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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 for the selection of antibiotic resistance caused by the use of biocidal products. We aimed to complete the 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 most clinically-relevant antibiotics was determined according to the standardized methodology for over 1600 clinical *S. aureus* isolates and compared to susceptibility profiles of benzalkonium chloride, chlorhexidine, triclosan, and sodium hypochlorite. The relationship between antibiotic and biocide susceptibility profiles was evaluated using non-linear correlations.

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

Our data do not support any selective pressure for association between biocides and antibiotics resistance and furthermore do not allow for a defined risk evaluation for some of the compounds. Importantly, our data clearly indicate that there does not involve any 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), also 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 exerted 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 into 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, national and international agencies and consumer associations have recently 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 has been witnessed by the 2009 report of the Scientific Committee on Emerging and Newly Identified Health Risks on biocides in general [3], the report of the Scientific Committee on Consumer Safety in 2010 [4], and the most recent report (December 2013) of the Food and Drug Administration (FDA) [5].

All these reports pose significant questions highlighting one of the main issues that, despite the information obtained *in vitro* on laboratory strains, there exists 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. Also biocides MBCs were evaluated. This is particularly relevant because biocides are usually utilized at bactericidal concentrations, and hence changes on MBC can reflect on the actual susceptibility to these antimicrobial agents.

MATERIALS AND METHODS

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

The evaluation of the relationship between biocide and antibiotic susceptibility profiles was performed using non-linear correlations. Bivariate correlations were calculated using Matlab® 7.10.0.499 (R2010a). Spearman's correlation coefficient was com-

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puted 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 the statistical association between each pair of variables and a p-value 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, no strong relationship between any biocide and antibiotic phenotypes was evidenced. Indeed, the data analysed showed weak to moderate bivariate correlations. The result of this study matches with that of a previous study of a smaller group of antibiotics where only the profiles 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 characterised the compounds of the same class to other compounds of the same class (i.e. resistance to one beta-lactam is correlated to resistance to another beta-lactam). As expected high correlation coefficients for macrolides (>0.86), quinolones (>0.77) and beta-lactams (>0.67) were yielded. The shading in Table **1** also clearly indicates 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]. With respect to the biocides, a series of observations have to be made which include (i) that whether 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 is in accordance with the 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 I). A previous study was conducted in this direction but with a smaller group of antibiotics [10]. The analysis of the same set of strains using machine learning techniques [10], has shown that a simultaneous reduced susceptibility to chlorhexidine and to benzalkonium chloride is associated with resistance to at least one antibiotic. However, using the same approach we were unable to find specific biocide/antibiotic associations [10]. In this study, 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 benzakonium.

The data obtained in this study did not show any strong or moderate statistically significant correlation when comparing the susceptibilities of either triclosan or sodium hypochlorite for any of the tested antibiotics. On the other hand, correlation coefficients for MICs of benzalkonium chloride were found to be above 0.4 associated with the susceptibility of 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 drug resistant *S. aureus* clones since early 50s and also among animal isolates of different staphylococcal species [8]. Similar data can be seen for chlorhexidine. The somewhat weaker data for chlorhexidine are due to the less evident phenotype conferred by the QacA efflux pump [8].

A large survey on over 3300 isolates of *S. aureus*, *Salmonella* spp., *Escherichia coli*, *Candida albicans*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Enterococcus faecium*, and *Enterococcus faecalis* has recently shown that biocide resistant subpopulations are uncommon in natural populations of clinically relevant microorganisms [7]. In this study, *S. aureus* exhibited discrete subpopulations with defined genetic mechanisms for reduced susceptibility to triclosan, benzalkonium chloride and chlorhexidine [7-9]. We here performed a correlation analysis of the biocide susceptibility profiles to antimicrobial drug resistance in general by testing over 1600 clinical *S. aureus* isolates. Data clearly show an absence of correlation of susceptibility profiles for the biocides triclosan or sodium hypochlorite and any clinically relevant antibiotic. In contrast, our data show a significant relationship with a moderate correlation between susceptibility profiles to chlorhexidine and benzalkonium chloride. Although a relationship between MDR efflux pumps and quinolone resistance is known, it should be noted that while *in vitro* mutants in the promoter of the *norA* efflux pump gene confer resistance to norfloxacin and fluoroquinolones, which 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 could be involved to some extent 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 [9] and plasmids which also harbour cadmium resistance pumps. 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 here show that in *S. aureus* there is no correlation of susceptibility profiles to triclosan or sodium hypochlorite and any clinically relevant antibiotic. The data further show that there is in contrast a significant relationship with a moderate correlation between susceptibility profiles to the bis-biguanide chlorhexidine and the quaternary ammonium compound benzalkonium chloride and some classes of antibiotics [10]. In the light of the recently published observations that most clinically relevant bacterial species do not show the presence of subpopulations with decreased biocide susceptibility [7], our data suggest that the global use of biocide to date 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 should answer some of the other questions relating to 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.

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. 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, Meticillin OXA, Moxifloxacin MFX, Penicillin P, Quinupristin/Dalfopristin SYN, Sulfamethoxazole with Trimethoprim SXT , Teicoplanin TEC, Telithromycin TEL, Tetracycline TE, Vancomycin VA.

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