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An update on microbiological causes of canine otitis externa in Campania Region, Italy

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ABSTRACT

Objective: To update the recent knowledge of the microbiological causes of canine otitis externa in Campania Region (Italy) and the antibiotic susceptibility patterns of the isolated strains.**Methods:** A total of 122 dogs were examined by otoscopy, and auricular swab samples were collected from both ears in 74 dogs presenting clinical bilateral otitis and from single ears in 48 dogs displaying clinical unilateral otitis. Cytological examination, bacteriological analysis and antimicrobial susceptibility tests were performed.**Results:** Thirty-one out of 122 dogs were positive for yeast species (25.4%, 95% confidence interval (CI): 18.2%–34.2%) with a higher prevalence of *Malassezia pachydermatis* (21/31 isolates, 67.7%, CI: 48.5%–82.7%), and a total of 91 out of 122 dogs were positive for bacterial species (74.6%; CI: 65.8%–81.8%) with a higher prevalence of *Staphylococcus pseudintermedius* (45/143 isolates, 31.5%, CI: 24.1%–39.8%). These results are the first description of *Streptococcus agalactiae*-associated otitis. The yeasts isolated showed high levels of susceptibility to all antifungal agents tested; on the contrary all the isolated bacterial strains were highly resistant to at least four out of ten antimicrobial classes. Both Gram-positive and Gram-negative bacteria showed high resistance to amoxicillin/clavulanate and kanamycin hence they are not recommended as initial empirical therapy for the otitis treatment.**Conclusions:** This update illustrates an increase in antibiotic resistances providing an insight into the current knowledge of the therapeutic procedures followed on canine otitis externa in Italy. It also emphasizes the importance of considering the results of the microbiological and sensitivity tests to decide on an appropriate antibiotic therapy.

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All experimental procedures involving animals were conducted in accordance to guide for use and care of animals that visit our clinic and approved by The Local Ethical Committee of The Faculty of Veterinary Medicine, University of Naples "Federico II" (authorisation reference number Prot. 2011/0123330).

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1. Introduction

Otitis externa is the most common ear disease of dogs, being up to 20% of the dog population affected by this disease. It has a multifactorial etiology but it is predominantly a microbial infection. Clinical signs, such as exudates and frequently erythema, oedema, offensive odour and pruritus are seen. Microorganisms belonging to the normal microbial flora of the auricular area can become pathogenic when environmental and/or primary conditions determine faster expression of their virulence factors.

Malassezia genus are commonly isolated in auricular canal of dogs and cats, having otitis externa or in cases of dermatitis disorders. The yeasts of the *Malassezia* genus are

opportunistic microorganisms and can cause both human [1,2] and animal infections [3]. Particularly *Malassezia pachydermatis* (*M. pachydermatis*) has been reported as the predominant causative agent of canine otitis externa [4].

Furthermore, among bacterial agents, it is known that *Staphylococcus*, *Pseudomonas*, *Escherichia*, and *Proteus* species are considered important pathogens causing otitis externa in dogs [5]. Among Gram-positive bacteria, *Staphylococcus pseudintermedius*, (*S. pseudintermedius*) a coagulase-positive staphylococcal species, is frequently associated with pyoderma, otitis externa, urinary tract infections, and opportunistically infected sites in dogs [6]. Among Gram-negative bacteria, *Pseudomonas aeruginosa* (*P. aeruginosa*) plays a major role in otitis externa also because of the increasing number of multiresistant strains [7].

Regular treatment at home with disinfecting ear washes should become part of the pet's grooming-routine with a correct antimicrobial therapy. It has been described a high sensitivity to beta-lactams and aminoglycoside-aminocyclitols for Gram-positive bacteria, while for Gram-negative bacteria has been suggested the use of aminoglycoside-aminocyclitols, polymyxin B and enrofloxacin [8].

In recent decades it was reported that the high frequency of multidrug-resistant of the isolated bacteria could become a high risk factor for owners and veterinary professionals. Therefore, a rational policy of antibiotic prescription in order to prevent the selection of resistant strains is needed.

The purpose of the present study is to evaluate the presence of microorganisms involved in otitis externa in dogs from Campania Region, in southern Italy, in order to update data and to investigate the presence of multi-resistant bacterial strains [9].

2. Materials and methods

2.1. Samples

One hundred and twenty-two dogs with clinical signs of otitis externa, such as local pain, pruritus, erythema, ear discharge and desquamation of the epithelium, in at least one ear, were selected to collect auricular swabs, during a period of two years, and a total of 196 culture swabs were obtained. Precisely, samples were collected from both ears in 74 dogs with clinical bilateral otitis and unilaterally from 48 dogs with clinical unilateral otitis. Sterile cotton-tipped applicator was used to collect samples of ear exudates by inserting swabs into ear canal, rotating once through 360° and then rolling it out, and immediately transferred to Amies transport medium (Oxoid Ltd, UK) and maintained at 4 °C (not longer than 24 h) until processing. The samples were presented for screening at the Microbiology Laboratory of the Department of Veterinary Medicine and Animal Production, University of Naples Federico II (Italy). The canine population studied aged from 2 months to 16 years and included 75 males and 47 females.

Dogs were excluded from the trial if they had previously received (i) topical treatment (within 10 days), (ii) systemic treatment with an antibiotic, antifungal or nonsteroidal anti-inflammatory (within 10 days), (iii) a steroidal anti-inflammatory treatment (within 14 days), or (iv) a long acting steroidal anti-inflammatory drug systemically (within 60 days). Informed consent was obtained from the owners of all dogs prior to their participation in the study. All experimental procedures were approved by The Local Ethical Committee of The Faculty of Veterinary Medicine, University of Naples Federico II (authorisation reference number Prot. 2011/0123330).

2.2. Cytological analysis

For cytological analysis cotton-tipped swabs of ear canal exudates were streaked onto glass slides, which were then heat fixed and stained with a modified Wright's stain (Diff Quik, Dade Behring, Deerfield, IL). All stained slides were examined under a Nikon Eclipse E600 Microscopy (Nikon Instruments Inc., Melville, NY) at 40× magnification. At least 10 fields were examined, and a number of yeast cells per field (> 5 yeast/field) represented excessive colonization by the organism and were considered positive (infection). A number of bacteria 25 per microscopy field (40×) were considered positive (infection) as previously described by Ginel *et al.* [10].

2.3. Microbiological analysis

Collected samples were plated on blood agar base supplemented with 5% sheep blood, selective medium used for the isolation of Gram-positive microorganisms, on mannitol-salt agar, selective medium to identify staphylococci, and on MacConkey agar, selective and differential medium to grow Gram-negative bacteria, which were incubated aerobically at 37 °C for 24–48 h. Sabouraud's dextrose agar with chloramphenicol was used to grow fungal flora and was kept at 30 °C for 7 days, then stained with Gram stain and examined microscopically. The plates were all microbiological media from Oxoid Ltd, UK. In the case of yeasts of the genus *Candida*, we performed subculture on Oxoid chromogenic candida agar media to differentiate species of *Candida*. Further confirmation of genus and species of yeasts were obtained by biochemical identification using Remel RapID™ yeast plus identification panel after pure culture growth on Emmons agar (Oxoid).

Bacteria were identified by macroscopic observation of the colonies, Gram staining, standard laboratory methodologies (catalase, staphylocoagulase tube test, aesculin), and miniaturized biochemical tests API system (bioMérieux SA, Marcy L'Etoile, France). The species identification by miniaturized biochemical tests was accepted when probability was > 88%. β-haemolytic cocci strains were identified as group B [*Streptococcus agalactiae* (*S. agalactiae*)] or group C *Streptococcus dysgalactiae* (*S. dysgalactiae*), using a streptococcus grouping kit (Streptex, Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The presence of the *mecA* gene was detected by growth on oxacillin-containing media (2 mg/L), agar diffusion with oxacillin disks (5 µg) and positive latex agglutination test (PBP2' Test. Oxoid Ltd, UK).

2.4. Antimicrobial susceptibility testing

The antimicrobial susceptibility patterns of isolated bacterial strains were determined by disk diffusion test using Mueller–Hinton agar (Oxoid Ltd, UK). The inhibitory zone diameters obtained around the antibiotic disks were measured after incubation for 24 h at 37 °C and evaluated according to the Clinical and Laboratory Standards Institute [11]. An oxacillin (methicillin) susceptibility test of all isolates was performed by oxacillin disk diffusion and if assayed, the minimal inhibitory concentration was determined by standard methods.

Ten drug classes were included in this study, using commercial disks containing the drugs (Oxoid Ltd, UK). Penicillins were represented by benzylpenicillin (penicillin G) (10 IU),

ampicillin (10 µg), amoxicillin-clavulanic acid combination (20 + 10 µg), and oxacillin (1 µg). Furthermore, cephalosporins (first generation) were represented by cefadroxil (30 µg), cephalosporins (third generation) were represented by ceftriaxone (30 µg), fluoroquinolones by enrofloxacin (5 µg) and norfloxacin (10 µg), aminoglycosides by gentamicin (10 µg), kanamycin (30 µg), neomycin (30 µg) and streptomycin (10 µg), glycopeptides by vancomycin (30 µg), macrolides by erythromycin (15 µg), and polypeptides by bacitracin (0.05 µg) and colistin sulfate (10 µg). In addition, tetracyclines were assayed as doxycycline (30 µg) and tetracycline (10 µg) and lincosamides as lincomycin (2 µg). Finally, we assayed also a sulfonamide (sulfamethoxazole/trimethoprim, 50 µg). The choice of drugs depended by strains and clinical use. The strains were categorized as sensitive or resistant to the drug, intermediate susceptibility was regarded as resistant, and only the resistances are reported in the results.

Antifungal susceptibility was performed by disc diffusion method using antimycotic sensitivity test agar. Discs used were amphotericin B (100 IU), fluconazole (10 µg), clotrimazole (10 µg), itraconazole (10 µg), miconazole (10 µg) was measured as for the instruction manual (HiMedia). ATCC strain of *Candida albicans* was used as control.

2.5. Statistical analysis

Statistical analysis of bacteriological examination results was performed by One-way ANOVA with Bonferroni post-test using GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software Inc., La Jolla, CA). *P* value of less than 0.05 was regarded as statistically significant.

3. Results

The screening in 196 samples performed by cytological examination showed a prevalence significantly higher ($P < 0.001$) of bacterial compared to fungal otitis externa, both in monolateral (37/11) and bilateral infection (53/21). Furthermore, the cases of bilateral otitis (74/122) was significantly higher ($P < 0.001$) respect to monolateral otitis (48/122) regardless of the microorganism that causes disease.

Yeasts were detected in a total of 53 swabs samples with *M. pachydermatis* presenting the highest prevalence (67.74%) followed by *Candida parapsilosis* (12.90%), as shown in Table 1. These diseased dogs after local therapy with miconazole and prednisolone acetate, for at least 2 weeks, showed a remission of symptoms.

Table 1

Number and percentage of isolated yeasts from dogs with otitis externa.

Yeasts	No. of isolates	Percent (%)	95% CI
<i>M. pachydermatis</i>	21	67.74	48.54–82.68
<i>Candida parapsilosis</i>	4	12.90	4.22–30.76
<i>Candida tropicalis</i>	2	6.45	1.13–22.84
<i>Candida glabrata</i>	1	3.23	0.17–18.51
<i>Candida albicans</i>	1	3.23	0.17–18.51
<i>Aspergillus niger</i>	1	3.23	0.17–18.51
<i>Exophiala</i> spp	1	3.23	0.17–18.51
Total	31		

CI: Confidence interval.

Table 2 shows the number and percentage of isolated bacteria. The most frequently isolated strain was *S. pseudintermedius*

(31.5%; 95% CI: 24.1%–39.8%) followed by *P. aeruginosa* (16.1%; 95% CI: 10.7%–23.4%), *Proteus mirabilis* (*P. mirabilis*) (6.3%; 95% CI: 3.1%–12.0%), and *Staphylococcus aureus* (*S. aureus*) (4.9%; 95% CI: 2.2%–10.2%). Moreover, a total of four out of forty-five *S. pseudintermedius* (8.9%; 95% CI: 2.9%–22.1%) were positive for the *mecA* gene as confirmed by oxacillin agar screening test, oxacillin diffusion test and latex agglutination test. A total of 10 bacteria belonging to Streptococcaceae group

Table 2

Number and percentage of isolated bacteria from dogs with otitis externa.

	Bacteria	No. of isolates	Percent (%)	95% CI	
Staphylococcaceae	<i>S. pseudintermedius</i>	45	31.5	24.1–39.8	
	<i>S. aureus</i>	7	4.9	2.2–10.2	
	<i>S. xylosus</i>	7	4.9	2.2–10.2	
	<i>Staphylococcus chromogenes</i>	4	2.8	0.9–7.4	
	<i>S. hyicus</i>	3	2.1	0.5–6.5	
	<i>Staphylococcus capitis</i>	2	1.4	0.2–5.5	
	<i>Staphylococcus epidermidis</i>	1	0.7	0.0–4.4	
	<i>Gemella morbillorum</i>	2	1.4	0.2–5.5	
	Pseudomonadaceae	<i>P. aeruginosa</i>	23	16.1	10.7–23.4
		<i>Pseudomonas putrida</i>	4	2.8	0.9–7.4
<i>Pseudomonas luteola</i>		2	1.4	0.2–5.5	
<i>Pseudomonas fluorescens</i>		1	0.7	0.0–4.4	
Enterobacteriaceae		<i>P. mirabilis</i>	9	6.3	3.1–12.0
	<i>Escherichia coli</i>	6	4.2	1.7–9.3	
	<i>Enterobacter cloacae</i>	1	0.7	0.0–4.4	
	<i>Citrobacter braakii</i>	1	0.7	0.0–4.4	
	<i>Klebsiella pneumoniae</i>	1	0.7	0.0–4.4	
	Streptococcaceae	<i>S. mitis</i>	4	2.8	0.9–7.4
		<i>Streptococcus canis</i>	1	0.7	0.0–4.4
		<i>S. agalactiae</i>	1	0.7	0.0–4.4
		<i>S. dysgalactiae</i>	1	0.7	0.0–4.4
		<i>Streptococcus sanguinis</i>	1	0.7	0.0–4.4
<i>Streptococcus equinus</i>		1	0.7	0.0–4.4	
<i>Streptococcus simulans</i>		1	0.7	0.0–4.4	
Enterococcaceae		<i>Enterococcus faecalis</i>	4	2.8	0.9–7.4
	<i>Enterococcus avium</i>	1	0.7	0.0–4.4	
	<i>Enterococcus durans</i>	1	0.7	0.0–4.4	
	<i>Enterococcus faecium</i>	1	0.7	0.0–4.4	
	Other	<i>Micrococcus</i> spp.	1	0.7	0.0–4.4
		<i>Kocuria varians</i>	3	2.1	0.5–6.5
<i>Aerococcus viridans</i>		1	0.7	0.0–4.4	
<i>Pasteurella pneumotropica</i>		1	0.7	0.0–4.4	
Total		143			

S. mitis: *Streptococcus mitis*; *S. xylosus*: *Staphylococcus xylosus*; *S. hyicus*: *Staphylococcus hyicus*.

were identified and an interesting case of *S. agalactiae*-associated otitis was observed.

Table 3 shows the results of the antimicrobial susceptibility tests of the most frequently isolated strains from dogs with otitis externa. However, *S. pseudintermedius*, *S. aureus* and *S. xylosus*, which represented 85.5% of staphylococcal strains, were variously resistant to all antimicrobial classes. Furthermore, 4/45 (8.9%; 95% CI: 2.9%–22.1%) *S. pseudintermedius*, 6/7 (85.7%; 95% CI: 42.0%–99.2%) *S. xylosus*, 3/4 (75.0%; 95% CI: 21.9%–98.7%) *Staphylococcus chromogenes*, and 3/3 (100%; 95% CI: 31.0%–96.8%) *S. hyicus* strains isolates were methicillin/oxacillin and penicillin resistant using disk diffusion tests and could be cultivated on oxacillin resistant screening agar base (data not shown). Among thirty *Pseudomonas* spp strains, the most frequently isolated *P. aeruginosa* (23 strains) presented evident multidrug resistant profiles, with over 73% resistance to penicillins (ampicillin and amoxicillin-clavulanate), over 65% to aminoglycosides (kanamycin, neomycin, streptomycin) and tetracyclines (doxycycline and tetracycline), and over 43% resistance to enrofloxacin, 100% to bacitracin and over 69% to lincomycin. Two isolates of *Pseudomonas luteola* and one of *Pseudomonas fluorescens* showed a substantial multi-resistance (data not shown). It is noteworthy that multidrug resistant *P. mirabilis* strains were observed.

The most of streptococcal strains (*S. mitis*, *S. agalactiae* and *Streptococcus canis*) were also multi-resistant. In addition, surprisingly, the only *S. agalactiae* strain, generally considered uniformly susceptible to penicillins, was not susceptible to penicillin G with a minimal inhibitory concentration of 128 µg/L, and showed simultaneous resistance to ceftriaxone, erythromycin, tetracycline, lincomycin, sulfamethoxazole/trimethoprim, other than others β-lactams tested. *S. dysgalactiae* strain isolated was resistant to erythromycin and tetracycline (data not shown).

4. Discussion

Ear disease is one of the most common conditions present in pets, and the infections can be caused by both bacterial and fungal origin. *Malassezia* spp are normal commensals and occasional pathogens of the skin for many veterinary species, present in up to 40% of canine otitis cases [12]. Herein, we reported a prevalence of yeast species of 25.4% with a higher prevalence of *M. pachydermatis* (67.74%).

S. pseudintermedius represents the majority of all coccal microorganism isolated, whereas Gram-negative bacteria as *P. aeruginosa*, *P. mirabilis* and others, are also included among the most common isolates that may cause canine otitis externa [5,12]. In the present study, in agreement with the above mentioned findings, the most commonly identified bacteria were *S. pseudintermedius*, *P. aeruginosa* and *P. mirabilis*. Furthermore, most *S. pseudintermedius*, *S. xylosus*, and *S. hyicus* strains isolates were methicillin/oxacillin and penicillin resistant. We reported no isolation of *Staphylococcus schleiferi* which is considered as a potential pathogen in dogs and is often oxacillin resistant [13].

The members of the *Staphylococcus* genus have a high frequency of conjugation and frequently acquire plasmids that encode antimicrobial resistance, and the production of the enzyme β-lactamase is the major mechanism by which staphylococci acquire resistance [14]. The reservoir of *mecA* gene is likely larger in coagulase-negative-staphylococci than in *S. aureus* [15], as more times reported, both in human clinical isolates [16] and in various animal species [17,18].

The literature reports a higher interest for coagulase-positive staphylococci, but also the coagulase-negative staphylococci, which have long been considered as nonpathogenic, recently have assumed an important role as pathogens because of their increasing incidence as cause of bacteremia. Interestingly, among

Table 3

Antimicrobial susceptibility of the most frequently isolated strains from dogs with otitis externa.

Drug	Percentage of resistance				
	<i>S. pseudintermedius</i> (n = 45)	<i>P. aeruginosa</i> (n = 23)	<i>P. mirabilis</i> (n = 9)	<i>S. mitis</i> (n = 4)	<i>Enterococcus faecalis</i> (n = 4)
Penicillin G	57.7	NA	NA	0.0	50.0
Oxacillin	8.9	NA	NA	0.0	25.0
Ampicillin	NA	73.9	33.3	NA	75.0
Amoxicillin-clavulanic acid	35.5	78.3	33.3	0.0	50.0
Ceftriaxone	11.1	39.1	0.0	25.0	NA
Norfloxacin	11.1	0.0	0.0	25.0	50.0
Enrofloxacin	15.5	43.5	0.0	25.0	50.0
Gentamicin	11.1	8.7	11.1	25.0	75.0
Kanamycin	62.2	87.0	22.2	50.0	50.0
Neomycin	31.1	65.2	33.3	25.0	50.0
Streptomycin	46.6	78.3	55.5	25.0	75.0
Vancomycin	11.1	52.2	44.4	0.0	25.0
Erythromycin	31.1	NA	NA	25.0	NA
Bacitracin	68.8	100.0	66.6	75.0	50.0
Doxycycline	15.5	78.3	55.5	25.0	50.0
Tetracycline	35.5	69.6	77.7	50.0	75.0
Lincomycin	20.0	69.6	66.6	50.0	75.0
Sulfamethoxazole/ trimethoprim	46.6	17.4	55.5	25.0	NA

NA: Not assayed.

our isolates we identified only one strain of methicillin/oxacillin-resistant *Staphylococcus epidermidis* resistant to 6/17 antimicrobial drugs, which represents serious evidence of emergence of multidrug-resistant strains in dog infections. A substantial multi-resistance was also found in others Gram-positive bacteria isolates, as streptococci and enterococci. The only *S. agalactiae* strain showed a reduced susceptibility to penicillin G. There is little information on the nature of mechanism of resistance. The most likely hypothesis is the modification of amino acid sequence of penicillin-binding protein [19]. However, *S. agalactiae* (group B streptococci) is an emerging pathogen in immunocompromised adults and a major cause of neonatal pneumonia, sepsis, and meningitis; it has been isolated in bovine mammary gland infections, and in companion animals. Its surface-localized penicillin-binding protein 1a, encoded by *ponA*, is known to be an important virulence factor in infection [20]. Traditionally, β -lactams are the first-line agents against *S. agalactiae* and resistance is rarely reported [21,22]. Our case demonstrated that the resistances, overall to β -lactams and cephalosporins, of this strain request further studies.

The rods involved in canine otitis externa are frequently *P. aeruginosa*, although *P. mirabilis*, or other Gram-negative bacteria such as *Escherichia coli*, *Corynebacterium* species or, infrequently, *Klebsiella* and *Enterobacter* spp., may occur. Our results indicated that in our region, the predominant Gram-negative bacteria were *P. aeruginosa* and *P. mirabilis* displaying a high rate of resistance to each of the antimicrobials tested. The most frequent antimicrobial classes used in small animals in veterinary medicine include penicillins, cephalosporins, macrolides, lincosamides, fusidic acid, tetracyclines, chloramphenicol, potentiated sulphonamides, aminoglycosides and fluoroquinolones. Aminoglycosides, such as gentamicin and neomycin, are routinely used for topical therapy in canine otitis mainly caused by *P. aeruginosa* [5,7,12,23], but our *Pseudomonas* spp. isolates presented multidrug resistant profiles with very high resistance to aminoglycosides, other than penicillins and tetracyclines. Our results combined with data from the literature support the premise that antimicrobials should be selected basically on bacterial culture and antimicrobial susceptibility test. Until recently, multi-resistance was overall seen in Gram-positive bacteria [24,25]. However, the recent rapid international spread of multi-resistant Gram-negative bacteria [26], is much more ominous, and the optimal management of serious infections with these bacteria remains to be determined. A recent study has reported that administration of a topical bacteriophage mixture leads to lysis of *P. aeruginosa* in the ear without apparent toxicity and that might potentially become a convenient and effective treatment for *P. aeruginosa*-associated otitis in dogs [27].

Consistent with previous reports, this study is a further confirmation that coagulase-positive species of staphylococcal strains are the most causative microorganisms of otitis externa in dogs. In particular, both *S. pseudintermedius* and methicillin/oxacillin-resistant *S. pseudintermedius* strains represent a group of emerging pathogens to be monitored as opportunistic animal pathogens with zoonotic capabilities. In addition, to our knowledge, this is the first report of canine otitis correlated to *S. agalactiae*, a bacteria isolated mainly from humans and bovine sources. Surprisingly, this isolate presented a simultaneous resistance to more different classes of antibiotics other than β -lactams. Thus, our data emphasize the importance: (i) to study profiles and genetic characteristics of GBS colonizing the pet animals, (ii) to investigate the possible transmission from

human to dog, and (iii) to focus on the prevention and treatment of GBS infections.

Conflict of interest statement

We declare that we have no conflict of interest.

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