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In vitro antiseptic susceptibilities for *Staphylococcus pseudintermedius* isolated from canine superficial pyoderma in Japan

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Background – Topical therapy, particularly with chlorhexidine, is becoming increasingly common as a treatment option for canine pyoderma; however, there are limited studies on the susceptibility of *Staphylococcus pseudintermedius* to chlorhexidine compounds.

Objectives – To determine the *in vitro* susceptibility of both meticillin-resistant and meticillin-susceptible *S. pseudintermedius* isolates to chlorhexidine and other antiseptic agents and the presence of multidrug efflux pump genes.

Samples – One hundred *S. pseudintermedius* isolates from 23 initial and 77 recurrent cases of canine pyoderma.

Methods – After bacterial identification and *mecA* testing, minimal inhibitory concentrations (MICs) of antiseptic agents were determined. Multidrug efflux pump genes, including *qacA*, *qacB* and *smr*, were identified.

Results – Of the 100 isolates, 57 were identified as meticillin-resistant *S. pseudintermedius*. The MIC_{90} of chlorhexidine acetate, chlorhexidine gluconate, acriflavine, ethidium bromide and benzalkonium chloride were 1, 1, 2, 0.5 and 2 μg/mL, respectively. Multidrug efflux pump genes *qacA*, *qacB* and *smr* were not detected in any of the isolates.

Conclusions and clinical importance – The MICs for chlorhexidine and other antiseptics remain low, and multidrug efflux pump genes were not found in the tested isolates.

Introduction

Canine superficial pyoderma is a common skin disease of dogs, and the primary pathogen is *Staphylococcus pseudintermedius*.[1] In addition to addressing the underlying cause, treatment of superficial pyoderma includes the use of systemic antimicrobials and topical therapy. Following the emergence of multidrug-resistant *mecA*-positive *S. pseudintermedius*, there has been increased interest in the use of topical antiseptics as sole or adjuvant therapy. In previous studies, we demonstrated the efficacy of 2% chlorhexidine acetate (Nolvasan® Surgical Scrub; Fort Dodge Animal Health, Fort Dodge, IA, USA) as monotherapy for canine superficial pyoderma associated with meticillin-resistant (MR) *S. pseudintermedius* group (SIG), but not in all cases.[2–4] In those studies, 2–4% chlorhexidine acetate resulted in a positive clinical response in 60–70% of the dogs.[2–4] In humans, there are reports of topical treatment with chlorhexidine not being successful in eliminating meticillin-resistant *Staphylococcus aureus* (MRSA),[5] and a low level of resistance to chlorhexidine is a concern.[6] A possible explanation is the existence of multidrug efflux pump genes, which have been detected in *S. aureus* isolates, that confer resistance to antiseptic agents, including chlorhexidine, quaternary ammonium compounds (e.g. benzalkonium chloride) and dyes (e.g. acriflavine and ethidium bromide).[7–10] The aims of this study were to assess the *in vitro* susceptibilities of both meticillin-resistant and meticillin-susceptible (MS) *S. pseudintermedius* to chlorhexidine acetate, as well as other antiseptics, and to determine whether multidrug efflux pump genes (*qacA*, *qacB* and *smr*) were present.

Materials and methods

Bacterial isolates

One hundred clinical isolates were obtained from dogs with either a first occurrence of pyoderma (n = 23) or recurrent pyoderma (n = 77) between May 2009 and September 2010. Samples were collected from dogs presented to the ASC Dermatology Service, Tokyo, Japan. These samples have been used in previous studies.[2–4] Pyoderma was confirmed based on clinical signs, cytological findings and bacterial culture. Samples for bacterial culture and susceptibility testing were collected from skin lesions compatible with pyoderma. Swabs

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(Sterile BBL CultureSwab; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) were rubbed vigorously against the sampling site and stored at 4°C, and were processed within 7 days. Each swab was inoculated onto blood agar (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) and mannitol salt agar (Nissui Pharmaceutical Co., Ltd), and incubated aerobically at 37°C for 18–24 h. Staphylococcal isolates were putatively identified using colony morphology, the ability to grow on mannitol salt agar, Gram-stain characteristics and coagulase reaction. The strains were stored in skimmed milk at −80°C until further use.

Identification of *S. pseudintermedius*

Bacterial speciation was performed as previously described using a multiplex PCR method.[11] (See Table S1 in Supplementary material for primers).

Determination of meticillin resistance by detection of *mecA*

Primers for *mecA* PCRs, mA1 (5′-TGCTATCCACCCTCAAACAGG-3′) and mA2 (5′-AACGTTGTAACCACCCCAAGA-3′), were used in the present study with a previously published method.[12] All results were confirmed by at least two independent experiments.

Antimicrobial and antiseptic susceptibility testing

Antimicrobial susceptibility testing was used to identify MR and MS staphylococci. The minimal inhibitory concentrations (MICs) of antimicrobial and antiseptic agents were determined by the standard agar plate dilution method according to the Clinical and Laboratory Standards Institute (CLSI) document M31-A3.[13] Isolates with a MIC for oxacillin ≥0.5 μg/mL or positive for *mecA* by PCR were regarded as MR strains.[14] The antiseptic agents tested included chlorhexidine acetate and chlorhexidine gluconate, acriflavine, ethidium bromide and benzalkonium chloride.

Determination of multidrug efflux pump genes, including *qacA, qacB* and *smr*

A search for *qacA/B* and *smr* genes was performed by multiple PCRs with the following sets of primers: 5′-GCAGAAAGTGCAGAGTTCG-3′ and 5′-CCAGTCCAATCAGCCTG-3′ for *qacA/B* (product size 361 bp);[15] and 5′-GCCATAAGTACTGAAGTTATTGGA-3′ and 5′-GACTACGGTTGTTAAGACTAAACCT-3′ for *smr* (product size 195 bp).[16] The PCR assays were performed using the modified colony direct method described by Tsuchizaki *et al.*[17] *Staphylococcus aureus* JCM16555 (TS77) in *qacA/B* and *S. aureus* JCM16554 (L20A) in *smr* were used as reference strains in the phenotypic and genotypic tests. All results were confirmed by at least two independent experiments.

Statistical analysis

The chi-square test was used to analyse differences between *mecA* presence in initial and recurrent cases. Noncontinuous and categorical, matched-pair Mann–Whitney *U*-tests were used to analyse differences in susceptibility to chlorhexidine between *mecA*-positive and *mecA*-negative *S. pseudintermedius*. Values of $P < 0.05$ were considered statistically significant. All analyses were performed using the software package Stata® version 11 (StataCorp LP, College Station, TX, USA).

Results

Species identification and detection of *mecA*

All 100 isolates were identified as *S. pseudintermedius*. Of these, 57 of 100 were *mecA* positive. The *mecA* was detected in 13 isolates from 23 dogs with first-time pyoderma and in 44 of 77 isolates from dogs with recurrent pyoderma. There was no significant difference between *mecA* presence in initial and recurrent cases ($P = 0.96$).

Antiseptic susceptibility testing

The MICs of antiseptic agents are shown in Table 1. The MIC_{90} of chlorhexidine acetate, chlorhexidine gluconate, acriflavine, ethidium bromide and benzalkonium chloride was 1, 1, 2, 0.5 and 2 μg/mL, respectively, for all 100 isolates. There were no significant differences in MICs of chlorhexidine acetate ($P = 0.32$) and chlorhexidine gluconate ($P = 0.54$) between *mecA*-positive and *mecA*-negative *S. pseudintermedius*. (See Table S2 in Supplementary material for details of antimicrobial susceptibility testing for the 100 isolates).

Detection of multidrug efflux pump genes

Multidrug efflux pump genes, including *qacA*, *qacB* and *smr*, were not detected in any isolates.

Discussion

Staphylococcus aureus can develop antiseptic resistance by multidrug efflux pumps in the bacterial cell membrane, which are encoded by some antiseptic resistance genes.[7–10] Two major groups of resistance genes, one group including *qacA* and *qacB* and another including *smr*, which is identical to *qacC*, *qacC9*, *qacD* or *ebr*, are associated with high-level and low-level resistance to antiseptics, respectively.[8] The multidrug efflux pump genes are more frequently isolated in MRSA than in MS *S. aureus* (MSSA).[18,19] Apart from *S. aureus* strains isolated from humans, multidrug efflux pump genes have been found in equine, bovine and feline staphylococcal strains, as well as in staphylococcal strains from unpasteurized milk from dairy cattle and dairy goat herds.[20–23] In the present study, *qacA*, *qacB* and *smr* were evaluated in 100 isolates of *S. pseudintermedius* from dogs, and none of these efflux pump genes was identified in either MS or MR *S. pseudintermedius*. Of the strains, 57 of 100 were *mecA*-positive and 50 of 57 were multidrug-resistant, i.e. they were resistant to at least three classes of antimicrobials in addition to the β-lactams (see Table S2 in Supplementary material).

Table 1. Minimal inhibitory concentrations (MICs) of antiseptic agents for meticillin-susceptible *Staphylococcus pseudintermedius* (MSSP) and meticillin-resistant *S. pseudintermedius* (MRSP) strains

	CHA		CHG		AF		EB		BKC	
MIC (μg/ml)	MSSP (*n*)	MRSP (*n*)	MSSP (*n*)	MRSP (*n*)	MSSP (*n*)	MRSP (*n*)	MSSP (*n*)	MRSP (*n*)	MSSP (*n*)	MRSP (*n*)
0.5	23	26	19	21	31	28	43	57	0	0
1	18	25	22	34	6	18	0	0	1	18
2	2	4	0	2	6	11	0	0	40	32
4	0	2	2	0	0	0	0	0	2	7

Abbreviations: AF, acriflavine; BKC, benzalkonium chloride; CHA, chlorhexidine acetate; CHG, chlorhexidine gluconate; EB, ethidium bromide.

One of the most commonly used topical antiseptics in small animal dermatology is chlorhexidine. The MICs of chlorhexidine are reportedly different in MRSA and MSSA, although no MIC standardization for chlorhexidine has yet been proposed.[24] The MIC of chlorhexidine for MSSA is considered to be 1 μg/mL,[7] but other sources cite 4 in MRSA or 8 μg/mL in MSSA.[25,26] In the present study, four isolates had a MIC of 4 μg/mL for chlorhexidine, although MIC standardization for chlorhexidine has yet to be proposed for *S. pseudintermedius*. Previous studies have shown that 2–4% chlorhexidine is an effective treatment for superficial pyoderma in some, but not all, dogs.[2–4,27] There are a number of possible explanations for discrepancies between *in vivo* antiseptic efficacy and *in vitro* susceptibility. Chlorhexidine might not have good penetration into the skin. Its concentration at 100 μm depth following 2 and 30 min exposure to human skin was reportedly 0.157 ± 0.047 and 0.077 ± 0.015 μg/mg, respectively.[28] Thus chlorhexidine might fail to reach the deeper skin layers and the hair follicles. Another explanation for lack of response may be related to biofilm development, which has been found in cases of *S. aureus* impetigo and furuncles in humans.[29] *Staphylococcus aureus* within a biofilm reportedly decreases the efficacy of chlorhexidine as a disinfectant.[30] A recent report suggests that *S. pseudintermedius* has the potential to form a biofilm.[31] Another explanation could be related to the fact that organic matter decreases the efficacy of disinfectants, including chlorhexidine.[32] In the case of skin disease, it might be less efficacious due to poor penetration into papules, pustules, crusts and/or exudate. Prewashing patients to remove gross debris may increase its efficacy. Finally, the most important factors for unsuccessful topical therapy are likely to be a lack of owner compliance and underlying or concurrent primary skin diseases which favour the persistence of pyoderma.[33] Further investigation will be warranted to identify whether clinical chlorhexidine resistance exists in some dogs with pyoderma.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotide primers for multiplex-PCR for species identification of coagulase-positive staphylococci and *S. schleiferi* subsp. *schleiferi*.

Table S2. Antimicrobial susceptibility of meticillin-susceptible *Staphylococcus pseudintermedius* (MSSP) and meticillin-resistant *S. pseudintermedius* (MRSP) strains.