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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 $p < 0.05$.