PAPER

The susceptibility of *Pseudomonas spp.* isolated from dogs with otitis to topical ear cleaners

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Objective: **To investigate the in vitro efficacy of commercially available topical otic preparations ("ear cleaners") against *Pseudomonas spp*. isolated from canine ear infections.**

Methods: **Between January and May 2011, 50 isolates that were morphologically and phenotypically confirmed as *Pseudomonas spp*. were isolated from 48 dogs that had been identified with clinical signs of otitis externa and media at a referral dermatology clinic in the north west of the UK. The in vitro efficacy of eight different topical preparations against these isolates was investigated using an in-agar inhibition test.**

Results: **Of the eight preparations tested, three showed consistently good in vitro activity against *Pseudomonas spp*., while a further three were consistently ineffective. For the remaining two preparations, in vitro efficacy was variable and inconsistent.**

Clinical Significance: **Topical treatment with ear cleaners is considered to be a valuable adjunct in the treatment of canine otitis that involves multi-antimicrobial-resistant organisms such as *Pseudomonas spp*. Where treatment with antimicrobials is not an option, the use of these preparations, as a sole form of therapy, may be effective in some cases. As a comparison with other similar studies looking at the activity of ear cleaners against bacterial isolates from otitis, this study uses isolates from 50 ears from 48 dogs providing a significant number of isolates for analysis.**

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INTRODUCTION

Otitis externa and otitis media are relatively common canine diseases, occurring in approximately 10 to 20% of dogs presented to veterinary practices (Scott and others 2001). There are numerous primary causes of the disease. In younger dogs, these are most often related to allergy, including atopic dermatitis, while in adult dogs, they are more usually associated with endocrine problems, including hypothyroidism (Griffin and DeBoer 2001, Olivry and others 2010).

Predisposing factors that contribute to otitis, but which do not cause it, include the conformation of the ear canal (recognised in a variety of breeds such as the spaniel and poodle), treatment effects (usually due to the application of topical drugs) and spending considerable time in water (macerating the inside of the ear canal) (Yoshida and others 2002).

Infection when it occurs is recognised as a perpetuating secondary trigger that drives the disease process and is not a primary factor (Rosser 2004, Zur and others 2011). These secondary pathogens include gram-positive organisms, chiefly *Staphylococcus pseudintermedius* and other species of staphylococcus, including coagulase-negative strains, *Streptococcus canis, Enterococcus spp.* and *Corynebacterium auriscanis;* yeasts, in particular, *Malassezia pachydermatis,* and gram-negative bacteria, most often *Pseudomonas spp*. and *Proteus spp.* (Cole and others 1998, Morris 2004).

The most significant pathogen found on the skin of dogs is *S. pseudintermedius*, and this is also the most common bacterial pathogen involved in canine otitis. In the acute stage of the disease, the changing environment within the ear canal predisposes to the development of infection involving this and other gram-positive bacteria. However, as the disease becomes more chronic,

there is often stenosis of the ear canal, with an increase in humidity and pH. At this time, there is also a corresponding shift in microflora from predominantly gram-positive bacteria to initially a mixed population of gram-positive and gram-negative bacteria with Malassezia yeasts, and then, as disease progresses further, to a predominantly, or often pure, gram-negative population (McCarthy and Kelly 1982, Uchida and others 1990, Graham-Mize and Rosser 2004).

Although the incidence of antimicrobial-resistant gram-positive bacteria (chiefly meticillin-resistant strains of *S. pseudintermedius* (MRSP) and enterococci) that can be associated with otitis is increasing (Malik and others 2005, Morris and others 2006), antimicrobial-resistant isolates from ears are more usually gram-negative bacteria, primarily, *Pseudomonas spp.*

Members of the genus Pseudomonas display a wide spectrum of innate resistance to several classes of antimicrobials. In addition, they demonstrate acquired resistance to antimicrobials often considered the treatment of choice, notably fluoroquinolones and aminoglycosides, in part because of the increasing frequency with which antimicrobials are prescribed (Graham-Mize and Rosser 2004). The aim of this study was to investigate the in vitro efficacy of a range of commercially available otic preparations in inhibiting the growth of *Pseudomonas spp.*

MATERIALS AND METHODS

Transport swabs from dogs with clinical evidence of otitis were submitted to one author (SIS) for bacterial culture and susceptibility testing. Swabs were cultured onto blood agar, colistin-nalidixic acid agar (CNA, which is selective for gram-positive organisms), MacConkey agar and Saboraud's agar (Oxoid, Basingstoke, UK). After incubation for 15 to 18 hours at 35 to 37 °C in 5% CO2, colonies of putative pathogens were selected for in vitro susceptibility testing. Such testing was performed in accordance with British Society for Antimicrobial Chemotherapy (BSAC) guidelines, using Iso-Sensitest agar containing 4% horse blood (Oxoid, Basingstoke, UK).

Any isolate with the characteristic colonial morphology of a Pseudomonad was tested for its oxidase reaction using a filter paper impregnated with "oxidase reagent" (*N,N,N',N'*-tetra methyl-*p*-phenylenediamine). All species within the genus Pseudomonas yield a positive oxidase test, wherein the colour of the reagent rapidly changes from colourless to deep blue. In addition, all isolates were tested for their susceptibility to a range of antimicrobials that are commonly chosen by practitioners to treat otitis: framycetin, fuscidic acid, polymyxin B, amoxycillin with clavulanic acid, cephalexin, cefovecin, doxycycline, gentamicin, marbofloxacin, orbifloxacin and pradofloxacin, and biochemically phenotyped using the API 20 NE identification system (Biomerieux, Basingstoke, UK).

Isolates were stored frozen on Microbank™ beads (Pro-Lab Diagnostics, Neston, UK) in the event any further studies were required. Any isolate that gave a phenotypic identification as a member of the genus Pseudomonas was entered into the study regardless of the actual species within that genus, although the majority proved to be *Pseudomonas aeruginosa* or *Pseudomonas fluorescens.*

Eight topical ear cleaning preparations were used in the study and the pH of each of these was measured using a Jenway 3510 pH meter (Camlab Ltd., Cambridge, UK). The preparations used were Sancerum, MSD Animal Health (ear cleaner 1); CerumAural, Dechra Veterinary Products (ear cleaner 2); Malacetic otic, Dechra Veterinary Products (ear cleaner 3); Cleanaural, Dechra Veterinary Products (ear cleaner 4); Surosolve, Fidavet (ear cleaner 5); Otodine, Vetruus (ear cleaner 6); Otoclean, Elanco Animal Health (ear cleaner 7) and Epiotic, Virbac (ear cleaner 8). For each isolate, two Iso-Sensitest agar plates (Oxoid, Basingstoke, UK) were inoculated with a suspension of the organism under test. The inoculum was prepared in accordance with BSAC standards for susceptibility testing, at a density equivalent to a 0·5 McFarland standard, such that following incubation, a semi-confluent lawn of growth was evidenced (Fig 1).

Wells of 10 mm diameter were then cut into the agar using a sterile cork borer. Four wells were cut into each plate, giving eight wells in total. Volume of 200 µl of each preparation was then pipetted into the designated well of the plate, after which plates were incubated for 15 to 18 hours at 35 to 37 °C.

Following incubation, the diameter of the zone around each of the wells was measured. Two separate measurements were taken diagonally across the well, from one leading zone edge to the other. The average zone diameter was then calculated for each of the preparations.

Statistical analysis was performed using one-way ANOVA to test the hypothesis that all products were equal. Where the overall test of product was statistically significant ($P<0·05$), suggesting that at least two of the products were not equal, all pairs of means were compared within this analysis. The difference between the products was deemed significant when P was <0·05.

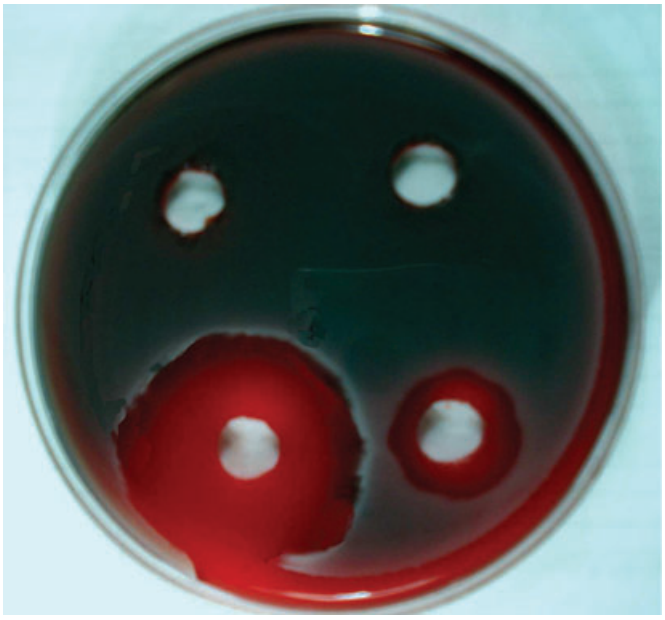

FIG 1. Zones of inhibition of Pseudomonas around four topical ear cleaners

RESULTS

The pH of each preparation was not provided by the manufacturers and was measured and is shown in Table 1. The zone of inhibition was measured for each isolate so that the mean zone of inhibition could be calculated for each ear cleaner (1 to 8). Ear cleaner 2 demonstrated no inhibition for any of the isolates and so was not included in the statistical analysis (Table 2). Ear cleaners 4 and 5 showed poor inhibition for most isolates but did show signs of good inhibition of small numbers of isolates (Table 3) and so were included in the analysis. Of the eight preparations tested, three ear cleaners, numbers 1, 3 and 6, consistently inhibited growth of the test organism in 50 of 50 (100%) cases, with a mean zone of inhibition of 20.5, 22.1 and 23.6 mm, respectively. Conversely, three other ear cleaners, numbers 2, 4 and 5, failed to inhibit the growth of Pseudomonas in 50 of 50 (100%) cases, with mean zone sizes of 10.0, 10.7 and 10.8 mm, respectively. The remaining two cleaners, 7 and 8, showed variable and inconsistent inhibition of the Pseudomonas isolates, with mean zone sizes of 16.8 and 15.0 mm, respectively (Table 3). To ensure that all ear cleaners demonstrated the ability to diffuse across the plates, the maximum and minimum values for each cleaner was measured. A difference in the measurement of the maximum and minimum values clearly demonstrated that the cleaner was able to diffuse across the plate, only cleaner 2 showed no zone of inhibition for any isolate (Table 4).

Table 1. Topical otic preparations used in the study

n	Product name	pH	Components (as listed by the manufacturer)
1	Sancerum	2.5	Lactic acid 2.5%, salicyclic acid 0.1%
2	CerumAural	5.9	Squalene in isopropyl myristate, liquid petroleum
3	Malacetic otic	4.4	Acetic acid 2%, boric acid 2%
4	Cleanaural	6.3	Propylene glycol, isopropyl alcohol, citric acid, L-menthol, chlorothymol, thomethamine
5	Surosolve	6.4	Salicyclic acid, tris EDTA, chlroxylenol, sodium docusate, propylene glycol
6	Otidin	7.1	Propylene glycol, chlorhexidine gluconate 0.15%, Tris EDTA
7	Otoclean	2.8	Salicyclic acid, propylene glycol, polyethylene glycol, ethoxydiglycol, glycerol, lactic acid, oleic acid, plant extracts
8	Epiotic	6.9	Salicyclic acid, PCMX, EDTA, monosaccharides, docusate sodium

EDTA, Ethylenediaminetetraacetic acid; PCMX, para-Chloro-meta-xylenol

Table 2. One-way ANOVA statistical analysis of the difference in inhibition zone size for each product

Ear cleaner number	Values *a*, *b*, *c*, *d*, *e*, *f*: values with different letters are significantly different at P<0.05	Mean	SEM
6	*a*	23.64	0.4002
3	*b*	22.10	0.4002
1	*c*	20.54	0.4002
7	*d*	16.78	0.4002
8	*e*	15.32	0.4002
5	*f*	10.82	0.4002
4	*f*	10.74	0.4002

SEM, Standard error of the mean

Table 4. Maximum and minimum values of zone diameter for all eight otic preparations

Ear cleaner number	Minimum value (mm)	Maximum value (mm)
1	15	29
2	10	10
3	10	30
4	10	25
5	10	20
6	16	29
7	11	25
8	10	28

DISCUSSION

This study clearly demonstrates that ear cleaners show variable antimicrobial activity against different isolates of Pseudomonas. In previous studies that have been performed looking at the antimicrobial properties of topical products, small numbers of isolates have been used to investigate the activity of the preparations (Lloyd and Lamport 2000, Cole and others 2003, Reme and others 2006, Swinney and others 2008, Guardabassi and others 2010). To the author's knowledge, this study is unique in that 50 isolates of Pseudomonas, drawn from clinical cases taken from a referral clinic in the North of England, were used. It shows that there is a variability in the sensitivity of these isolates to topical therapy, suggesting that smaller studies may not be representative of the true activity of a topical product.

The methodology employed in this study uses a Kirby-Bauer type technique using wells filled with ear cleaner, rather than impregnated discs. Susceptibility to a particular ear cleaner is measured as the diameter of the zone of inhibition around the well. Bacterial susceptibility will therefore be dependent on both the antibacterial action of the cleaner and also the diffusion of it through the agar. It is therefore conceivable that a cleaner that diffuses poorly may appear to exhibit reduced antibacterial activity. However, this study evaluated the maximum and minimum zone diameters for each cleaner and suggests that all except cleaner 2 (which is not recognised as having any antibacterial activity)

Table 3. Values of the mean, standard deviation and variance of the zone of inhibition for each ear cleaner (1 to 8)

Ear cleaner	1	2	3	4	5	6	7	8
Mean	20.54	10.00	22.14	10.74	10.82	23.64	16.78	14.96
Standard deviation	3.06	0	3.75	2.43	2.29	2.75	2.72	4.19
Variance (Standard deviation)	9.36	0	14.04	5.91	5.25	7.54	7.40	17.59

were capable of diffusing at least 20 mm into the plate. Unlike studies that involve shampoo therapy, where the active antibacterial components can be clearly identified, there is considerable debate as to which components of an ear cleaner provide its antibacterial properties.

The pH of a product is thought to be an important factor in determining the antibacterial properties of it. The three cleaners that displayed the poorest activity had a pH value, as measured in the laboratory, of between 5.9 and 6.4. However, the two products with the lowest pH values showed variable antipseudomonal activity. Cleaner 1, with a pH of 2.5, showed consistently excellent activity, but cleaner 7, with a similar pH (of 2.8), had very variable activity, suggesting that, in vitro, Pseudomonas is not affected by pH.

Isopropyl alcohol (IPA) is known to have excellent antibacterial properties (Larson and Morton 1991). In previous studies (Swinney and others 2008), it has been proposed that IPA is one of the components of ear cleaners that provides good antibacterial properties. These results, in part, contradict that premise, as it would appear that Pseudomonas is not consistently sensitive to IPA-based based products, as is demonstrated by the poor activity of ear cleaner 4. Monosaccharides in ear cleaners are said to work by preventing bacterial adherence (Reme and others 2006). The only ear cleaner that contained monosaccharides was cleaner 8, which showed variable activity against Pseudomonas. However, it may be that the true benefits of monosaccharides as antiadhesive agents are assessed poorly by the in vitro methodology employed here. *para*-Chloro-meta-xylenol (PCMX) is a broad spectrum phenolic germicide that has bactericidal properties. Unfortunately, however, Pseudomonas organisms are reported to be resistant to it (Denyer and Stewart 1998). While the mechanism of action of PCMX is not known, its phenolic nature may exhibit an effect on bacterial membranes (Denyer and Stewart 1998). In this study, ear cleaner 8, which contains PCMX, showed inconsistent results against Pseudomonas, suggesting that it may have a poor mode of action against Pseudomonas.

Numerous authors have described the antipseudomonal activity of acetic acid (Griffin 1993, Rosychuk 1994, van Balen and others 2003, Thorp and others 1998). Its actual mode of action is unknown but is thought to be linked to a unique property of the acid that is not pH dependent. Acetic acid is also reported to be safe as a middle ear flush, making it a good choice of therapy when the ear drum is ruptured (Rosychuk 1994). The antipseudomonal effect of ear cleaner 3 may therefore be due to its 2% acetic acid content. Four of the ear cleaners (1, 5, 7 and 8) contain salicylic acid (SA). The SA is recognised as having an excellent cleaning activity because of its keratolytic properties (Waller and others 2006), which is why it is generally included in ear cleaners. It is not recognised as having antibacterial properties, suggesting that this component of ear cleaners 1, 5, 7 and 8 was not responsible for its antipseudomonal activity. If SA did have a significant direct action against Pseudomonas, it would have been expected that cleaners 5, 7 and 8 would have performed better.

The lack of efficacy of SA and the inconsistent effect of pH would also suggest that the activity of cleaner 1 was possibly due to its lactic acid component. Lactic acid has a dual mode of action when included in topical therapy; it will reduce the pH of the product and is also known to be effective in disrupting the outer cell membrane of gram-negative bacteria (Alakomi 2000). It may be this second property that rendered cleaner 1 so effective, rather than its low pH. Owing to the unknown ototoxic potential of lactic acid, its use in cases where the ear drum is ruptured has not been described.

Chlorhexidine at high concentrations is recognised at being ototoxic and can affect both the vestibular and cochlear systems (Harvey and others 2005). While concentrations of 0.05% chlorhexidine can display broad spectrum antibacterial properties (Harvey and others 2005) and have low levels of ototoxicity, Pseudomonas may be resistant to chlorhexidine at this concentration or lower (Little 1996). A study by Merchant and others (1993) demonstrated that a 0.2% chlorhexidine solution produced no adverse effects when instilled into the middle ear of dogs by myringotomy, suggesting that concentrations of 0.2% or less chlorhexidine is a safe choice as a flush in otitis even when the ear drum is damaged. Ear cleaner 6 contains 0.15% chlorhexidine and showed excellent activity against Pseudomonas, suggesting that chlorhexidine at this concentration is an excellent safe choice as a flush in otitis.

Tris EDTA is a component of ear cleaners 5, 6 and [illegible]. The poor activity of ear cleaner 5 suggests that Tris EDTA in itself may have limited efficacy against Pseudomonas, which is contrary to findings published by some authors (Stinnet and others 1973, Kirkland 1983, Foster and DeBoer 1998, Nuttall and Cole 2004, Nuttall and Cole 2007). However, Tris EDTA has also been demonstrated by Wooley and others (1984) and Farca and others (1991) to have synergistic effects with many antibiotics, in particular, aminoglycosides and fluoroquinolones, and other work has shown it to have synergy with chlorhexidine (Guardabassi and others 2010). The fact that ear cleaner 6 had excellent activity against Pseudomonas would support this previous work.

In addition, Tris EDTA has been proposed as a safe flush in cases of otitis media (Foster and DeBoer 1998, Nuttall and Cole 2004, Nuttall and Cole 2007), which suggests that cleaner 6 would also be a suitable product to use when the ear drum cannot be visualised. This study highlights a number of factors. It suggests that, in future, for the results of studies assessing topical therapy to be meaningful, they should be undertaken using large numbers of isolates. It appears that Pseudomonas is not a pH-dependent bacterium, in that it does not consistently show sensitivity to a low pH. Some of the components of ear cleaners, notably isopropyl alcohol, PCMX, monosaccharides, SA and EDTA, have inconsistent activity against Pseudomonas. Cleaners containing acetic acid, lactic acid and chlorhexidine at a concentration of 0.15%, in combination with EDTA Tris, consistently manifested excellent activity against Pseudomonas.

Clearly, this work is based on in vitro methodology and does not take into account the activity of any of these products within the ear itself. It may be that the effect of pH and the action of many of the components, including monosaccharides, may be different in vivo, and there is a real need to extend such work to assess the activity of topical products on clinical cases in the in vivo setting.

Conflict of interest

Mrs Susan Paterson is a Veterinary Consultant for Dechra Animal Health and Veterinary Dermatologicals.

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