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Qac genes and biocide tolerance in clinical veterinary methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* and *Staphylococcus pseudintermedius*

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ABSTRACT

Qac genes are associated with increased tolerance to quaternary ammonium compounds and other cationic biocides such as chlorhexidine. This study aimed to determine whether *qac* genes and increased biocide tolerance were present in 125 clinical methicillin-resistant and susceptible veterinary staphylococci. A total of 125 methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant and -susceptible *Staphylococcus pseudintermedius* (MRSP and MSSP) from three archived Australian veterinary staphylococci collections underwent whole genome sequencing, multilocus sequence typing and *qac* gene screening. Two MRSA isolates (12%) harboured *qacA/B* genes; both isolates were ST8 from horses. *QacJ, qacG* and *smr* genes were identified in 28/90 (31%) MRSP and 1/18 (6%) MSSP isolates. ST71 MRSP was significantly more likely to harbour *qac* genes than other MRSP clones ($p < 0.05$). A random subset of 31 isolates underwent minimum bactericidal concentration (MBC) testing against F10SC^{TM} (benzalkonium chloride and biguanide), and Hexacon^{TM} (chlorhexidine gluconate), with and without the addition of bovine serum albumin (BSA) as an *in vitro* substitute for organic matter contamination. *Qac* genes were not associated with increased phenotypic biocide tolerance but biocide efficacy was significantly affected by the presence of BSA. In the absence of BSA, all MBC values were well below the recommended usage concentration. When BSA was present, regardless of *qac* gene presence, 50% of MRSA and 43% of MRSP had an F10SC^{TM} MBC above the recommended concentration for general disinfection. *Qac* genes did not confer increased *in vitro* biocide tolerance to veterinary staphylococci. Organic matter contamination must be minimized to ensure the efficacy of biocides against MRSA and MRSP.

1. Introduction

Staphylococcus spp. are part of the normal microbiota of humans and animals and while their presence is generally innocuous, they can cause serious opportunistic infections. *Staphylococcus pseudintermedius* is a common veterinary pathogen that is also an occasional zoonotic pathogen (Stegmann et al., 2010). The recent and rapid rise of multidrug and methicillin-resistance in *S. pseudintermedius* has led to heightened interest in the use of topical biocides to treat canine skin conditions (Loeffler et al., 2011; Valentine et al., 2012; Uri et al., 2016). *In vivo* and *in vitro* studies have shown promising results for topical treatment of methicillin-susceptible and -resistant *S. pseudintermedius* infections with biocides such as chlorhexidine gluconate (Loeffler et al., 2011; Uri et al., 2016; Valentine et al., 2012). However, there is growing concern in the human medical literature about the presence of genetic determinants of biocide tolerance in *Staphylococcus* species, such as the quaternary ammonium compound (QAC) resistance gene group (Tennent et al., 1989; Paulsen et al., 1996). QAC resistance proteins are inducible efflux pumps that are encoded by plasmid-borne genes (Bjorland et al., 2003). These proteins appear to aid extraction of cationic substances such as quaternary ammonium compounds and can protect against certain host-derived antimicrobial peptides (Paulsen et al., 1996; Kupferwasser et al., 1999; Couto et al., 2008; Liu et al., 2009; Wassenaar et al., 2015). QAC proteins are found in several bacterial genera and can be divided into two broad groups: the Major Facilitator Family, which includes *qacA* and *qacB*, and the Small Multidrug Resistance protein family, which includes *qacG, qacH, qacJ,* and *qacC/smr* (Wassenaar et al., 2015). The prevalence and distribution of

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qac genes varies with geography, *Staphylococcus* species, and the host species of the isolate (Wassenaar et al., 2015). *In vitro* studies have shown that *qac* genes can increase biocide tolerance amongst *Staphylococcus* isolates, but efflux capability varies depending on the specific *qac* gene and the compound being tested (Littlejohn et al., 1992; Bjorland et al., 2003). *QacA*-positive isolates have higher tolerance for biocides than *qacB*-positive isolates, while isolates harbouring *qacJ* demonstrate increased biocide tolerance compared to *qacG*- and *smr*-positive isolates (Bjorland et al., 2003). The QAC, benzalkonium chloride, and the bisbiguanide, chlorhexidine, are two cationic biocides commonly used in human and veterinary medicine. Several studies have found that *Staphylococcus aureus* isolates harbouring *qac* genes demonstrate higher tolerance to benzalkonium chloride and chlorhexidine, evidenced by a significantly higher minimum bactericidal concentration (MBC) in *qac* gene-positive isolates compared to *qac* gene-negative isolates (Smith et al., 2008; Liu et al., 2015). *Qac* genes have historically been termed biocide 'resistance' genes, but most studies find that while isolates with *qac* genes tend to have a higher MBC than isolates without, the MBC for all isolates is still much lower than the recommended concentrations for practical biocide use as disinfectants in hospitals (Vali et al., 2008; Liu et al., 2015). Therefore, it is more appropriate to refer to biocide 'tolerance' rather than resistance; if used at their recommended concentration, biocides are generally still effective at killing isolates with *qac* genes.

Biocide tolerance has important implications for infection control, particularly for difficult-to-treat organisms like methicillin-resistant *Staphylococcus* spp. Several studies in human medicine have examined *qac* genes in MRSA and demonstrated that their presence is associated with increased *in vitro* biocide tolerance (Smith et al., 2008; Otter et al., 2013; Liu et al., 2015), but similar studies in veterinary medicine are lacking. *Qac* genes have been found in low numbers of methicillin-susceptible *S. pseudintermedius* (MSSP) from dogs (Couto et al., 2013a) and a range of *Staphylococcus* species from horses (Bjorland et al., 2003; Sidhu et al., 2007; Couto et al., 2013b), but they have not yet been reported in methicillin-resistant *S. pseudintermedius* (MRSP). Given the rising prevalence of MRSP in veterinary medicine (Moodley et al., 2014) and its growing profile as a potential zoonotic pathogen (Stegmann et al., 2010), the possible presence of *qac* genes in MRSP needs to be addressed. Consequently, this study screened 125 *S. pseudintermedius* and *S. aureus* clinical veterinary isolates for *qac* genes. It also examined phenotypic biocide tolerance in a subset of 31 isolates by measuring the minimum bactericidal concentration of a quaternary ammonium compound, F10SC^{TM} (benzalkonium chloride and polyhexamethylene biguanide hydrochloride) and a bisbiguanide, Hexacon^{TM} (chlorhexidine gluconate).

2. Materials and methods

2.1. Bacterial isolates

One hundred and eight clinical isolates of *S. pseudintermedius* (90 MRSP, 18 MSSP) and 17 methicillin-resistant *S. aureus* (MRSA) were included in the study. Bacterial isolates were obtained from three collections stored at the Sydney School of Veterinary Science, The University of Sydney, NSW, Australia. Collection A came from an Australia-wide surveillance study that collected all clinical veterinary isolates of coagulase-positive *Staphylococcus* from January 2013 to January 2014 (Saputra et al., 2017; Worthing et al., 2018a; Worthing et al., 2018b). Collection B were clinical *Staphylococcus* isolates from canine pyoderma cases in Sydney, NSW, that were collected as part of a research project in 2013 (Ravens et al., 2014). Collection C were freeze-dried archived clinical *Staphylococcus* isolates collected by the Veterinary Pathology Diagnostic Services, University of Sydney, NSW, between 1999 and 2002. The MRSP originated from dogs (n = 89) and a cat (n = 1), while the MRSA isolates came from dogs (n = 7), cats (n = 3), horses (n = 6) and a kangaroo (n = 1). The MSSP originated from dogs (n = 16) and cats (n = 2). The speciation of all isolates was determined by standard phenotypic tests and MALDI-TOF MS (Bruker, USA), and was confirmed *via* identification of the species-specific thermonuclease gene, *nuc,* in sequenced data.

2.2. In silico analysis and typing

All isolates underwent whole genome sequencing and multilocus sequence typing (MLST), as previously described (Worthing et al., 2018a,b). *De novo* contigs for each isolate were BLAST screened for *qac* genes against reference sequences (*qacA/B, qacJ, qacG, qacH,* and *qacC/smr;* NCBI accession numbers: NC_007931.1, NG_048046.1, NG_051904.1, NC_019081.1, and GQ900464.1, respectively) using CLC Genomics Workbench (Qiagen, USA). Isolates with ≥90% similarity to a reference sequence were deemed to be positive for that gene.

2.3. Biocide tolerance testing

Minimum bactericidal concentration values were determined for two veterinary biocides, the quaternary ammonium and biguanide compound, F10SC^{TM} (5.4% w/w benzalkonium chloride, 0.4% w/w polyhexamethylene biguanide hydrochloride; batch number: 170922, Health and Hygiene, South Africa) and 5% w/v chlorhexidine gluconate (Hexacon^{TM}, batch number: 12355, Apex Laboratories, Australia) as previously described (Vali et al., 2008; Liu et al., 2009; Couto et al., 2013a; Liu et al., 2015), with the following modifications. Isolates were subcultured onto tryptose soy agar, incubated at 37 °C overnight and then inoculated into 0.9% saline to obtain 0.5 McFarland standard turbidity, yielding an estimated 1.5×10^8 CFU/mL suspension. Two-fold dilutions of each biocide were prepared in sterile water. The range of dilutions tested was 1:50 to 1:25600, which equated to benzalkonium chloride concentrations of 0.5–1080 mg/L and chlorhexidine concentrations of 0.5–1000 mg/L. Biocide dilutions were prepared in two protein conditions: with and without a total concentration of 30 g/L (3%) bovine serum albumin (BSA; Sigma Aldrich, USA). BSA was used to replicate the effect of protein contamination *in vitro* (Liu et al., 2015). Therefore, isolates were tested against four biocide preparations: benzalkonium chloride and biguanide with 3% BSA (F10SC + BSA), benzalkonium chloride and biguanide without 3% BSA (F10SC–BSA), chlorhexidine gluconate with 3% BSA (chlorhex + BSA) and chlorhexidine gluconate without 3% BSA (chlorhex–BSA). To expose the bacteria to each biocide, 100 µl of colony suspension was inoculated into 900 µl of each diluted biocide and left at room temperature for 5 min. To inactivate the biocide, 100 µl of the biocide/bacteria mix was then transferred to 900 µl sterile neutralizer (3 g/L lecithin and 30 g/L tween 80 in phosphate buffered saline; pH 7.4 ± 0.4) and left at room temperature for 5 min. Two 25 µl drops of neutralized sample were then plated onto sheep blood agar (Oxoid, Basingstoke) and incubated for 18–24 h at 37 °C. Survivors were enumerated using the drop plate method as previously described (Vali et al., 2008). Negative controls used sterile saline instead of biocide. The MBC was determined by the concentration of biocide that yielded a 5-logarithmic reduction in bacterial survivors when compared to saline controls. Samples were run in duplicate. If duplicates returned a different MBC value, the higher value was designated as the MBC for that isolate. Duplicate results that were more than two-fold different from each other were repeated in triplicate; the median triplicate result was then recorded. ATCC *S. aureus* 29,213 was used as an internal control strain.

2.4. Statistical analysis

For comparisons between groups of more than 10, the Mann-Whitney U test was used to assess differences in median MBC values (GraphPad Prism 7, USA). Categorical comparisons were undertaken by constructing contingency tables and performing Fisher's exact test. Results were considered significant if $p < 0.05$.

Table 1
Qac genes and multilocus sequence types (MLST) of coagulase-positive staphylococci from Australian animals.

MLST	Number of *Qac*-positive isolates/total number of isolates	*Qac* genes present
MRSA		
ST8	2/2	*qacA/B*
Others	0/15	None
MRSP		
ST71	16/26	*qacG, qacJ, smr*
ST64	4/4	*qacG*
ST45	2/6	Unnamed putative *qac* gene[a]
ST496	1/8	Unnamed putative *qac* gene[a]
ST525	1/5	*qacG*
ST498	1/3	*smr*
ST25	1/1	*qacJ*
ST544	1/1	*qacJ*
ST537	1/1	*qacG*
ST544	1/1	*qacG*
Others	0/34	None
MSSP		
ST538	1/1	*qacJ*
Others	0/17	None

[a] Accession number: U81980.1.

3. Results

3.1. Frequency of qac genes amongst Staphylococcus isolates

A total of 31/125 (25%) *Staphylococcus* isolates harboured *qac* genes, which consisted of 2/17 (12%) MRSA, 28/90 (31%) MRSP, and 1/18 (6%) MSSP isolates. The range of sequence types examined and the *qac* genes that they harboured, are shown in Table 1. *qacA/B* genes were only found amongst MRSA isolates, both of which were ST8 from horses. The most common *qac* gene amongst MRSP was *qacJ* (n = 15, 54%), followed by *qacG* (n = 8, 29%) and *smr* (n = 2, 7%). MRSP isolates from the same sequence type generally harboured the same *qac* gene, but ST71 MRSP isolates harboured either *qacJ* (n = 13), *qacG* (n = 2), or *smr* (n = 1). ST71 isolates were significantly more likely to harbour *qac* genes than other MRSP sequence types (OR = 6.9, CI = 2.5–19.0, $p < 0.01$). Three MRSP isolates (one ST496 and two ST45) harboured a putative novel *qac* gene with only 83% sequence homology to *qacJ*. This 324bp *qac* sequence had 100% homology to the sequence from the NCBI database of an unnamed *qac* gene from the *S. aureus* plasmid, pKH4 (Accession number: U81980.1).

3.2. Biocide tolerance

The MBC values for benzalkonium chloride/biguanide and chlorhexidine gluconate were determined for a randomly selected subset of 31 *qac*-positive and *qac*-negative isolates (Table 2). Fourteen of the tested isolates were *qac*-positive (MRSP, n = 12; MRSA, n = 2) while 17 were *qac*-negative (MRSP, n = 11; MRSA, n = 6). Of the *qac*-positive isolates that underwent MBC testing, most harboured *qacJ* (9/12; Table 2). The range and frequency of benzalkonium chloride/biguanide MBC values for MRSP isolates is shown in Fig. 1. The MBC values for benzalkonium chloride/biguanide without BSA (F10-BSA) ranged from 1.05 mg/L to 16.87 mg/L while they ranged from 16.87 mg/L to 135 mg/L for F10SC + BSA. The MBC values for chlorhexidine gluconate without BSA (chlorhex-BSA) ranged from 7.81 mg/L to 31.25 mg/L while they ranged from 125 mg/L to 500 mg/L for chlorhex + BSA. The median MBC values of benzalkonium chloride/biguanide were not different between *qac*-positive and *qac*-negative MRSP isolates, but the presence of BSA significantly increased the median MBC (F10SC-BSA = 4.21 mg/L, F10SC + BSA = 67.5 mg/L; $p < 0.0001$). The range and frequency of chlorhexidine MBC values for MRSP isolates is shown in Fig. 2. Similar to benzalkonium chloride/biguanide, the median MBC values of chlorhexidine gluconate were not significantly different between *qac*-positive and *qac*-negative MRSP isolates (chlorhex + BSA: *qac*-positive MBC = 250 mg/L, *qac*-negative = 125 mg/L; $p = 0.4$. chlorhex-BSA: *qac*-positive and *qac*-negative = 15.63 mg/L). The presence of BSA significantly increased the median MBC (chlorhex-BSA MBC = 15.63 mg /L, chlorhex + BSA = 250 mg/L; $p < 0.0001$).

For MRSA, the F10SC + BSA MBC was 67.5 mg/L for one *qac*-positive isolate and 135 mg/L for the other, while the median MBC was 135 mg/L for the *qac*-negative isolates (range = 33.75–135 mg/L). The F10SC-BSA MBC was 8.43 mg/L for one *qac*-positive MRSA isolate and 16.87 mg/L for the other while the median MBC for *qac*-negative isolates was 4.21 mg/L (range = 2.1–16.87 mg/L). The chlorhex + BSA MBC was 500 mg/L for both *qac*-positive MRSA isolates while the median MBC for *qac*-negative isolates was 250 mg/L (range = 250–500 mg/L). The chlorhex-BSA MBC for *qac*-positive MRSA isolates was 31.25 and 125 mg/L while for *qac*-negative isolates, the median MBC was 46.9 mg/L (range = 31.25–250 mg/L).

4. Discussion

Biocide tolerance genes have previously been reported in MRSA from humans (Smith et al., 2008; Otter et al., 2013; Liu et al., 2015) MSSA from horses (Bjorland et al., 2003) and MSSP from dogs (Couto et al., 2013a); but here we report the first instance of *qac* genes in MRSP from dogs and MRSA from horses. Of the 90 MRSP isolates surveyed, 31% harboured either *qacJ, qacG* or *smr* while 2/17 (12%) of MRSA isolates harboured *qacA* genes. *In vitro* testing found no significant differences in the MBC of benzalkonium chloride/biguanide or chlorhexidine between *qac*-positive and *qac*-negative MRSP isolates. The lack of a significant difference between *qac* phenotypes in this study could be due to a number of reasons including small sample size, lack of *qac* gene expression, or differences in study design compared to previous studies. Cervinkova et al. (2012) suggested that stage of bacterial growth is one of the key drivers in expression of *qac* genes, with expression being highest in the exponential growth stage (Cervinkova et al., 2012). The isolates in the current study were exposed to biocides after 18–24 h incubation, which likely means that they were in the post-exponential (stationary) phase of growth. *Qac* gene expression may consequently have been lower in this study compared to other studies that have examined isolates in the exponential phase of growth. A future study that uses real-time quantitative PCR to compare differential growth phase expression of *qac* genes amongst veterinary staphylococci would therefore be useful.

Both *qacA*-positive MRSA isolates were ST8 from horses. The ST8 MRSA lineage is more commonly associated with horses than most other MRSA lineages (Moodley et al., 2006; van Duijkeren et al., 2010) and while equine-specific markers have not yet been detected, it appears that ST8 MRSA has greater affinity for equine hosts than other MRSA lineages. Screening of a larger sample of equine-derived MRSA will help to determine whether ST8 is more likely to carry *qac* genes than other lineages.

We found that ST71 MRSP isolates were significantly more likely to harbour *qac* genes than other MRSP clones. ST71 was the most common MRSP lineage in a recent Australia-wide survey of veterinary staphylococci (Worthing et al., 2018a), which is consistent with previous reports (Perreten et al., 2010; Couto et al., 2016; Worthing et al., 2018a). It is tempting to suggest that the presence of *qac* genes has conferred biocide tolerance and a subsequent fitness advantage to the ST71 clone, as has been observed in an epidemic clone of *qac*-positive ST22 MRSA that outcompeted other MRSA clones in a human hospital setting (Otter et al., 2013). However, we found no significant difference in benzalkonium chloride/biguanide or chlorhexidine gluconate MBC values between *qac*-positive and *qac*-negative MRSP isolates. Previous studies have found that *qacJ*-positive *S. aureus* isolates showed higher

Table 2
Minimum bactericidal concentration (MBC) values of benzalkonium chloride/biguanide (F10SC™) and chlorhexidine gluconate (Hexacon™) for MRSA and MRSP isolates with and without *qac* genes.

Isolate ID	Species	MLST	*Qac* gene present	MBC (mg/L)			
				Benzalkonium chloride/biguanide		Chlorhexidine gluconate	
				With BSA[a]	Without BSA	With BSA	Without BSA
N13/1/408	MRSA	ST8	*qacA/B*	67.5	16.87	500	125
N13/1/396	MRSA	ST8	*qacA/B*	135	8.43	500	31.25
N13/1/17	MRSA	ST22	None	135	4.21	250	31.25
Q13/1/145	MRSA	ST22	None	135	2.1	250	62.5
N13/1/715	MRSA	ST22	None	67.5	2.1	250	31.25
N13/1/648	MRSA	ST612	None	135	16.87	250	250
V13/2/458	MRSA	ST612	None	67.5	4.21	500	31.25
N13/4/96	MRSA	ST612	None	33.75	4.21	250	62.5
ATCC29213	MSSA	Control	None	135	16.87	500	125
N13/4/25	MRSP	ST25	*qacJ*	67.5	2.1	125	15.63
Q13/1/190	MRSP	ST496	Unnamed *qac*[b]	135	8.43	250	15.63
V13/2/470	MRSP	ST71	*qacG*	135	8.43	250	7.81
V13/2/18	MRSP	ST71	*qacJ*	135	16.87	250	31.25
V13/2/18	MRSP	ST71	*qacJ*	67.5	4.21	500	31.25
V13/6/4	MRSP	ST71	*qacJ*	135	4.21	250	31.25
V13/6/5	MRSP	ST71	*qacJ*	67.5	4.21	250	31.25
N13/1/103	MRSP	ST71	*qacJ*	33.75	2.1	125	15.63
V13/2/133	MRSP	ST71	*qacJ*	16.87	4.21	250	15.63
V13/2/152	MRSP	ST71	*qacJ*	135	2.1	125	15.63
N13/1/480	MRSP	ST71	*qacJ*	67.5	2.1	250	15.63
Q13/1/311	MRSP	ST71	*smr*	33.75	2.1	250	15.63
N13/4/115	MRSP	ST496	None	135	4.21	250	15.63
V13/2/83	MRSP	ST497	None	67.5	4.21	500	15.63
V13/2/173	MRSP	ST497	None	67.5	67.5	250	15.63
V13/2/242	MRSP	ST497	None	33.75	1.05	125	31.25
V13/2/92	MRSP	ST497	None	16.87	1.05	125	15.63
V13/2/393	MRSP	ST71	None	67.5	2.1	125	7.81
V13/2/475	MRSP	ST71	None	135	4.21	125	7.81
V13/2/413	MRSP	ST71	None	135	4.21	250	7.81
V13/2/440	MRSP	ST71	None	135	4.21	250	7.81
V13/2/441	MRSP	ST71	None	16.87	2.1	250	15.93
V13/2/488	MRSP	ST71	None	135	2.1	125	7.81

[a] BSA = 3% bovine serum albumin.
[b] Accession number: U81980.1.

benzalkonium chloride tolerance than *qacJ*-negative isolates and those with other Small Multidrug Resistance protein family genes (*qacG* or *smr*) (Bjorland et al., 2003). Our limited sample size precluded comparing the MBC values of *qacJ*-positive isolates with other types of *qac* genes, so it is possible that significant differences exist that could not be detected. A future study that utilized plasmid vector transformation of *qac* genes to a *qac*-negative *S. pseudintermedius* recipient would help to determine the phenotypic efflux capability of the various SMR genes, as would measuring ethidium bromide minimum inhibitory concentrations to identify isolates with increased efflux capability (Tennent et al., 1989; Couto et al., 2008; Couto et al., 2013b).

Alternatively, *qac* genes may confer a biological advantage to ST71 by mechanisms other than biocide resistance such as conferring resistance to host-derived antimicrobial peptides. For example, *qacA*-positive *S. aureus* isolates survived exposure to thrombin-induced platelet microbicidal protein 1a (tPMP-1), a cationic peptide release by rabbit platelets (Kupferwasser et al., 1999). In acting as cationic compounds that disrupt the bacterial cell membrane (Yeaman et al., 1998), host antimicrobial peptides may act in a similar manner to cationic biocides like benzalkonium chloride or chlorhexidine (Gilbert and Moore, 2005). Staphylococci have been in contact with their hosts' antimicrobial peptides for many thousands of years more than manmade cationic

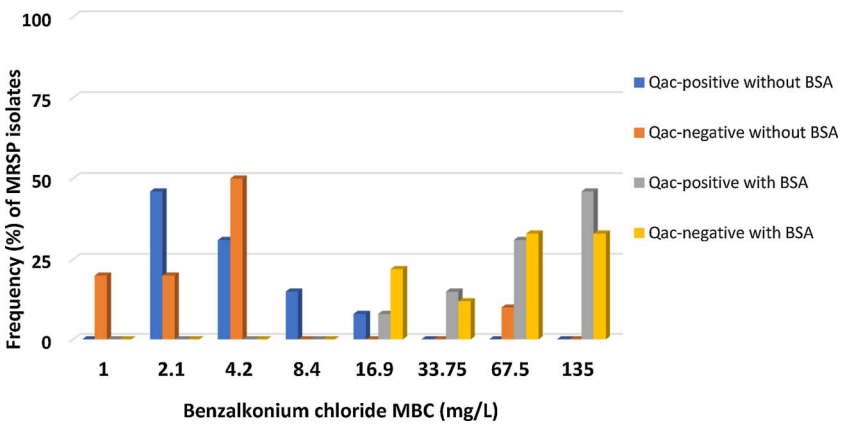

Fig. 1. Frequency of minimum bactericidal concentration (MBC) of benzalkonium chloride/biguanide (F10SC™) for *qac*-positive and *qac*-negative methicillin-resistant *S. pseudintermedius* (MRSP) isolates in the presence and absence of 3% bovine serum albumin (BSA). Manufacturer's minimum recommended in-use concentration = 108 mg/L (Health and Hygiene Pty Ltd).

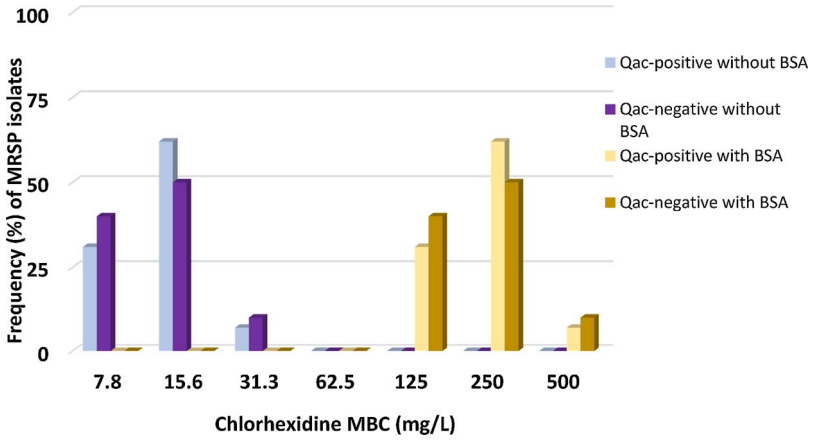

Fig. 2. Frequency of minimum bactericidal concentration (MBC) of chlorhexidine gluconate (Hexacon™) for *qac*-positive and *qac*-negative methicillin-resistant *S. pseudintermedius* (MRSP) isolates in the presence and absence of 3% bovine serum albumin (BSA). Manufacturer's recommended in-use concentration = 1000–5000 mg/L (Apex Laboratories, Australia).

biocides, so it is quite probable that any bacterial efflux effect originally evolved with a physiological role (Hassan et al., 2015) such as extrusion of antimicrobial peptides and/or other natural biocides. If the function of *qac* genes was indeed originally to defend against mammalian cationic proteins, it makes sense that their coincidental efflux capacity for cationic biocides is variable, depending on the original mammalian protein targeted. This could explain why different *qac* genes appear to vary in their efflux capacity against various biocides (Littlejohn et al., 1992; Bjorland et al., 2003). Future studies could examine whether *qac*-positive MRSP lineages such as ST71 display higher tolerance to canine-derived antimicrobial peptides when compared to *qac*-negative isolates. Screening of archival veterinary staphylococcal isolates could also help to determine whether any evolutionary correlations exist between the occurrence of *qac* genes and the use of biocides in veterinary practice.

A bimodal distribution of MBC values of benzalkonium chloride/biguanide and chlorhexidine was evident in this study, but rather than reflecting wild type and resistant subpopulations due to the absence and presence *qac* genes, the bimodality of MBC values reflected the presence (higher MBC) and absence (lower MBC) of protein contamination. This study used the addition of 3% bovine serum albumin as an *in vitro* indicator of organic matter contamination that would be present on mammalian skin and likely also in the veterinary hospital environment, where benzalkonium chloride and chlorhexidine-based disinfectants are commonly used. We found that the addition of BSA yielded a statistically significant increase in the median benzalkonium chloride and chlorhexidine MBC values for MRSP. Similarly, Liu et al. (2015) compared chlorhexidine MBC values for MRSA isolates with and without 3% BSA, and found that the presence of BSA caused a four-fold increase in the chlorhexidine MBC of *qacA*-positive MRSA isolates. The labelled concentration for Hexacon™ 5% chlorhexidine gluconate is 1000 mg/L for general antisepsis and 5000 mg/L for surgical skin preparation (Apex Laboratories, Australia). The chlorhexidine gluconate MBC values for MRSP and MRSA we report are well below the recommended usage concentration and thus all isolates would likely be killed 'in the field' if the product is used appropriately. Meanwhile, the labelled recommended concentration of F10SC™ benzalkonium chloride/biguanide is 1:500 (~108 mg/L) for general disinfection, 1:250 (~432 mg/L) for high level disinfection, and 1:125 (~432 mg/L) for resistant viruses such as parvovirus (Health and Hygiene, South Africa). In the presence of bovine serum albumin, 50% of MRSA isolates and 43% of MRSP isolates had an MBC of 135 mg/L, which is above the recommended 108 mg/L concentration for general disinfection. Bovine serum albumin is merely an *in vitro* substitute for protein contamination; it is probable that the real organic contamination found in a veterinary environment would have a greater effect on biocide efficacy than that identified in the current study. Overall, these results reinforce the importance of removing gross contamination and organic matter prior to disinfection, particularly with disinfectants such as benzalkonium chloride/biguanide.

In this study, we used a biocide exposure time of 5 min, which replicates the approximate time that a topical treatment such as a chlorhexidine-based shampoo may be applied to a dog (Borio et al., 2015). However, *in vitro* biocide testing may otherwise not appropriately model what happens *in vivo* and cautious interpretation and application of results is warranted. Firstly, 3% BSA is a poor surrogate for the complex organic mixture of hair, skin cells and debris that would be on the skin of a dog with staphylococcal pyoderma, likely underestimating the true inhibitory organic effect. Secondly, poor compliance of animal owners and veterinary personnel to label instructions can considerably affect the efficacy of infection control and treatment measures; an aspect beyond the scope of *in vitro* studies such as this. For example, an observational study found that veterinarians used a contact time of as low as seven seconds for a chlorhexidine-based surgical cleaning product during pre-operative hand scrubbing, despite the labelled recommendation being at least two minutes (Anderson et al., 2013). Future studies and guidelines should consider that users may not adhere to scientifically-proven biocide contact times, which could account for anecdotal reports of failure of topical therapies and potentially the emergence of bacterial resistance. Additional *in vivo* and prospective studies are required to establish the true clinical efficacy of biocides against MRSP and MRSA.

5. Conclusions

This pilot found that 31% of MRSP and 12% of MRSA isolates harboured *qac* genes. Although our sample size was larger than previous studies (Couto et al., 2013a; Uri et al., 2016), our study was still limited by the relatively small sample size of isolates that underwent MBC testing. Future studies could be strengthened by undertaking MBC testing on a large sample size and ideally compare MBC values both between and within clonal types. Now that *qac*-MRSP veterinary clinical isolates have been detected, ongoing surveillance studies will no doubt procure more *qac*-positive MRSP isolates that will provide a larger sample pool for future studies. Despite rising levels of resistance to systemic antimicrobials, it is heartening to know that MRSP and MRSA can still be killed by commonly used veterinary biocides, as long as they are used at their recommended concentration and organic matter contamination is minimized. We suggest that it may be prudent to use the 'high level disinfection' concentration for F10SC as a minimum concentration in environments where organic contamination is likely. Although we have documented the first report of *qac* genes in MRSP isolates, the biological significance of *qac* genes in veterinary medicine is not yet fully understood. Consequently, this *in vitro* study is being followed by a prospective *in vivo* study by our research team,

investigating clinical outcomes for dogs who carry or are infected by *qac*-positive MRSP.

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Conflict of interest statement

Sam Abraham and Darren Trott have previously received funds from Zoetis Pty Ltd. All other authors, none to declare.

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