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Role of resident and acquired multi-drug efflux pumps in reduced susceptibility to cationic biocides in Staphylococcus aureus

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Objectives: In view of the requirements posed by licensing process of disinfectants according to the EC biocide directive, we evaluated the possibility to devise an *in vitro* test for evaluation of resistance to cationic antibacterial compounds including quaternary ammonium compounds and bisbiguanides.
Methods: Three strains of *S. aureus* mutants were selected *in vitro* with benzalkonium chloride (BZC; quaternary ammonium compound), chlorhexidine (CHX; bisbiguanide), acriflavine (AF) and ethidium bromide (EB) and their phenotypes and *norA* promoter sequences were determined. Data on laboratory mutants was compared to molecular data from of 245 clinical *S. aureus* strains showing decreased susceptibility to BZC or CHX.
Results: A survey for susceptibility to EB, AF, BZC and CHX on 58 clinical *S. aureus* isolates showed a bimodal distribution of susceptibility profiles for EB and AF and a normal distribution of susceptibility profiles to CHX and BZC. Mutation frequency in vitro to these compounds was found to be around 1E-10 for EB and AF, while no mutants could be selected in a single step protocol for BZC and CHX. Multiple passages on selective plates allowed to select also mutants with BZC and CHX. Irrespective the selective agent all mutants showed important increases in MIC and MBC to norfloxacin, ciprofloxacin, EB and AF. For BZC and CHX the MIC and MBC did either not change or increased by a single dilution. All mutans showed mutations in the promoter region of the NorA MDR efflux pump. Upon the 245 clinical isolates with reduced susceptibility to BZC or CHX, 78 were positive for qacA, 13 for qacC and 1 for qacG and all of these had increased MIC for EB. For 39 clinical strains with reduced EB or BZC susceptibility the *norA* promoter was sequenced. Out of these 9 had a short duplication, 5 a mutated and 25 a *wt norA* promoter region. In only two cases a clinical strain matched to a mutation also generated *in vitro*.
Conclusion: Our data show (1) that EB and AF are suitable agents for monitoring efflux phenotypes and related genotypes, while BZC and CHX not, (2) that standard mutation selection assays cannot be performed for BZC and CHX (3) and that the mutations selected in vitro by BZC and CHX do not match those detected in clinical isolates. Summarised these data indicate that for *S. aureus* an in vitro test for prediction of resistance development to BZC and CHX is not feasible and, in any case, would yield results of no clinical relevance.

RESULTS

- 1668 *Staphylococcus aureus* clinical isolates from a world wide collection where screened for their MIC and MBC to the biocides benzalkonium chloride and chlorhexidine. Both the MIC and MBC value showed an uni-modal distribution (Fig. 1A and 1B). 245 of these strains, which showed high biocide MICs and MBCs were further analysed (Figure 2).
- The ethidium bromide (EB) MICs of these 245 strains showed a clear bi-modal distribution (Fig. 2a). The EB resistant subpopulation was positive for *qacA* in 92,6% of isolates (Fig. 2a dashed). QacC carrying strains had an intermediate MIC of 32 (Fig. 2a black).
- Mapping of the *qac* determinants according to the MIC and MBC to biocides did not produce a significant overlap. Interestingly the strains with the highest MBC to biocides tend to be *qac* negative.
- In vitro selected mutants showed irrespective the selective agent the same phenotypes with significant increase in quinolone and EB and acriflavine (AF) resistance and only minor decreases in biocide susceptibility (Table I). All mutants were mutated in the *norA* promoter region.
- Mutations in the *norA* promoter region of clinical isolates and laboratory mutants showed very limited overlap (Fig. 3).

CONCLUSIONS

- In view of the possible risk of biocide use on antibiotic resistance (2) it has been proposed to introduce into the licensing process a test to evaluate resistance generation (1). Given the fact that only few of the clinical strains of *S. aureus* showed reduced susceptibility to benzalkonium chloride or chlorhexidine due to mutations and since these mutations poorly overlapped to those selected in vitro, our data indicate that such an *in vitro* test may be of low predictive value for biocides resistance in *S. aureus*.
- The weak changes in biocide susceptibility which *qac* genes and *norA* mutations confer to *S. aureus* strains does not rule out that biocides could select for resistance-plasmid maintenance, but this fact and the lack of efficient mutant selection strategies in vitro hamper data collection for a risk analysis.

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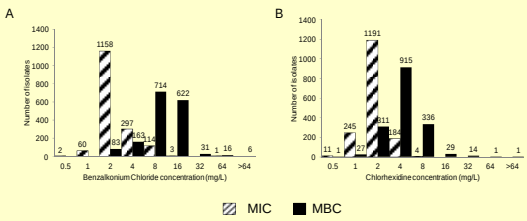

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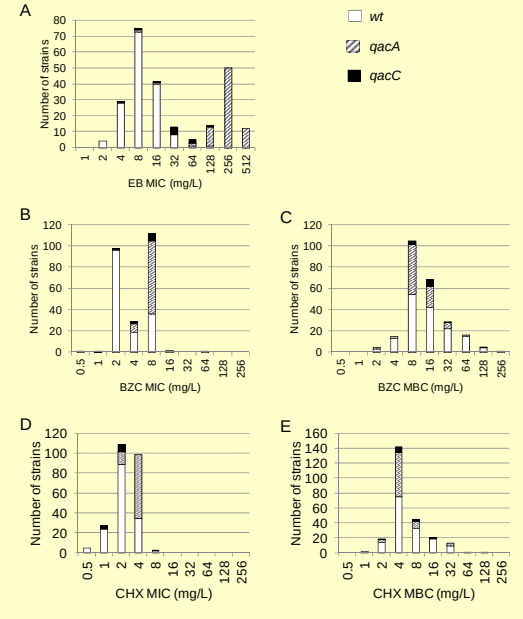

Strain	Genetic background	Selective agent	*norA* mutations	MIC (mg/L)						MBC (mg/L)					
				NOR	CIP	EB	AF	CHX	BZC	NOR	CIP	EB	AF	CHX	BZC
ATCC25923		-	wt	1	1	16	32	2	2	8	1	16	64	2	4
MO060	ATCC25923	AF	A94T	16	8	64	256	4	4	16	8	128	256	8	8
MO061	ATCC25923	AF	T91A	8	4	32	256	4	4	8	4	64	256	4	4
MO062	ATCC25923	AF	T126A	8	4	128	256	4	4	16	4	128	256	32	8
MO072	ATCC25923	EB	T126A	8	4	128	256	4	4	8	4	128	256	4	4
MO058	ATCC25923	EB	T126A	8	2	128	256	4	4	8	16	128	256	32	16
MO059	ATCC25923	EB	T126A	8	4	128	256	4	4	8	4	128	256	4	4
ATCC6538		-	wt	1	0.5	4	16	4	2	2	0.5	16	32	4	8
MO063	ATCC6538	AF	A107G	8	4	32	128	4	4	8	4	32	256	4	4
MO064	ATCC6538	AF	T126A	8	2	32	128	4	4	8	4	64	256	4	8
MO065	ATCC6538	AF	T126A	8	2	32	128	4	4	8	4	64	256	8	4
MO037	ATCC6538	CHX	T89G	16	4	32	64	8	4	16	4	64	128	128	4
MO038	ATCC6538	CHX	T89G	16	4	256	256	4	4	16	4	256	256	8	8
MO039	ATCC6538	CHX	T90G	8	4	256	256	4	4	8	4	256	256	16	8
MO040	ATCC6538	CHX	T90G	8	4	32	64	4	4	16	4	128	64	32	4
MO041	ATCC6538	CHX	T90G	8	4	16	256	4	4	8	4	64	256	64	4
RN4220		-	wt	1	0.5	8	16	8	2	2	1	16	32	8	2
MO069	RN4220	AF	A107G	8	4	64	256	4	4	8	4	128	256	8	8
MO070	RN4220	AF	A107G	16	8	64	256	4	4	16	8	128	256	8	8
MO071	RN4220	AF	T126A	8	4	64	128	4	8	16	4	64	256	4	8
MO066	RN4220	EB	T126A	16	4	128	256	4	4	16	8	128	256	8	8
MO067	RN4220	EB	A107G	8	4	64	256	4	4	16	4	128	256	8	8
MO068	RN4220	EB	T126A	16	4	64	256	4	4	16	8	128	256	32	8
MO042	RN4220	CHX	A130C T126A	8	4	256	256	8	4	16	8	256	256	8	4
MO043	RN4220	CHX	A130C T126A	16	4	256	256	8	4	16	8	256	256	32	4
MO044	RN4220	BZC	A130C T126A	16	4	128	256	4	8	16	16	256	256	8	8
MO045	RN4220	BZC	A130C T126A	16	8	128	256	8	4	16	8	128	256	8	8

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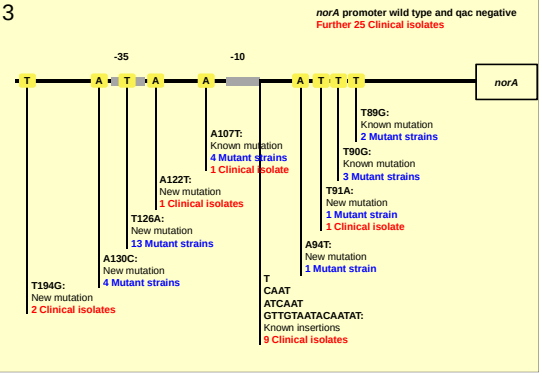

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