1.00																		
CXM	0.30	0.40	0.04	0.09	0.00	0.03	0.13	0.13	0.38	0.66	1.00																	
CEC	0.28	0.37	0.01	0.06	0.00	0.02	0.11	0.09	0.71	0.81	0.67	1.00																
CPD	0.34	0.44	0.01	0.06	0.02	0.01	0.08	0.10	0.41	0.67	0.78	0.70	1.00															
SXT	0.07	0.12	0.06	0.00	0.06	0.06	0.06	0.01	0.12	0.21	0.22	0.22	0.23	1.00														
CC	0.29	0.33	0.03	0.05	0.02	0.01	0.07	0.09	0.28	0.46	0.51	0.46	0.55	0.20	1.00													
E	0.27	0.33	0.02	0.02	0.01	0.03	0.06	0.04	0.25	0.47	0.48	0.45	0.49	0.20	0.54	1.00												
CLR	0.24	0.29	0.03	0.03	0.01	0.01	0.02	0.03	0.24	0.44	0.45	0.42	0.47	0.19	0.53	0.86	1.00											
AZM	0.23	0.30	0.07	0.02	0.03	0.02	0.01	0.02	0.25	0.45	0.44	0.40	0.47	0.18	0.54	0.89	0.86	1.00										
TEL	0.33	0.35	0.01	0.07	0.03	0.02	0.08	0.10	0.26	0.46	0.50	0.45	0.54	0.23	0.73	0.59	0.56	0.59	1.00									
SYN	0.11	0.10	0.03	0.00	0.03	0.01	0.02	0.04	0.11	0.22	0.26	0.23	0.25	0.16	0.22	0.22	0.21	0.22	0.23	1.00								
TEC	0.08	0.02	0.05	0.02	0.04	0.02	0.02	0.03	0.00	0.03	0.04	0.08	0.03	0.05	0.09	0.11	0.10	0.09	0.11	0.33	1.00							
VA	0.37	0.46	0.01	0.02	0.04	0.05	0.10	0.10	0.28	0.48	0.60	0.52	0.62	0.25	0.50	0.51	0.48	0.48	0.54	0.18	0.01	1.00						
CIP	0.40	0.49	0.01	0.05	0.02	0.06	0.10	0.11	0.35	0.62	0.70	0.63	0.73	0.28	0.60	0.60	0.56	0.56	0.63	0.20	0.03	0.82	1.00					
LVX	0.34	0.40	0.00	0.05	0.01	0.02	0.07	0.09	0.26	0.50	0.60	0.51	0.61	0.27	0.52	0.49	0.46	0.46	0.54	0.19	0.03	0.78	0.77	1.00				
GAT	0.33	0.41	0.01	0.02	0.02	0.01	0.05	0.08	0.26	0.48	0.58	0.50	0.59	0.25	0.51	0.51	0.48	0.49	0.53	0.21	0.04	0.83	0.78	0.79	1.00			
MFX	0.13	0.15	0.02	0.02	0.02	0.04	0.05	0.05	0.14	0.23	0.27	0.28	0.28	0.26	0.29	0.26	0.25	0.24	0.25	0.22	0.17	0.23	0.25	0.28	0.26	1.00		
TE	0.26	0.30	0.04	0.03	0.03	0.06	0.11	0.09	0.27	0.36	0.45	0.42	0.46	0.13	0.51	0.40	0.38	0.37	0.54	0.21	0.18	0.44	0.49	0.40	0.41	0.27	1.00	
LZD	0.08	0.13	0.09	0.02	0.07	0.10	0.09	0.03	0.06	0.13	0.14	0.10	0.13	0.16	0.04	0.12	0.08	0.08	0.11	0.06	0.01	0.08	0.12	0.20	0.09	0.09	0.12	1.00

* marks MBC. All shaded cells report statistically significant correlations. The darker the shading the higher the correlation coefficient; all shaded correlation coefficients are statistically significant. Amoxicillin with Clavulanic Acid AMC, Azithromycin AZM, Cefaclor CEC, Cefpodoxime CPD, Cefuroxime CXM, Ciprofloxacin CIP, Clarithromycin CLR, Clindamycin CC, Erythromycin E, Gatifloxacin GAT, Levofloxacin LVX, Linezolid LZD, Moxifloxacin MFX, Penicillin P, Quinupristin/Dalfopristin SYN, Sulfamethoxazole with Trimethoprim SXT , Teicoplanin TEC, Telithromycin TEL, Tetracycline TE, Vancomycin VA.

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