

ORIGINAL ARTICLE

First identification of methicillin-resistant *Staphylococcus pseudintermedius* strains among coagulase-positive staphylococci isolated from dogs with otitis externa in Trinidad, West Indies

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Background: Otitis externa is a common inflammatory ear disease in dogs caused by a variety of pathogens, and coagulase-positive staphylococci are frequently isolated from such infections.

Objective: To identify antimicrobial susceptibility profiles and methicillin-resistant strains among coagulase-positive staphylococci isolated from otitis externa in dogs.

Methods: A cross-sectional study was performed over 2 years on 114 client-owned dogs presented to the Veterinary Teaching Hospital with a primary complaint of ear infections. Swabs were obtained from both ears and cultured for staphylococci which were subsequently confirmed as coagulase-positive using rabbit plasma. Antimicrobial susceptibility assays were assessed on all isolates followed by subsequent genetic analysis for species identification and detection of the *mecA* gene.

Results: Sixty-five coagulase-positive staphylococci were isolated from 114 client-owned dogs. The isolates exhibited resistance against neomycin (58.5%), streptomycin (49.2%), penicillin (49.2%), polymyxin B (44.6%), tetracycline (36.9%), sulphamethoxazole/trimethoprim (33.8%), kanamycin (33.8%), doxycycline (32.3%), norfloxacin (23.1%), amoxicillin/clavulanate (20%), ciprofloxacin (20%), enrofloxacin (18.5%), gentamicin (16.9%), and cephalothin (9.2%). Forty (61.5%) of the isolates were resistant to at least three or more antimicrobials and 10 were sensitive to all. Using a multiplex polymerase chain reaction assay based on species-specific regions of the thermonuclease (*nuc*) gene, 38/65 (58.5%) isolates were classified as *Staphylococcus aureus*, 23/65 (35.4%) as *S. pseudintermedius*, 2/65 (3.1%) as *S. intermedius*, and 2/65 (3.1%) as *S. schleiferi*. Analysis for the *mecA* gene revealed two positive isolates of *S. pseudintermedius* which were oxacillin-resistant, representing a first report of such organisms in the Caribbean.

Conclusion: Despite the relatively high prevalence of multidrug-resistant coagulase-positive staphylococci in Trinidad, these are largely susceptible to gentamicin consistent with use in clinical practice. The first detection of methicillin-resistant *S. pseudintermedius* (MRSP) in dogs is likely to have implications on the treatment options for otitis externa in dogs and potential public health significance.

Keywords: *staphylococci*; *antimicrobials*; *otitis externa*; *mecA*; PCR

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Otitis externa is frequently encountered in small animal practice, where it is associated with a wide variety of aetiologies. The leading antecedents to

this inflammatory ear disease are excessive moisture, ear canal maceration (trauma), and anatomical conformation of the ears (1, 2). Recent evidence indicates that the

shar-pei, German shepherd, and cocker spaniel breeds are more prone to otitis externa than other breeds (2, 3). And for undefined reasons, the severity of the infection appears greater in older than in younger dogs (2, 3). Parasites, foreign bodies, hypersensitivity, and allergic and autoimmune diseases, singly or in combination, are the primary cause(s) of otitis externa in dogs, and the incidence of the secondary pathogen may be related to the initiating factor (3). Although each of these primary aetiologies can induce inflammation of the external auditory meatus of dogs, the situation is worsened by secondary infections caused by bacteria with or without yeasts (4–7). Thus, bacteria and yeasts are recognised perpetuating agents of otitis externa. Common clinical signs are head shaking and scratching of ears (acute cases), and abnormal and/or malodorous discharge may be seen after headshaking (1).

Of the secondary invaders, bacteria are by far commonly isolated from cases of otitis externa, and different genera and species have been reported (4–7). *Staphylococcus* species (coagulase-positive and -negative) are frequently isolated, either singly or in combination with β -haemolytic streptococci, *Pseudomonas* spp., *Proteus* spp., and other bacterial pathogens or yeasts, from cases of otitis externa (6, 7). Eliminating these infectious microorganisms from the ear canal is a key therapeutic target to resolve the clinical signs. The use of antimicrobials in veterinary medicine is widely believed to accelerate the evolution of multidrug-resistant bacterial strains, and this has become a major global concern. Here, we assess the antimicrobial susceptibilities of coagulase-positive staphylococci isolated from client-owned dogs presented at a Veterinary Teaching Hospital in Trinidad. We employ a species-specific multiplex polymerase chain reaction (PCR) for species identification of coagulase-positive staphylococci. Because methicillin-resistant *Staphylococcus aureus* (MRSA) is highly prevalent in the human population in Trinidad (8, 9), and an animal-to-human transmission and *vice versa* is documented (10, 11), we further examine if any of the staphylococci carried the *mecA* and *femA* genes that are associated with MRSA (12).

Materials and methods

Sample collection

In total, 114 client-owned dogs, of mixed breeds, were presented to the Veterinary Teaching Hospital, The University of the West Indies (Trinidad), over a 2-year period with primary presenting complaints of head shaking, ear scratching, and abnormal or malodorous ear discharges. The Veterinary Teaching Hospital in Trinidad serves as a primary and referral centre for medical and surgical cases. Following routine clinical examination, the animals were aseptically swabbed in both ear canals prior to confirmation of otitis externa by otoscopic examination. Samples were transported to the bacteriology laboratory

at ambient temperature and processed within 1 h of collection.

Isolation and presumptive identification of pathogens

Swabs from both ears were inoculated in parallel on Columbia-Agar supplemented with 5% sheep blood agar (Oxoid, Basingstoke, UK), streaked for single colonies and incubated at 37°C for 18–24 h in 5% carbon dioxide. Streaked agar plates were examined for growth and, when necessary, a further incubation for 24–72 h was performed. Specimens from animals with prior or current antibiotic treatment (as indicated on the submission form) were pre-incubated in brain heart infusion broth at 37°C for 18–24 h before streaking on sheep blood agar.

Presumptive identification of staphylococci was performed to a genus level using standard techniques: colonial characteristics, cellular morphology (after Gram's staining technique), and minimal biochemical tests as recommended (13). Briefly, suspected staphylococci were tested for catalase production and positive isolates were subjected to slide and tube coagulase tests using rabbit plasma per standard protocol. Where both ears of the same dog yielded presumptive staphylococci, only one coagulase-positive isolate was preserved at –20°C in 15% (v/v) glycerol for further analysis.

Antimicrobial susceptibility testing of coagulase-positive staphylococci

Antimicrobial susceptibilities of coagulase-positive staphylococci to 14 commonly used drugs were determined by the Kirby–Bauer disc diffusion test on Mueller–Hinton agar (Oxoid) and interpreted as recommended (14). *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 29213 were routinely used as controls. All antimicrobial discs were purchased from Oxoid. Briefly, isolates were grown to mid-exponential phase, adjusted to 0.5 MacFarland opacity tube, and overlaid on Mueller–Hinton agar. Excess inoculum was aseptically removed from the agar plate. The following antimicrobial agents were tested: gentamicin (10 μ g), tetracycline (30 μ g), sulphamethoxazole/trimethoprim (23.25 μ g/1.75 μ g), streptomycin (10 μ g), penicillin (10 U), doxycycline (30 μ g), amoxicillin–clavulanic acid (30 μ g), enrofloxacin (5 μ g), neomycin (30 μ g), cephalothin (30 μ g), ciprofloxacin (5 μ g), norfloxacin (5 μ g), kanamycin (30 μ g), and polymyxin B (300 U). Susceptibility to oxacillin (1 μ g) was tested in *mecA*-positive isolates using *S. aureus* ATCC 35419 as a positive control. Oxacillin is a common antimicrobial used for testing for methicillin resistance, which is encoded by the *mecA* gene.

After measuring zones of inhibition, the isolates were classified as susceptible, intermediate, or resistant to antimicrobials tested in accordance with the breakpoints proposed by the Clinical Laboratory Standards Institute (14). Isolates in the intermediate range were reclassified as susceptible or resistant depending on whether the

measurement was closer to the susceptibility or resistance breakpoints as previously done (15, 16).

Genetic analysis of coagulase-positive staphylococci

To determine the distribution of the species among coagulase-positive isolates, a species-specific multiplex PCR based on the thermonuclease (*nuc*) gene was performed essentially as described (17), with a slight modification so that *S. hyicus* was excluded from the panel as it is often associated with exudative epidermitis in pigs. To detect the presence of MRSA, the isolates were screened for the *mecA* and *femA* genes using colony PCR. Primer sequences, annealing temperatures, and the targeted genes are given in Table 1 (18, 19). *S. aureus* ATCC 35419 was used as positive control for the *mecA* and *femA* genes. The reaction mixture for PCR consisted of 12.5 µl of GoTaq[®] PCR Mastermix (Promega, Madison, WI, USA), 10 pmol of each primer (forward and reverse), and bacterial DNA (template) in a total volume of 25 µl. The amplification protocol was as follows: an initial denaturing cycle at 94°C for 5 min followed by 25 cycles at 94°C for 30 s, annealing at respective temperatures (Table 1) for 45 s and 72°C for 90 s, with a final extension step at 72°C for 5 min. Amplicons were resolved and detected on Gelred-stained (Biotium Inc. Hayward, CA, USA) 1% agarose gel in tris-acetate-EDTA buffer. The validity of the representative amplicons was ascertained by DNA sequencing. Amplified products were either submitted for sequencing as crude PCR products or after cloning into a commercial plasmid vector. Resolved amplicons were alternatively purified of agarose gel using a Qiagen gel extraction kit (Qiagen, Hilden, Germany), cloned into TOPO[®] TA vector (Life Technologies, Sao Paulo, Brazil), and then submitted to

Macrogen Korea (Seoul, South Korea) for sequencing. Sequences were confirmed with BLAST analysis at the National Centre of Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST).

Results

Antimicrobial susceptibility patterns of coagulase-positive staphylococci

The susceptibility profiles of the staphylococcal isolates were determined using the disk diffusion test. All coagulase-positive staphylococci isolated from ears of the same dog exhibited a similar antibiotic susceptibility profile and were considered to represent a single isolate. In total, 65 isolates from 65 dogs were subjected to further analysis. To assess the presence of clonal spread among the dogs, all coagulase-positive staphylococci were examined for susceptibility against common antimicrobials routinely used for the treatment of other medical conditions in dogs. The relative proportions of the isolates resistant to the various antimicrobials as determined by the disc diffusion test and interpreted according to the Clinical Laboratory Standards Institute guidelines is shown in Table 2. In decreasing order, resistance was recorded as follows: neomycin (58.5%), streptomycin (49.2%), penicillin (49.2%), polymyxin B (44.6%), tetracycline (36.9%), sulphamethoxazole/trimethoprim (33.8%), kanamycin (33.8%), doxycycline (32.3%), norfloxacin (23.1%), amoxicillin/clavulanate (20%), ciprofloxacin (20%), enrofloxacin (18.5%), gentamicin (16.9%), and cephalothin (9.2%). Within this scenario, there was a high level of multidrug-resistant combinations, with 40/65 (61.5%) of the isolates being resistant to at least three or more antimicrobials.

Table 1. Oligonucleotide primers used for genetic characterisation and species identification of coagulase-positive staphylococci from otitis externa in dogs

Primer name	Target gene	Sequence (5' to 3')	Annealing T (°C)	Species	Reference
mecA-F	<i>mecA</i>	TGGCTATCGTGTCACAATCG	58	<i>S. aureus</i> (MRSA)	(18)
mecA-R		CTGGAACCTGTTGAGCAGAG			
femA-F	<i>femA</i>	CTTACTTACTGGCTGTACCTG	53	<i>S. aureus</i>	(19)
femA-R		ATGTCGCTTGTTATGTGC			
pse-F2	<i>nuc</i>	TAGGCAGTAGGATTCGTTAA	56	<i>S. pseudintermedius</i>	(17)
pse-R5		CTTTTGTGCTTCCTTTTGG			
au-F3	<i>nuc</i>	TCGCTTGCTATGATTGTGG	56	<i>S. aureus</i>	(17)
au-nucR		GCCAATGTTCTACCATAGC			
in-F	<i>nuc</i>	CATGTCATATTATTGCGAATGA	56	<i>S. intermedius</i>	(17)
in-R3		AGGACCATCACCATTGACATATTGAAACC			
sch-F	<i>nuc</i>	AATGGCTACAATGATAATCACTAA	56	<i>S. schleiferi</i>	(17)
sch-R		CATATCTGTCTTTTCGGCGCG			
dea-F	<i>nuc</i>	TGAAGGCATATTGTAGAACAA	56	<i>S. delphini</i> group A	(17)
dea-R		CGRTACTTTTCGTTAGGTCG			
deb-F	<i>nuc</i>	GGAAGRTTTCGTTTTCTAGAC	56	<i>S. delphini</i> group B	(17)
deb-R4		TATGCGATTCAAGAACTGA			

Table 2. The frequency of resistance of coagulase-positive staphylococci isolates from otitis externa to common antimicrobials used for the treatment of medical conditions (including otitis externa) in dogs at the Veterinary Teaching Hospital, Trinidad

Antimicrobial	Number of resistant isolates (expressed as % of total)
Tetracycline (TET)	24/65 (36.9)
Sulphamethoxazole/trimethoprim (TRI)	22/65 (33.8)
Gentamicin (GEN)	11/65 (16.9)
Cephalothin (CEP)	6/65 (9.2)
Streptomycin (STR)	32/65 (49.2)
Enrofloxacin (ENR)	12/65 (18.5)
Amoxicillin/clavulanate (AMC)	13/65 (20.0)
Penicillin (PEN)	32/65 (49.2)
Ciprofloxacin (CIP)	13/65 (20.0)
Neomycin (NEO)	38/65 (58.5)
Doxycycline (DOX)	21/65 (32.3)
Norfloxacin (NOR)	15/65 (23.1)
Polymyxin B (POL)	29/65 (44.6)
Kanamycin (KAN)	22/65 (33.8)

There was a wide variation in the antibiotic resistance profiles, with no clearly discernible pattern across the combinations being observed (Table 3); the presence of one isolate resistant to 11 and another to 12 antimicrobial agents, respectively, was noteworthy.

Genetic analysis of coagulase-positive staphylococci

Using a species-specific multiplex, 38/65 isolates (58.5%) were identified as *S. aureus*, 23/65 (38.4%) as *S. pseudintermedius*, 2/65 (3.1%) as *S. intermedius*, and 2/65 (3.1%) as *S. schleiferi*. To detect methicillin-resistant staphylococci, all isolates were further screened for the *mecA* and *femA* genes, but none carried the *femA* gene. Only two *S. pseudintermedius* isolates were positive for the *mecA* gene. To confirm phenotypic expression of the *mecA* gene, the two isolates were resistant to oxacillin by the disc diffusion method. Further, the *mecA*-positive isolates were also susceptible to fluoroquinolones (enrofloxacin, ciprofloxacin, and norfloxacin), cephalothin, and gentamicin, but resistant to penicillin, tetracycline, doxycycline, neomycin, polymyxin B, kanamycin, and amoxicillin.

Discussion

Antimicrobial resistance has become a global concern, and there is a need to continuously monitor for the evolution of resistant strains. Our studies revealed that most of the coagulase-positive staphylococci were susceptible to cephalothin and gentamicin and by comparison, the majority were resistant to neomycin. Not surprisingly, the susceptibility of staphylococci from dogs to cephalothin and gentamicin has previously been reported as relatively

Table 3. Multidrug-resistant combinations among coagulase-positive staphylococci from dogs with otitis externa

Multidrug-resistant combinations (number of antimicrobials)	Number of isolates
CIP/ENR/NOR* (3)	2
NEO/POL/STR (3)	9
NOR/POL/STR (3)	3
AMC/KAN/POL (3)	3
NEO/PEN/STR/TRI (4)	2
NEO/PEN/POL/STR (4)	4
AMC/POL/TET/TRI (4)	1
KAN/NEO/POL/STR (4)	2
DOX/PEN/STR/TET (4)	3
DOX/NEO/PEN/TET (4)	1
AMC/DOX/PEN/TET (4)	1
DOX/PEN/STR/TET/TRI (5)	1
DOX/GEN/POL/STR/TET (5)	1
DOX/KAN/NEO/PEN/POL/STR (6)	1
AMC/KAN/NEO/PEN/POL/STR/TRI (7)	1
ENR/GEN/NEO/POL/STR/TET/TRI/CEP (8)	1
AMC/CIP/ENR/KAN/NEO/NOR/PEN/STR/TRI (9)	2
CIP/ENR/GEN/KAN/NEO/NOR/PEN/POL/STR/TRI/CEP (11)	1
DOX/CIP/ENR/GEN/KAN/NEO/NOR/PEN/POL/STR/TET/CEP (12)	1
Total	40

*For full names of antimicrobials, refer to Table 2.

high in earlier studies (5, 7, 20, 21). A high degree of resistance to neomycin was seen in this study, although there was no information implicating its routine use in the treatment of otitis externa. Nevertheless, these findings were consistent with a previous report on coagulase-positive staphylococci isolated from canine otitis externa (22). Although the development of antimicrobial resistance can be linked to usage in animals, we were unable to establish such an association with respect to gentamicin. At our Veterinary Teaching Hospital, Otomax[®] Ointment (Merck Animal Health, Milton Keynes, UK), which contains gentamicin (antibacterial), clotrimazole (antifungal), and betamethasone (steroid), is routinely used for the treatment of otitis externa with a very good success rate. Despite this, resistance against multiple antimicrobials was noted (Table 2). The occurrence of multidrug-resistant isolates should be a major concern, because this leaves limited antibiotic treatment options for otitis externa. The variation in the antibiotic resistance patterns found in our study reflects a lack of clonal spread of the isolates among the dogs presented to the teaching hospital, despite the two MRSP isolates showing a similar antibiotic resistance profile. Resistance to multiple antimicrobial agents in *S. aureus* often arises from the acquisition of discrete genetic elements, such as plasmids, transposable insertion

sequences, and genomic islands (reviewed in Ref. 23). Such elements are exchanged via horizontal gene transfer between interrelated bacterial strains, species, and even different genera. Mixed-culture transfer or 'phage-mediated conjugation' also mediates a high-frequency transfer of plasmid DNA in *S. aureus* (reviewed in Ref. 23). The existence of such a wide variety of genetic evolutionary mechanisms in bacterial pathogens clearly warrants the development of an antimicrobial resistance monitoring program to enable an early detection of the emergence of 'superbugs', which would render the use of antimicrobials ineffective.

The possibility that dogs could act as a source for zoonotic staphylococcal infections in humans was suggested many years ago (24). With an increasing prevalence of methicillin-resistant strains associated with animals and their potential to transmit to humans and *vice versa* being reported (11, 12, 25), it was imperative to examine for the presence of such zoonotic strains among our isolates. It is clear that MRSA is prevalent in the human population of Trinidad and Tobago, observed at a prevalence rate of 12.8% between 2000 and 2001 (9), and rising to 20.8% in 2004 (8). Only two *S. pseudintermedius* isolates carried the *mecA*, but not the *femA* gene. The *mecA* gene encodes for a penicillin-binding protein, which mediates resistance to methicillin, and in MRSA, this is often associated with the presence of the *femA* gene (12), which is required for the expression of resistance to methicillin (26). However, the *mecA* gene can also be exclusive to the *femA* gene, particularly in coagulase-negative *S. aureus* (12) and other staphylococci, albeit at low frequency. The PCR techniques adapted in the present study have previously been endowed with 100% sensitivity for both *mecA* and *femA* genes and a specificity of 98% (*mecA*) and 100% (*femA*) (18). It is therefore unlikely that the PCR assay failed to detect *mecA*-positive isolates among the coagulase-positive staphylococci. The relatively low occurrence of MRSP among staphylococci from dogs in this study (2 out of 65 isolates) is consistent with previous reports (27, 28). Furthermore, a recent study in Grenada reported a lack of methicillin resistance in 28 isolates of *S. pseudintermedius* from cases of canine pyoderma (21). Indeed, MRSP has previously been detected at veterinary teaching hospitals (28–30), and evidence indicates that they can cause infections in humans (11, 25). Therefore, screening for such organisms helps to inform on the potential zoonotic threats associated with superficial infections of dogs, such as otitis externa.

In conclusion, antimicrobial susceptibility patterns were determined for coagulase-positive staphylococci from cases of otitis externa and numerous multidrug-resistant combinations were noted. Although MRSA is prevalent in the human population in Trinidad, we were unable to detect any MRSA in dog isolates implying a low risk of human-to-pet transmission. The two *mecA*

coagulase-positive MRSPs represented the first such report in Trinidad and the Caribbean region. And with the transmission of *S. pseudintermedius* between humans and animals now well documented (11, 25), these findings have potential public health implications.

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Conflict of interest and funding

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