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In vitro antimicrobial activity of a commercial ear antiseptic containing chlorhexidine and Tris–EDTA

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Abstract

Minimum bactericidal concentrations (MBCs) of a commercial ear antiseptic containing chlorhexidine 0.15% and Tris–EDTA (Otodine®) were determined by broth microdilution for 150 isolates representing the most common pathogens associated with canine otitis. The microorganisms were classified into three groups according to their levels of susceptibility. The most susceptible group included *Staphylococcus pseudintermedius, Malassezia pachydermatis, Streptococcus canis* and *Corynebacterium auriscanis,* which were generally killed by 1 : 64 dilution of the antiseptic product (MBC = 23/0.8 μg/mL of chlorhexidine/Tris–EDTA). The most resistant organism was *Proteus mirabilis,* which survived up to 1 : 8 dilution of the product (MBC = 375/12 μg/mL). *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* displayed intermediate MBCs ranging between 188/6 and 47/1.5 μg/mL. Interestingly, *S. pseudintermedius* was more susceptible than *S. aureus,* and no significant difference was observed between meticillin-resistant and meticillin-susceptible isolates within each species, indicating that antiseptic use is unlikely to co-select for meticillin resistance. Although the concentrations required for killing (MBCs) varied considerably with microorganism type, the combination of chlorhexidine 0.15% and Tris–EDTA was active against all the pathogens most commonly involved in canine otitis.

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Introduction

The recent emergence of multiresistant bacteria in companion animals, especially meticillin-resistant staphylococci,[1–3] has evidenced a need for alternative therapeutic approaches to eliminate bacterial strains that are virtually resistant to all oral antibiotics, while minimizing further selection of multiresistant strains. Among the various recommendations, topical use of antimicrobial products, including antiseptics, has been recommended to treat surface skin infections.[4] This approach is less likely to favour selection of antimicrobial resistance in the commensal microflora as topical products, in contrast with systemic antibiotics, act primarily on the site of infection. Furthermore, the antimicrobial concentrations obtained at the infection site by topical formulations may be effective against strains defined as resistant by susceptibility testing,[5] because the resistance breakpoints used for defining *in vitro* resistance are based on the concentrations achieved by systemic therapy, which are markedly lower than those obtained by local treatment.

In canine otitis externa, systemic antimicrobials are unlikely to achieve therapeutic concentrations in the waxy exudate present in the infected ear canal. Instead, local antimicrobial therapy, including flushing with antiseptic products alone or combined with topical antibiotics, is the recommended treatment.[4,6] Most products contain one or more ingredients with antibacterial, antifungal or anti-inflammatory activity and other components (ceruminolytics, astringents, stabilizers and surfactants) that increase the solubility and the activity of the antimicrobial ingredients. Topical antiseptic products used for management of canine otitis, generally referred to as ear cleaners, may contain different molecules with antimicrobial activity, such as for example chlorhexidine and ethylene diamine tetra acetic acid-tromethamine (Tris–EDTA). Chlorhexidine is a biguanide compound that exerts bactericidal activity on both Gram-positive and Gram-negative bacteria by membrane disruption.[7] This antiseptic is currently used with success for decolonization of meticillin-resistant *Staphylococcus aureus* (MRSA) carriage in humans.[8] In veterinary medicine, local treatment with chlorhexidine has been reported to be successful as an alternative or complimentary approach to antibiotic treatment of meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) infections in small animals.[2] Tris–EDTA is a chelating agent affecting permeability of the outer membrane in Gram-negative bacteria by removing Ca^{2+} and Mg^{2+}.[9] This mechanism of action results in a synergistic effect when Tris–EDTA is administered together with other antimicrobials, including antiseptics and systemic antibiotics,[10,11] most likely by enhancing their penetration into the bacterial cell.

A recent study by Swinney *et al.*[12] compared the antimicrobial efficacy of nine commercially available ear cleaners against *S. pseudintermedius, Pseudomonas*

aeruginosa and *Malassezia* spp. As pointed out by the authors, a weakness of the study was the use of a single strain for each microorganism, which was selected to avoid any unusual antimicrobial resistance pattern. In the present study, the antimicrobial efficacy of a commercial ear antiseptic (Otodine®; ICF, Cremona, Italy) containing chlorhexidine digluconate 0.15% and Tris–EDTA was tested on a large collection of clinical skin and ear isolates representing the most common microorganisms associated with otitis in dogs, including meticillin-resistant staphylococci displaying multiple resistance to systemic antibiotics. The primary objective was to assess the *in vitro* efficacy of this product against a wide range of pathogens and strains. As a secondary objective, the levels of susceptibility were compared among meticillin-resistant and meticillin-susceptible staphylococci to detect possible associations between multiple antibiotic resistance and reduced susceptibility to the combination of chlorhexidine and Tris–EDTA.

Materials and methods

Bacterial isolates and media

A collection of 150 bacterial and yeast isolates of canine origin were selected to include the most common species associated with otitis. Based on standard phenotypic identification, the collection was composed of the following species: *Corynebacterium auriscanis* (n = 12), *Escherichia coli* (n = 12), *Malassezia* spp. (n = 9), *Proteus mirabilis* (n = 11), *P. aeruginosa* (n = 19), *S. aureus* (n = 22), *S. intermedius* (n = 53) and *Streptococcus canis* (n = 12). *S. intermedius* isolates from dogs are here referred to as *S. pseudintermedius* according to the recent changes in the taxonomy of this staphylococcal group.[13] Most (n = 121) were isolated in Denmark from dogs with otitis or other dermatological infections over the period 1988–2009 at the Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen. The remaining isolates included 21 MRSP and 10 MRSA displaying multiple antimicrobial resistance patterns from infected or healthy dogs in Denmark, Finland, Germany, Italy, the Netherlands, Sweden, Switzerland, the UK and the USA. MRSP and MRSA isolates were selected on the basis of epidemiological data generated by previous studies,[1–3] and represented distinct genotypes and countries. Bacteria were grown overnight at 37 °C on meat gar agar (Oxoid, Basingstoke, UK) supplemented with 5% calf blood and *M. pachydermatis* was grown for 3 days at 37 °C on Sabouraud dextrose agar (Oxoid).

In vitro susceptibility testing

Otodine® is a commercial antiseptic product containing 1.5 mg/mL of chlorhexidine digluconate, 0.048 mg/mL of Tris–EDTA, 100 mg/mL of propylene glycol, and water. The *in vitro* antimicrobial activity of this commercial product was evaluated by broth microdilution following essentially the protocol described by Swinney *et al.*[12] Colony material from each isolate was suspended in 8-mL phosphate-buffered saline (PBS; pH 7.2) and the mixture was adjusted to a turbidity of 0.5 McFarland using a nephelometer (Trek Diagnostics, East Grinstead, UK). Preliminary experiments on a subset of isolates showed that this concentration corresponded to approximately 10^8 and 10^6 CFU/mL for bacteria and *Malassezia* respectively. Bacterial suspensions were further diluted 1 : 100 to obtain concentrations of 10^6 CFU/mL. One-hundred microlitres of undiluted and twofold dilutions of Otodine® in PBS were transferred to sterile 96-well microtitre plates (TPP, Trasadingen, Switzerland) and 100 μL of adjusted microbial suspensions (~10^5 CFU) were added to yield final dilutions from 1 : 2 to 1 : 256. As positive controls, microbial suspensions were also added to wells containing 100-μL PBS without antiseptic. After 30-min incubation at 37 °C, an aliquot of 10 μL from each well was plated onto blood agar (bacteria) or Sabouraud dextrose agar (*Malassezia*) and incubated at 37 °C for one and 3 days respectively.

A selection of seven representative strains (i.e. one per species) was also incubated for 10 min prior to plating on agar for comparison of the results obtained by the two exposure times. The numbers of colonies (CFU) growing at each dilution was recorded visually and classified as confluent growth when colonies were too numerous (over 50) to be counted. The minimum bactericidal concentration (MBC) was defined as the lowest concentration (i.e. the highest dilution) required for killing of the strain, i.e. no visible growth on the agar plate. The combined MBCs of chlorhexidine digluconate and Tris–EDTA were calculated based on the product composition provided by the manufacturer (1.5 and 0.048 mg/L respectively). A *P. aeruginosa* isolate (C22406) was included in all microtitre plates to test the reproducibility of the method.

Statistical analysis

The Fisher's exact test was used to detect statistically significant differences in the MBC distribution between meticillin-resistant and meticillin-susceptible isolates of staphylococci.

Results

The product showed excellent antimicrobial activity against all pathogens tested as shown in Figure 1. MBCs differed substantially depending on the species of microorganism but usually varied by two- or fourfold dilutions only among strains belonging to the same species. All isolates were completely killed by a 1 : 4 dilution of the antiseptic product, corresponding to an MBC of 375/12 μg/mL of chlorhexidine digluconate/Tris–EDTA (Figure 1). Three groups were defined according to their levels of susceptibility; (i) *Proteus mirabilis* constituted the most resistant group (MBC ≥ 94/3 μg/mL of chlorhexidine/Tris–EDTA); (ii) *E. coli*, *P. aeruginosa* and *S. aureus* generally displayed lower MBCs ranging between 188/6 and 47/1.5 μg/mL; (iii) the most susceptible group included *S. pseudintermedius*, *Malassezia* spp., *S. canis* and *C. auriscanis*. With very few exceptions, all strains belonging to these four species were completely eliminated at concentrations of 23/0.8 μg/mL (1 : 64 dilution of the product). *Staphylococcus pseudintermedius* appeared to be more susceptible than *S. aureus* irrespective of meticillin susceptibility (Figure 2).

No significant difference was observed between meticillin-resistant and meticillin-susceptible strains within the two staphylococcal species (Fisher's exact test, $P > 0.05$). MBCs for MRSA and MRSP were comparable with those of meticillin-susceptible *S. aureus* and meticillin-susceptible *S. pseudintermedius* respectively (Figure 2).

The MBC for the *P. aeruginosa* strain used as internal control differed by a maximum of twofold following repeated measurement. Reduction of the incubation time to 10 min yielded MBCs comparable with those obtained after 30-min incubation, i.e. within a single twofold variation (data not shown).

Discussion

The antiseptic product was shown to be active *in vitro* against the most common pathogens involved in canine otitis. Even Gram-negative bacteria and *S. aureus*, which

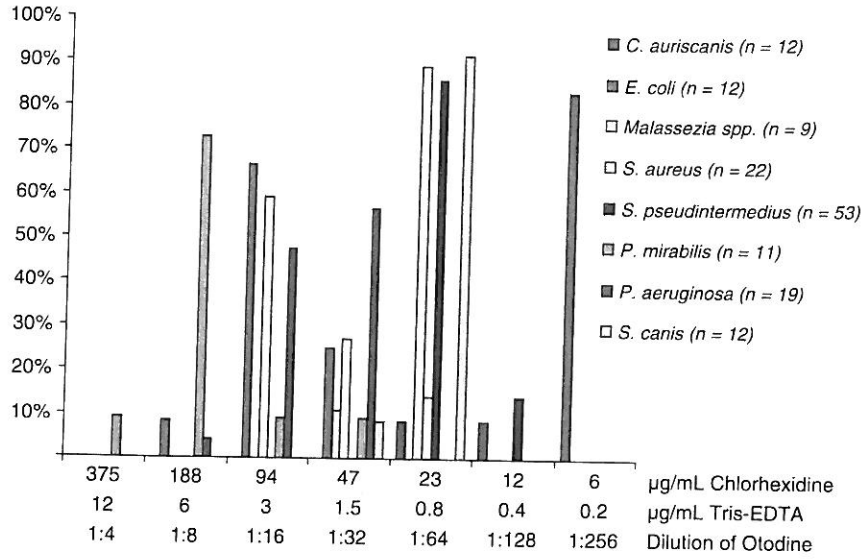

Figure 1. Distribution of minimum bactericidal concentrations (MBCs) of chlorhexidine digluconate and Tris–EDTA among canine isolates of *Corynebacterium auriscanis, Escherichia coli, Malassezia* spp., *Proteus mirabilis, Pseudomonas aeruginosa, Streptococcus canis, Staphylococcus aureus* and *Staphylococcus pseudintermedius*. The vertical axis indicates for each species the percentage of test isolates displaying a given MBC.

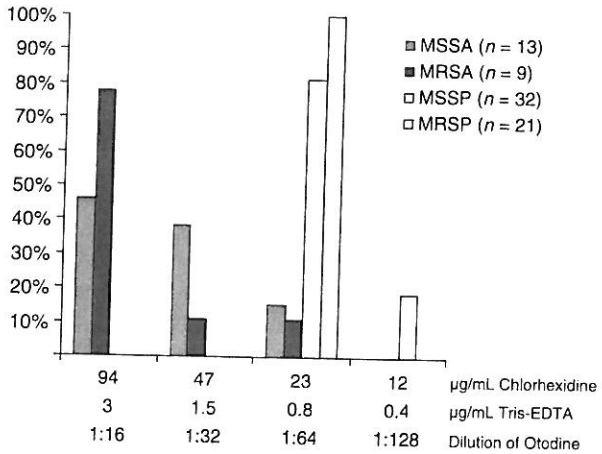

Figure 2. Distribution of minimum bactericidal concentrations (MBCs) of chlorhexidine digluconate and Tris–EDTA among meticillin-resistant *Staphylococcus aureus* (MRSA), meticillin-susceptible *S. aureus* (MSSA), meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) and meticillin-susceptible *S. pseudintermedius* (MSSP). The diagram shows that most *S. aureus* isolates displayed higher MBCs (94/3 or 47/1.5 µg/mL) compared with *S. pseudintermedius* isolates (23/0.8 or 12/0.4 µg/mL). The MBC distribution was similar for meticillin-resistant and meticillin-susceptible isolates within each species.

can display mechanisms of resistance to chlorhexidine,[10] were all completely killed following 30-min exposure to 1 : 4 dilution of the test product (MBC ≤ 375/12 µg/mL). In comparison with a similar formulation tested by Swinney *et al.*[12] using the same method, Otodine® was more active against *S. pseudintermedius* as a 1 : 64 dilution of the product resulted in complete killing of any isolates, including MRSP. As the same methodology was used in the two studies, this incongruence may be explained by either unusual resistance properties of the strain tested by Swinney *et al.* or by differences in the formulation of the two products.

Although strains with reduced susceptibility to chlorhexidine have been described in various Gram-negative species,[9] all *P. mirabilis* and *P. aeruginosa* tested in this study were killed by 375 µg/mL of chlorhexidine digluconate in combination with Tris–EDTA. This result is in line with previous data on chlorhexidine susceptibility reported for human isolates. Minimum inhibitory concentrations (MICs) ranging from 10 to 800 µg/mL have been reported for clinical human isolates of *P. mirabilis*.[14] Human *P. aeruginosa* isolates have been classified as resistant to chlorhexidine for MICs ranging from 50 to 400 µg/mL, in contrast to susceptible isolates with MICs from 3 to 25 µg/mL.[15] *In vitro* efficacy of the product tested in this study cannot be attributable solely to the effect of chlorhexidine as Tris–EDTA is known to potentiate the effects of various antimicrobials.[10,11] Tris–EDTA alone has previously been shown to be ineffective against either *P. aeruginosa, S. pseudintermedius* and *Malassezia* spp.[12] However, the combination of Tris–EDTA and chlorhexidine is likely to result in a synergistic effect, allowing the use of low doses of chlorhexidine that are not ototoxic.[16,17]

In addition to antibacterial activity against both Gram-negative and Gram-positive species, Otodine® showed an excellent fungicidal activity against *Malassezia*. Such a broad spectrum of antimicrobial activity is particularly advantageous in the treatment of canine otitis externa, which often involves different bacterial species and microorganisms. Despite their broad spectrum of activity, antiseptics do not seem to directly enhance selection of antibiotic resistance and, above all, bacteria do not seem to acquire resistance to the antiseptic at the concentrations used for topical treatment.[18,19] Interestingly, this study shows that MRSA and MRSP were as susceptible to the combination of chlorhexidine and Tris–EDTA as meticillin-sensitive isolates. Similar studies on human MRSA isolates have proved that meticillin-resistance is not associated with reduced susceptibility to chlorhexi-

dine and other biocides.[20] Therefore, it is reasonable to conclude that the antiseptic combination tested in this study can be successfully used for topical treatment of surface skin infections caused by meticillin-resistant staphylococci without any consequences on their selection. In the authors' opinion, rational use of antiseptics could also have a positive effect on prevention of antimicrobial resistance by reducing the use of systemic antibiotics and consequently by reducing antibiotic selective pressure.

In vitro data on antimicrobial susceptibility should be interpreted with caution when inferring *in vivo* clinical efficacy and should be confirmed by *in vivo* studies. The presence of exudate in the infected ear canal may result in antimicrobial dilution. Cerumen and pus may affect the *in vivo* antimicrobial efficacy by interfering with antimicrobial activity as well as by providing physical protection to the target pathogen. Interestingly, reduction of the exposure time to 10 min, which mimics the conditions of use recommended by the manufacturer, did not affect the MBCs, indicating that short exposure times are needed to achieve killing of susceptible isolates. This result corroborates a previous study,[21] in which 1–8 min of exposure to commercial ear antiseptics were shown to be sufficient to eliminate all isolates tested. As it is impossible to draw conclusions about the relative importance of individual constituents, the results have been analysed and discussed on the basis of the current knowledge of the antimicrobial activities by the two main antiseptic compounds contained in Otodine®, chlorhexidine digluconate and Tris–EDTA. However, it should be noted that the solvent propylene glycol also possesses antimicrobial properties.[22]

There is a clear need for studies assessing the antimicrobial activity of the individual components of antiseptic formulations used for management of canine otitis. Only by using this approach, it will be possible to identify and quantify synergistic and antagonistic effects between substances included in commercial ear antiseptics, thereby enhancing optimization of their composition and clinical efficacy.

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Résumé Les concentrations bactéricides minimales (MBCs) d'un antiseptique auriculaire commercial contenant de la chlorhexidine 0.15% et du Tris–EDTA (Otodine®) ont été mesurées par microdilution sur 150 échantillons des plus fréquents agents pathogènes d'otite canine. Les microorganismes étaient répartis en trois groupes en fonction de leur niveau de susceptibilité. Le groupe le plus sensible comprenait *Staphylococcus pseudintermedius, Malassezia pachydermatis, Streptococcus canis* et *Corynebacterium auriscanis,* qui étaient généralement tués à une dilution de 1 : 64 de la solution antiseptique (MBC = 23/0.8 μg/mL de chlorhexidine/Tris–EDTA). L'organisme le plus résistant était *Proteus mirabilis,* qui survivait à une dilution du produit allant jusqu'à 1 : 8 (MBC = 375/12 μg/mL). *Escherichia coli, Pseudomonas aeruginosa* et *Staphylococcus aureus* présentaient des MBC intermédiaires entre188/6 et 47/1.5 μg/mL. Notons que *S. pseudintermedius* n'était pas plus susceptible que *S. aureus* et aucune différence significative n'a été observée entre les isolats de chaque espèce résistant ou sensible à la méthiciline, indiquant que l'utilisation d'un antiseptique n'est probablement pas à l'origine d'une sélection de résistance à la méthiciline. Bien que les concentrations bactéricides requises (MBCs) varient énormément avec le type d'organisme, l'association chlorhexidine 0.15% et Tris–EDTA était efficace contre tous les pathogènes les plus communément impliqués dans les otites canines.

Resumen Se determinaron las concentraciones mínimas bactericidas (MBCs) de un antiséptico ótico comercial que contiene clorhexidina 0,15% y Tris–EDTA (Otodine®) en microdilución de caldo de cultivo, para 150 aislados representando los patógenos mas comunes asociados con otitis canina. Los microorganismos fueron clasificados en tres grupos de acuerdo con sus niveles de susceptibilidad. El grupo más susceptible incluía *Staphylococcus pseudintermedius, Malassezia pachydermatis, Streptococcus canis* y *Corynebacterium auriscanis,* los cuales eran generalmente destruidos a una dilución 1 : 64 del producto antiséptico (MBC = 23/0,8 μg/mL de clorhexidina/Tris–EDTA). El organismo más resistente fue *Proteus mirabilis,* el cual sobrevivió hasta una dilución 1 : 8 del producto (MBC = 375/12 μg/mL). *Escherichia coli, Pseudomonas aeruginosa* y *Staphylococcus aureus* mostraron MBCs de rango intermedio entre 188/6 y 47/1,5 μg/mL. Curiosamente, *S. pseudintermedius* fue más susceptible que *S. aureus,* y no hubo diferencia significativa entre los aislados resistentes y sensibles a meticilina para cada especie, indicando que el uso de antisépticos no debe co-seleccionar para la resistencia a meticilina. Aunque las concentraciones requeridas para destruir las bacterias (MBCs) variaron de forma considerable con el tipo de microorganismo, la combinación de clorhexidina con Tris–EDTA fue activa contra todos los patógenos comúnmente implicados en la otitis canina.

Zusammenfassung Die minimalen bakteriziden Konzentrationen (MBCs) eines kommerziellen Ohr-Antiseptikums, welches 0,15%iges Chlorhexidin und Tris–EDTA (Otodine®) enthielt, wurden mittels Mikroverdünnungsbrühe für 150 Isolate bestimmt, die die häufigsten Erreger, die mit caniner Otitis im Zusammenhang stehen, repräsentierten. Die Mikroorganismen wurden je nach ihrer Empfindlichkeit in drei Gruppen eingeteilt. Die empfindlichste Gruppe beinhaltete *Staphylokokkus pseudointermedius, Malassezia pachydermatis, Streptococcus canis* und *Corynebacterium auriscanis,* die grundsätzlich bei einer Verdünnung von 1 : 64 des antiseptischen Produktes (MBC = 23/0,8 μg/mL Chlorhexidin/Tris–EDTA) abgetötet wurden. Der resistenteste Organismus war *Proteus mirabilis,* der bis zu einer Verdünnung von 1 : 8 des Produktes (MBC = 375/12 μg/mL) überlebte. *Escherichia coli, Pseudomonas aeruginosa* und *Staphylokokkus aureus* zeigten mittlere MCBs, die von 188/6 bis 47/1,5 μg/mL reichten. Interessanterweise war *S. pseudointermedius* empfindlicher als *S. aureus* und es wurde kein signifikanter Unterschied zwischen Methicillin-resistenten und Methicillin-emfindlichen Isolaten innerhalb jeder einzelnen Spezies festgestellt. Das weist darauf hin, dass es unwahrscheinlich ist, dass die Verwendung eines Antiseptikums gleichzeitig auf Methicillin-Resistenz selektiert. Obwohl die Konzentration zur Abtötung der Keime (MBCs) deutlich zwischen den einzelnen Typen der Mikroorganismen variierte, war die Kombination von 0,15%igem Chlorhexidin und Tris–EDTA gegen die meisten Erreger, die am häufigsten bei der caninen Otitis auftraten, wirksam.

