Interaction of chlorhexidine with trisEDTA or miconazole *in vitro* against canine meticillin-resistant and susceptible *Staphylococcus pseudintermedius* isolates from two UK regions

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Running Title: Activity of chlorhexidine combinations

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Abstract

Background – Topical therapy is an important alternative to systemic antibacterial therapy for treatment of canine superficial pyoderma in light of the emergence of multidrug-resistant staphylococci. Chlorhexidine is widely used in shampoo products alone or in combination with miconazole or tromethamine-ethylenediaminetetraacetic acid (trisEDTA). Comparisons of these combinations have not been made.

Hypothesis/Objectives – To determine minimum inhibitory concentrations (MICs) of combinations of chlorhexidine/miconazole and chlorhexidine/trisEDTA *in vitro* in a collection of *Staphylococcus pseudintermedius* (SP) from northern (NUK) and south east (SEUK) United Kingdom (UK).

Methods – MICs of chlorhexidine, miconazole, trisEDTA and combinations of chlorhexidine/miconazole (1:1) or chlorhexidine/trisEDTA (80:16:1 and 80:5:1) were determined for 196 canine SP isolates from NUK (49 meticillin-resistant [MRSP], 50 meticillin-susceptible [MSSP]) and SEUK (48 MRSP, 49 MSSP) using agar dilution.

Results – TrisEDTA alone did not inhibit growth. Chlorhexidine/miconazole MICs (median = 0.5 mg/L) were lower than those of either drug alone ($P<0.05$) and lower than chlorhexidine/trisEDTA MICs (median = 1 mg/L; $P<0.0005$) in each bacterial type and from both regions, except for miconazole in NUK MSSP. An additive interaction was noted between chlorhexidine and miconazole or trisEDTA (80:16:1 ratio) in 79 and 43 isolates, respectively, whereas antagonism between chlorhexidine and trisEDTA was noted for three isolates. NUK isolates were more susceptible than SEUK ($P<0.05$), except MRSP exposed to chlorhexidine and the chlorhexidine/trisEDTA (80:16:1) combination.

Conclusions and Clinical Importance – These low MICs are likely to be exceeded by topical therapy. Evaluation of the mechanisms by which chlorhexidine combinations interact to reduce MICs is warranted, in view of increasing concerns of biocide tolerance in staphylococci.

Introduction

In the light of the emergence of multidrug resistant (MDR) staphylococci, especially meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) in veterinary medicine,[1] the role of antiseptics is becoming increasingly important. Improved treatment strategies for staphylococcal skin disease are needed to limit the spread of MRSP and to reduce selective pressure from repeated systemic antimicrobial therapy. Topical therapy, in particular with chlorhexidine, has been recommended as an option for treatment of canine superficial pyoderma.[2]

Previous studies have shown that chlorhexidine shampoo is an effective monotherapy in canine superficial pyoderma,[3, 4] and *in vitro* data indicates that chlorhexidine is effective against MRSP as well as its meticillin-susceptible counterpart (MSSP), and the predominant human commensal (*S. aureus;* both meticillin-resistant and susceptible strains).[5,6] Genes that encode drug efflux proteins in *S. aureus*, including *qacA/B* and *smr* which confer low-level and high-level chlorhexidine resistance respectively,[7,8] have rarely been described in *S. pseudintermedius.*[9, 10] However, the clinical relevance of this has not been assessed. The development of biocide resistance in *S. pseudintermedius* might well be minimised by combining chlorhexidine with other agents with additive or synergistic effects. Current products used for canine superficial skin infections include shampoos containing chlorhexidine alone, or in combination with miconazole or tromethamine and ethylenediaminetetraacetic acid (trisEDTA). Minimum inhibitory concentrations (MICs) have been established for miconazole and chlorhexidine in combination, with synergistic and additive interactions being shown.[5] A shampoo formulation of chlorhexidine and miconazole was shown to significantly reduce coagulase-positive staphylococcal counts on seborrhoeic canine skin when determined by cup-scrub in a blinded study design.[11] In 1987 it was reported that chlorhexidine and trisEDTA was more active than chlorhexidine alone against *S. aureus*;[12] although fractional inhibitory concentration (FIC) indices were not used to assess the interaction between the drugs. TrisEDTA has been shown to enhance the efficacy of marbofloxacin and gentamicin[13] against *Pseudomonas aeruginosa,* and has been shown to act synergistically with cefalexin, oxytetracycline, ampicillin, streptomycin and sulfadimethoxine against *S. aureus*.[14] This is thought to be due to EDTA damaging the outer cell wall causing the bacteria to become more permeable to the antimicrobial agents that act intracellularly.[13]

There has been limited investigation into the potential synergistic activity of different ratios of trisEDTA amongst chlorhexidine products; furthermore MICs for chlorhexidine / miconazole have not yet been compared to chlorhexidine / trisEDTA. We therefore determined MICs of chlorhexidine, miconazole, trisEDTA and the combination of chlorhexidine with either miconazole or trisEDTA in two ratios, in a large collection of MRSP and MSSP isolates obtained from the Northern and South-Eastern regions of the United Kingdom.

Materials and methods

Bacterial Isolates

One hundred ninety six *S. pseudintermedius* isolated from dogs were tested; of these, 49 MRSP and 50 MSSP were from Northern UK (NUK; the southern border of which were SY, ST, DE, S and YO post codes) and 48 MRSP and 49 MSSP from the South East UK (SEUK; from CB, AL, UB and London post codes). All NUK isolates were collected from clinical cases obtained in 2011-2015. The SEUK isolates were from a collection stored at -80°C in brain-heart infusion broth with 20% glycerol (obtained in 2010-2015); 44 MSSP were skin and mucosal carriage isolates and 5 MSSP and 48 MRSP were collected from clinical cases.

All isolates had been identified to species level through phenotypic methods, either by a diagnostic microbiology laboratory (NUK isolates) or for a previous study (SEUK isolates).[15] Briefly, colony morphology and haemolytic properties were assessed through growth on blood agar base containing 5% sheep blood; coagulase positive staphylococci were identified by clumping factor activity using dog plasma and DNase production.[16] Phenotypic identification of *S. pseudintermedius* was confirmed by demonstration of the species-specific thermonuclease gene (*nuc*) by PCR.[17] Meticillin resistance was confirmed genotypically by demonstrating the presence of *mecA* by PCR and phenotypically through growth on mannitol salt agar containing 6 mg/L oxacillin.[18]

MIC Determination

MICs were determined by agar dilution according to CLSI guidelines.[19] Prior to MIC determination, isolates were subcultured twice on blood agar base (CM0271, Oxoid, Basingstoke, UK) containing 5% sheep blood (TCS Biosciences, Buckingham, UK) at 35°C for 24 hours. Stock solutions of antimicrobials were prepared at 10x final concentration in phosphate-buffered saline (chlorhexidine digluconate C9394, tromethamine PHR1347 and ethylenediaminetetraacetic acid (EDTA) EDS; Sigma-Aldrich Inc, Dorset, UK) or 1% DMSO (miconazole nitrate PHR1163; Sigma-Aldrich Inc) adjusted for drug potency.[19] Serial two-fold dilutions were prepared in molten Mueller-Hinton agar, pH 7.2, conforming to (CLSI. Protocols for evaluating dehydrated Mueller-Hinton agar; approved standard – second edition. CLSI document M06-A2. Wayne, PA: Clinical and Laboratory Standards Institute; 2006) (CM0337, Oxoid). In the first instance an 80:16:1 combination of

chlorhexidine digluconate, tromethamine and EDTA was tested, alongside a 1:1 chlorhexidine digluconate and miconazole nitrate combination, and each drug alone. Final concentrations of the active fraction ranged from 0.125 to 16 mg/L of chlorhexidine digluconate, 0.25 to 64 mg/L of miconazole nitrate, 0.06 to 8 mg/L of a 1:1 combination of chlorhexidine digluconate and miconazole nitrate, 0.25 to 512 mg/L of a 16:1 combination of tromethamine and EDTA, and 0.03 to 16 mg/L of a 80:16:1 combination of chlorhexidine digluconate, tromethamine and EDTA. The study was extended using 185 of the original isolates (NUK MRSP n=49, NUK MSSP n=50, SEUK MRSP n=45, SEUK MSSP n=41) to compare an 80:5:1 combination of chlorhexidine digluconate, tromethamine and EDTA (0.125 – 16 mg/L), as used in a commercial product, with the 1:1 combination of chlorhexidine digluconate and miconazole nitrate (0.125 - 4 mg/L) repeated for internal control purposes. Start and end control plates of Mueller-Hinton agar alone were also inoculated in each experiment. Plates were held at 4°C in plastic bags until used within seven days.

A MIC was recorded as the lowest concentration of antimicrobial agent which completely inhibited colony formation, disregarding single colonies or a faint haze of growth.[19] Discrepancy between duplicate MICs was accepted provided the duplicates varied by only one dilution; in such cases, the higher value was identified as the final MIC, as a conservative interpretation. For quality control purposes three reference strains (*S. pseudintermedius* LMG 22219 (Belgian Co-ordinated Collections of Micro-organisms, Ghent, Belgium), *S. aureus* American Type Culture Collection (ATCC, Teddington, UK) 25923 and *S. aureus* ATCC 29663) and one MSSA isolate previously reported with high MIC values for miconazole (B122)[5] were included.

Fractional inhibitory concentration (FIC) indices

The FIC index (FICi) was calculated to analyse drug interaction of chlorhexidine and either miconazole or trisEDTA (16:1) when used in combination, using the formula $\Sigma FIC = FIC_A + FIC_B = (MIC_{AB}/MIC_A) + (MIC_{AB}/MIC_B)$, where MIC_A and MIC_B are the MIC of the drugs when used alone, and MIC_{AB} is the MIC of the two drugs in combination. FICi were interpreted according to EUCAST guidelines, wherein FICi ≤0.5 represented synergy, >0.5 to 1 indicated additivity, >1 to <2 represented indifference, and ≥2 indicated antagonism.[20]

Statistical Analysis

MICs were compared using a linear mixed effect model (SPSS version 20, IBM United Kingdom Ltd, Portsmouth, UK) after $\log_2$ transformation, comparing drug, region (NUK and SEUK) and bacterial group (MRSP and MSSP) while accounting for repeated measures. Significance was set at $P<0.05$.

Results

TrisEDTA (16:1 ratio) alone did not inhibit growth (MIC ≥ 512 mg/L, n=196) and thus was omitted from analysis in the linear mixed effect model. MICs of the 1:1 combination of chlorhexidine and miconazole in the initial MIC determination ranged from 0.25 – 2 mg/L (Table 1), with 90.3% (177/196) of isolates having an MIC of 0.25 or 0.5 mg/L. Values were very closely similar in the repeat analysis of 185 isolates with a range of 0.25 – 1 mg/L and 75% of isolates (139/185) having an MIC of 0.25 or 0.5 mg/L (Table 3). For the whole collection of 196 isolates, chlorhexidine / miconazole MICs (MIC_{90} = 0.5 mg/L) were significantly lower than chlorhexidine alone ($P<0.0005$; MIC_{90} chlorhexidine = 2 mg/L), chlorhexidine / trisEDTA 80:16:1 combination ($P<0.0005$; MIC_{90} 80:16:1 2 mg/L) and miconazole alone ($P<0.005$; MIC_{90} miconazole = 1 mg/L; except in NUK MSSP). Miconazole MICs were significantly lower than chlorhexidine alone or in 80:16:1 combination with trisEDTA ($P<0.005$, Table 1).

Combination with miconazole reduced the chlorhexidine MICs by zero (n=1), one (n=100), two (n=83) or three dilutions (n=12). In 79 of 196 isolates an additive interaction was observed between miconazole and chlorhexidine (FICi > 0.5 ≤ 1; Table 2).[20] No antagonistic interactions were observed; all other isolates fell into the 'no interaction' group.

MICs for chlorhexidine / trisEDTA in 80:16:1 combination (MIC_{90} 2mg/L, range 0.5 – 16 mg/L, Table 1) were significantly lower than that of chlorhexidine alone in all bacterial groups ($P<0.05$) except SEUK MRSP. A 80:16:1 combination with trisEDTA reduced the chlorhexidine MICs by one dilution in 43 cases, and had no effect in 150. There was an additive interaction between chlorhexidine and trisEDTA in a 80:16:1 combination in 21.9% isolates (43/196) whereas 3 isolates (1.5%) showed an antagonistic interaction where the MIC was increased by one dilution (Table 2).

As with the chlorhexidine / miconazole combination, MICs for the 80:5:1 ratio of chlorhexidine / TrisEDTA were closely comparable to those seen with the 80:16:1 ratio; the MIC_{50} and MIC_{90} for both combinations were 1 and 2 mg/L, respectively, and the lowest MIC was 0.5 mg/L in both studies (Table 3). As before, the chlorhexidine / miconazole combination (MIC_{50}=0.5 mg/L; MIC_{90}= 1.0 mg/L) had greater *in vitro* activity ($P<0.0005$) than the combination of chlorhexidine and TrisEDTA.

Amongst the MSSP, NUK isolates were significantly more susceptible to all drugs (except 80:5:1 chlorhexidine / trisEDTA) than SEUK isolates ($P<0.05$; Table 4). In the MRSP groups, SEUK were significantly less susceptible to miconazole than NUK isolates ($P>0.0005$), however no significance was found in differences between NUK and SEUK MRSP for the other drugs.

MRSP had significantly higher MICs of miconazole (NUK MRSP MIC_{90} = 0.5 mg/L, SEUK MRSP MIC_{90} = 1 mg/L) than MSSP (NUK MSSP MIC_{90} = 0.5 mg/L, SEUK MSSP MIC_{90} = 0.5 mg/L; $P<0.05$; Table 4). NUK MRSP had significantly higher MICs of chlorhexidine and chlorhexidine / trisEDTA 80:16:1 combination when compared with MSSP; however no differences were seen in other MRSP / MSSP comparisons (Table 4). Reference strains had MICs equal to or within one dilution of previously established values;[5] the quality control MSSA isolate (B122) showed more susceptibility to miconazole than previously determined, reflecting the overall increased susceptibility to miconazole seen in these results. MICs varied by a single dilution between replicates on only six occasions.

Discussion

Continued spread of MRSP and the potential for the emergence of tolerance to chlorhexidine[8, 9] warrants the monitoring of the efficacy of these combination products. Further investigation into the interaction of these combinations may indicate whether additive or synergistic combinations can be used to delay development of resistance, as well as to improve the efficacy of chlorhexidine. The efficacy of miconazole in this study against staphylococci is not unexpected considering previous research documenting its anti-staphylococcal activity.[21] The good activity of chlorhexidine and miconazole in combination against MRSP and MSSP is in accordance with and supports the results of a study of isolates originating from both the UK and Germany.[5] Previously, both synergistic and additive effects have been observed with miconazole and chlorhexidine, whereas only additive effects were noted in the present study; these differences appear to reflect a somewhat higher activity of miconazole alone. A degree of variation is well recognised in MIC determinations even when standardised protocols are followed.[22] Otherwise, MICs of field and reference strains were similar to values reported previously and there was good repeatability in the data within this study. The lower MICs and increased rates of additivity in this study indicated that the combination of miconazole / chlorhexidine is more efficacious *in vitro* than both tested combinations of trisEDTA /chlorhexidine.

The combination of trisEDTA with chlorhexidine resulted in lower MICs than with chlorhexidine alone (in most cases), however this appears modest in comparison to the enhancement seen when trisEDTA is combined with marbofloxacin or gentamicin against *Pseudomonas aeruginosa*.[13] This may reflect a difference between the effect of TrisEDTA on Gram-positive and negative bacteria, or could indicate that the mechanism of activity of chlorhexidine is not improved by the increased permeability afforded to the bacteria by the action of TrisEDTA. The mechanisms of interaction of these combinations warrants further investigation. The emergence of MRSP is attributed to the expansion of a few dominant lineages, rather than the acquisition of resistance genes by multiple lineages;[1] this is similar to the clonal expansion of MRSA in human medicine. This may account for the lack of regional differences in MIC amongst this study's MRSP isolates. More regional variability in MSSP MICS likely reflects greater genetic diversity in this group,[9] but the overall low MICs for both NUK and SEUK regions indicates that there should be no difference in response to treatment with these combinations according to region.

The low MICs in this study showed that chlorhexidine shampoos are rational choices for treatment of canine superficial pyoderma associated with *S. pseudintermedius*, including MRSP. The combination of chlorhexidine with miconazole showed apparent superiority over combination with trisEDTA *in vitro*, though product formulation, ability of the antiseptic to penetrate into the infected area of skin and client compliance may have confounding effects on treatment that must be investigated through *in vivo* studies. The very limited differences in susceptibility amongst MSSP and the apparent absence of regional differences amongst MRSP indicates that topical therapy remains an important option for veterinarians in the treatment of canine superficial pyoderma.

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Table 1: MICs of chlorhexidine (alone), miconazole (alone), chlorhexidine and miconazole in a 1:1 combination and chlorhexidine, tromethamine and EDTA in an 80:16:1 ratio for 196 *Staphylococcus pseudintermedius* isolates from dogs[1]

Drug	Bacterial Type	MIC / mg/L												[2]MIC_{50}	MIC_{90}
		0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64		
Chlorhexidine	NUK MRSP	Not tested	Not tested	0	0	0	16	25	8	0	0	Not tested	Not tested	2	4
	NUK MSSP	Not tested	Not tested	0	0	0	36	14	0	0	0	Not tested	Not tested	1	2
	SEUK MRSP	Not tested	Not tested	0	0	0	21	24	2	1	0	Not tested	Not tested	2	2
	SEUK MSSP	Not tested	Not tested	0	0	1	25	18	2	3	0	Not tested	Not tested	1	4
	Total	Not tested	Not tested	0	0	1	98	81	12	4	0	Not tested	Not tested	1	2
Miconazole	NUK MRSP	Not tested	Not tested	Not tested	0	37	12	0	0	0	0	0	0	0.5	1
	NUK MSSP	Not tested	Not tested	Not tested	2	48	0	0	0	0	0	0	0	0.5	0.5
	SEUK MRSP	Not tested	Not tested	Not tested	0	1	44	2	1	0	0	0	0	1	1
	SEUK MSSP	Not tested	Not tested	Not tested	0	33	8	4	4	0	0	0	0	0.5	2
	Total	Not tested	Not tested	Not tested	2	119	64	6	5	0	0	0	0	0.5	1
1:1 Chlorhexidine / miconazole	NUK MRSP	Not tested	0	0	2	47	0	0	0	0	Not tested	Not tested	Not tested	0.5	0.5
	NUK MSSP	Not tested	0	0	8	42	0	0	0	0	Not tested	Not tested	Not tested	0.5	0.5
	SEUK MRSP	Not tested	0	0	0	41	6	1	0	0	Not tested	Not tested	Not tested	0.5	1
	SEUK MSSP	Not tested	0	0	4	33	9	3	0	0	Not tested	Not tested	Not tested	0.5	1
	Total	Not tested	0	0	14	163	15	4	0	0	Not tested	Not tested	Not tested	0.5	0.5
80:16:1 chlorhexidine / tromethamine / EDTA	NUK MRSP	0	0	0	0	0	23	20	6	0	0	Not tested	Not tested	2	4
	NUK MSSP	0	0	0	0	1	47	2	0	0	0	Not tested	Not tested	1	1
	SEUK MRSP	0	0	0	0	0	25	20	2	1	0	Not tested	Not tested	1	2
	SEUK MSSP	0	0	0	0	1	42	1	2	0	3	Not tested	Not tested	1	4
	Total	0	0	0	0	2	137	43	10	1	3	Not tested	Not tested	1	2

MRSP, meticillin-resistant *S. pseudintermedius*; MSSP, meticillin-susceptible *S. pseudintermedius*; NUK, isolates of Northern UK origin; SEUK, isolates of a South-Eastern UK origin.
NUK MRSP n=49, NUK MSSP n=50, SEUK MRSP n=48, SEUK MSSP n=49.
[1]MICs for trisEDTA (alone) are not included as MIC >512 mg/L was established for all 196 isolates.
[2]MIC_{50} is equal to the median value.

Table 2: Interpretation of fractional inhibitory concentration indices for a 1:1 combination of chlorhexidine and miconazole and an 80:16:1 combination of chlorhexidine, tromethamine and EDTA (trisEDTA) for 196 *Staphylococcus pseudintermedius* isolates from dogs

Drug Combination	Bacterial Group	Synergy ≤0.5	Additive >0.5-≤1	Indifference >1-≤2	Antagonism >2
1:1 chlorhexidine and miconazole	N MRSP (n=49)	0	14	35	0
	N MSSP (n=50)	0	6	44	0
	SE MRSP (n=48)	0	43	5	0
	SE MSSP (n=49)	0	16	33	0
	Total	0	79	117	0
80:16:1 combination of chlorhexidine, tromethamine and EDTA (trisEDTA)	N MRSP (n=50)	0	9	40	0
	N MSSP (n=50)	0	13	37	0
	SE MRSP (n=49)	0	4	44	0
	SE MSSP (n=49)	0	17	29	3
	Total	0	43	150	3

MRSP, meticillin-resistant *S. pseudintermedius*; MSSP, meticillin-susceptible *S. pseudintermedius*; N, isolates of Northern UK origin; SE, isolates of a South-Eastern UK origin

Table 3: MICs of chlorhexidine and miconazole in a 1:1 combination and chlorhexidine, tromethamine and EDTA in an 80:5:1 ratio for 185 *Staphylococcus pseudintermedius* isolates from dogs[1]

Drug	Bacterial Type	MIC / mg/L								[1]MIC_{50}	MIC_{90}
		0.125	0.25	0.5	1	2	4	8	16		
1:1 Chlorhexidine / miconazole	NUK MRSP	0	0	43	6	0	0	Not tested	Not tested	0.5	1
	NUK MSSP	0	1	49	0	0	0	Not tested	Not tested	0.5	0.5
	SEUK MRSP	0	0	16	29	0	0	Not tested	Not tested	1	1
	SEUK MSSP	0	0	30	11	0	0	Not tested	Not tested	0.5	1
	Total	0	1	138	46	0	0	Not tested	Not tested	0.5	1
80:5:1 chlorhexidine / tromethamine / EDTA	NUK MRSP	0	0	0	18	20	1	0	0	2	4
	NUK MSSP	0	0	0	35	14	1	0	0	1	2
	SEUK MRSP	0	0	0	21	22	2	0	0	2	2
	SEUK MSSP	0	0	1	23	15	2	0	0	1	2
	Total	0	0	1	97	71	16	0	0	1	2

MRSP, meticillin-resistant *S. pseudintermedius*; MSSP, meticillin-susceptible *S. pseudintermedius*; NUK, isolates of Northern UK origin; SEUK, isolates of a South-Eastern UK origin.
NUK MRSP n=49, NUK MSSP n=50, SEUK MRSP n=45, SEUK MSSP n=41.
[1]MIC_{50} is equal to the median value.

Table 4: Comparisons (P values) of MIC of chlorhexidine, miconazole and combinations of chlorhexidine with either miconazole or tromethamine and EDTA amongst *Staphylococcus pseudintermedius* isolates grouped by UK region (NUK and SEUK) and bacteria type (MRSP and MSSP)[1]

	Chlorhexidine[2]	Miconazole[2]	1:1 Chlorhexidine / miconazole[2]	80:16:1 Chlorhexidine / tromethamine / EDTA[2]	80:5:1 Chlorhexidine / tromethamine / EDTA[3]
NUK versus SEUK MRSP	0.128	**<0.0005**	0.098	0.470	0.254
NUK versus SEUK MSSP	**0.008**	**<0.0005**	**0.002**	**0.014**	0.118
MRSP versus MSSP NUK	**<0.0005**	**0.022**	0.337	**<0.0005**	0.496
MRSP versus MSSP SEUK	0.789	**<0.0005**	0.644	0.060	0.124

[1]P value < 0.05 is considered significant. Significant values are in bold. [2]n = 196 *S. pseudintermedius* isolates [3]n = 185 *S. pseudintermedius* isolates