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Methicillin-resistant *Staphylococcus schleiferi* subspecies *coagulans* associated with otitis externa and pyoderma in dogs

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Abstract

Background: Dermatological infections are the most common cases in the daily pet clinic. Since its discovery in 1990, *Staphylococcus schleiferi* subspecies *coagulans* have been reported more frequently in canine otitis externa and pyoderma and even in cases of zoonoses.

Aim: Detect the presence of *S. schleiferi* subsp. *coagulans* of canine otitis externa and pyoderma, its antimicrobial resistance, and the presence of *mec*Agen.

Methods: Three-hundred-thirty-one swabs from dogs with otitis externa and pyoderma were cultured on bacteriological agar for bacterial isolation and subsequent biochemical and molecular identification. The identified *S. schleiferi* subsp. *coagulans* were evaluated for their antimicrobial susceptibility using the Kirby–Bauer technique, including an oxacillin disk, and subsequently, a PCR was run to identify which ones had the *mec*A gene.

Results: Thirty-four (22.97%) and twelve (6.56%) isolates were identified as *S. schleiferi* subspecies *coagulans* from otitis externa and pyoderma, respectively. Fluoroquinolones, the most widely used group of antibiotics in Peru, showed a susceptibility of 58.82% (20/34) in cases of otitis externa and 50% (6/12) in cases of canine pyoderma. Meanwhile, nitrofurantoin was the antibiotic with the best efficacy in both cases, with 97% (33/34) in otitis externa and 83% (10/12) in pyoderma. Furthermore, 40% (13/34) of *S. schleiferi* subsp. *coagulans* isolated from otitis externa were resistant to methicillin, and 85.29% (29/34) had the *mec*A gene. On the other hand, the only methicillin-resistant isolate from pyoderma was also the only one with a *mec*A gene.

Conclusion: This study is the first report of *S. schleiferi* subsp. *coagulans* in Peru, finding a higher percentage than reported in other South American countries.

Keywords: *Staphylococcus schleiferi*, CoPS, Methicillin-resistant, Oxacillin, *mec*A gene.

Introduction

The cutaneous and ear microbiota of dogs is composed of bacteria, fungi, and parasites, which protect the ear surface from other environmental microorganisms. Lack of knowledge of this function and using antiseptic solutions as cleaners allows the entry and colonization of pathogenic microorganisms (Nuttall and Cole, 2004; Ngo *et al*., 2018).

Carrying out treatments without really knowing the primary cause or the related microorganism generates a delay in the recovery of the patient in addition to producing multidrug bacterial resistance or causing severe damage in the dog, such as a ruptured eardrum (Newman *et al*., 2015).

Staphylococci are the predominant bacteria in otitis external and pyoderma in dogs. Coagulase-positive *Staphylococcus* (CoPS) have been more clinically aggressive and share antimicrobial-resistant genes (Devriese *et al*., 2005; Griffeth *et al*., 2008; Sasaki *et al*., 2010).

Staphylococcus schleiferi subsp. *coagulans* are commonly confused with other CoPS due to their similar morphological and biochemical characteristics (Devriese *et al*., 2005). In this way, its identification and epidemiological importance are biased. Likewise, the differentiation between the subspecies of *S. schleiferi* is complex because they have polymorphisms of equal length and only performing a PCR is not enough; this must be complemented with other biochemical tests (Yamashita *et al*., 2005).

Additionally, human cases of *S. schleiferi* subsp. *coagulans* have been reported (Kumar *et al*., 2007; Thibodeau *et al*., 2012; Swe *et al*., 2016), and in one of the cases where infection by a dog with recurrent otitis and pyoderma was suspected (Kumar *et al*., 2007).

Strains of *S. schleiferi* subsp. Methicillin-resistant coagulans (Kania *et al*., 2004; Bemis *et al*., 2006; Kumar *et al*., 2007; Griffeth *et al*., 2008; Kawakami *et al*., 2010; Davis *et al*., 2013; Swe *et al*., 2016) and fluoroquinolones (Muñoz *et al*., 2012; Davis *et al*., 2013) have been reported.

The aim of the current study was to detect the presence of *S. schleiferi* subsp. *coagulans* of canine otitis externa

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and pyoderma, its antimicrobial resistance, and the presence of *mec*Agen.

Materials and Methods

Sampling and isolation

A collection of 331 samples were collected by ear (148) and skin (183) swabs from dogs with otitis externa and pyodermas. All dogs were examined by the San Marcos National University's Veterinarian Clinic from 2017 to 2018. The samples were cultured on tryptic soy agar (TSA; Merck, Germany) at 37°C for 24 hours.

Identification of Staphylococcus species

Gram-positive cocci, catalase-positive, and typical colonial morphology (whitish round colonies) were identified as *Staphylococci*. Tube coagulase test with rabbit plasma (Bactident coagulase; Merck, Germany) was performed, and only CoPS strains were selected for further investigation. Other biochemical tests were acetoin production, acid production from trehalose and mannitol, and clotting factor. These strains were stored in 15% glycerol at −20°C until use.

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed with a commercially paper disk diffusion method using Muller–Hinton Agar. The most popular antimicrobial agents used for the treatment of otitis externa and pyoderma were tested: gentamicin (CN), oxacillin (OX), cephalexin (CL), clindamycin (DA), nitrofurantoin (FD), ciprofloxacin (CIP) for otitis externa and enrofloxacin (ENR) for pyoderma, neomycin (N), doxycycline (DO), and amoxicillin with clavulanic acid (AMC).

16S rDNA-specific PCR for identification of S. schleiferi subsp. coagulans

All CoPS strains were subcultured on TSA with 5% sheep blood at 37°C overnight. Bacterial DNA was extracted following the instructions from the kit (GeneJET Genomic DNA purification; Thermo Fisher Scientific, USA). A pair of primers was designed to amplify the 1,369 bp region of the 16S rDNA of *S. schleiferi* subsp. *coagulans*. Primer-1 (5′–GAACGGACAAGGAGCTTGCTCCTTTGAA-3′) and primer-2 (5′–TTACAAACTCTCGTGGTGTGA A-3′) correspond to the nucleotide residues 61–88 and 1,407–1,429, respectively. The PCR was performed in a 20 µl reaction volume. Each reaction mixture contained 1 µl (17–240 ng) of the sample DNA solution, 10 µl of 1X MasterMix (2XPCR MasterMix; abm, Canada), 1 µl (0.5 µM) of each primer, and completed with 7 µl of ultra-pure water. The amplification reaction was carried out in a standard thermal cycler (GenAmp Biometra; Gmbb Analytik Jena AG, Germany), using the following program: 1 minute at 94°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 57°C and 2 minutes at 72°C. The program was completed with an additional 2 minutes at 72°C. After the amplification, 5 µl of the reaction mixture was analyzed by electrophoresis on a 2% agarose gel in Tris-borate-EDTA buffer at 90 V for 90 minutes. A charge buffer Safe-Green was used with all the samples and a 100 bp ladder of 1.5 kb.

Detection of the mecA gen

The identification of this gen was carried out using a similar PCR method as described previously. A pair of primers was designed to amplify the 533 bp region. Primer-1 (5′-AAAATCGATGGTAAAGGTTG GC-3′) and primer-2 (5′-AGTTCTGCAGTACCGGA TTTGC-3′). The PCR was performed in a 20 µl reaction volume. Each reaction mixture contained 3 µl of the sample DNA solution, 10 µl of 1X MasterMix, 0.4 µl of each primer, and completed with 6.2 µl of ultra-pure water. The PCR program was 45 seconds at 94°C, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 50°C, and 1 minute at 72°C. The program was completed with an additional 2 minutes at 72°C. After the amplification, 5 µl of the reaction mixture was analyzed by electrophoresis on a 1.5% agarose gel in Tris-borate-EDTA buffer at 70V for 100 minutes. The same charge buffer was used with all the samples and a 50 bp ladder of 1 kb.

Results

Staphylococci were isolated from external auditory meatus in 112 (76.87%) of 148 samples of otitis externa and from skin lesions in 141 (77.05%) of 183 samples of pyoderma.

The staphylococcal isolates from otitis externa (n = 112) and pyoderma (n = 141) analyzed by PCR for the amplification of a 1,369 bp product of the 16S rDNA showed that 30.34% (34/112) and 8.5% (12/141) were identified as *S. schleiferi* subsp. *coagulans,* respectively (Fig. 1).

Two DNA samples of isolates S027 and S058, positive for *S. schleiferi*subsp. *coagulans* were sent to the Macrogen Laboratory (Korea) for purification and sequencing. The sequences of isolates S027 and S058 are deposited in GenBank with the codes MN497847 and MN497848, respectively.

The results of the antimicrobial susceptibility of *S. schleiferi* subsp. *coagulans* isolated from otitis externa (n = 34) and pyoderma (n = 12) were favorable with an efficacy level greater than 70% for CN (25/34 and 9/12), DA (24/34 and 10/12), FD (33/34 and 10/12), N (26/34 and 10/12) and DO (27/34 and 9/12). Beta-lactams (CL, OX, and AMC) obtained a moderately effective response in cases of otitis externa (21/34) but favorable in cases of pyoderma (11/12). Fluoroquinolones showed the lowest value in terms of efficacy with 58.8% (20/34) and 50% (6/12) of the otitis externa and pyoderma isolates, respectively (Table 1).

Regarding multidrug resistance, it was found that 17/34 and 3/12 of the *S. schleiferi* subsp. *coagulans* isolated from otitis externa and pyoderma, respectively, were resistant to 3 or more antibiotic groups.

Furthermore, *mec*A gene was detected in 85.29% (n = 34) and 8.3% (n = 1) of *S. schleiferi* subsp. *coagulans*

from samples of otitis externa and pyoderma, respectively (Fig. 2).

Discussion

Except for the study carried out by Bugden (2013) in Australia, where 35% (1,256/3,541) of the bacteria isolated from otitis externa was *Pseudomonas aeruginosa*, several studies have shown that *Staphylococcus* sp. is the primary bacterium found in canine otitis externa, in a range of 50%–60% (Yamashita *et al*., 2005; Penna *et al*., 2010; Muñoz *et al*., 2012; Zur *et al*., 2016). The present study found that 75.68% (112/148) of the otitis externa samples and 77.05% (141/183) of the pyoderma samples were positive for the genus *Staphylococcus*.

The frequency of isolation of *S. schleiferi* subsp. *coagulans* from canine pyoderma in the present study was 6.56% (12/183), a value very similar to the 5.93% (8/135) reported Muñoz *et al*. (2012) in Chile; but with respect to the otitis externa samples, there is a significant difference of little more than double, wherein this study they were 22.97% (34/148) compared to that reported by Muñoz *et al*. (2012) where it was 10.78% (11/102). This

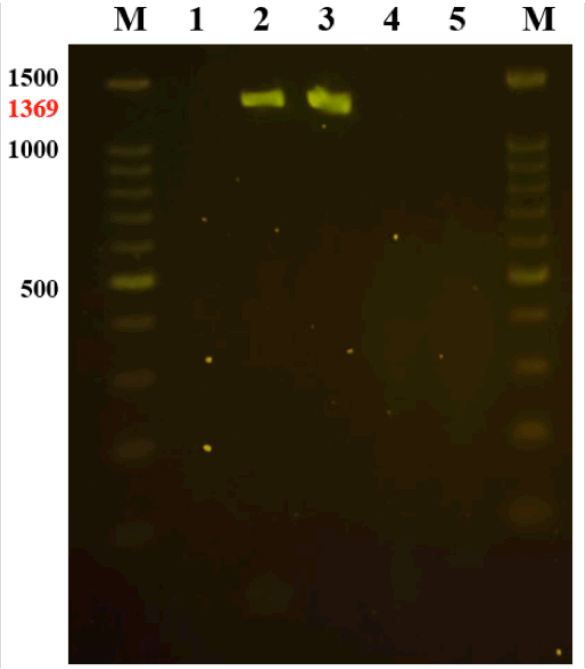

Fig. 1. Agarose gel electrophoresis 2% for the detection of *S. schleiferi* subsp. *coagulans*. Lanes: M, 1.5 kb marker; 1, blank; 2, isolate S027 (GenBank: MN497847)—1,369 bp; 3, isolate S058 (GenBank: MN497848)—1,369 bp; 4, strain of *S. aureus* (ATCC 4330); 5, strain of *S. pseudintermedius*.

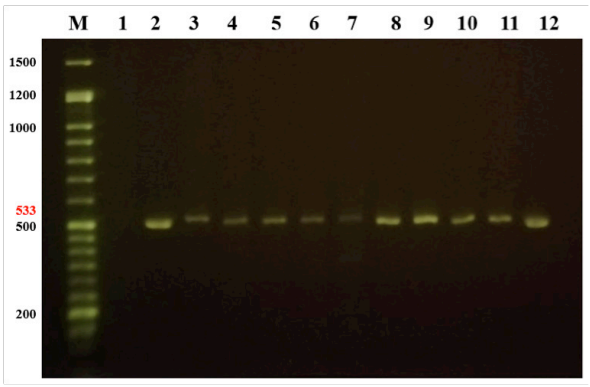

Fig. 2. Agarose gel electrophoresis 1.5% for the detection of *mecA* gen in *S. schleiferi* subsp. *coagulans*. Lanes: M, 1.5 kb marker; 1, blank; 2, strain of Methicillin-resistant *S. aureus* (ATCC 4330)—533 bp; 3, isolate S012; 4, isolate S017; 5, isolate S019; 6–10, isolates S027-31; 11, isolate S039; 12, isolate S040.

Table 1. Antibiotics susceptibility profile of *S. schleiferi* subspecies *coagulans* isolated from otitis externa and pyoderma in dogs.

	S. schleiferi* subspecies *coagulans	
Antibiotics	**Otitis externa isolates (*n* = 34), (%)**	**Pyoderma isolates (*n* = 12), (%)**
CN (30 µg)	25 (73.5)	9 (75.0)
OX (1 µg)	21 (61.8)	9 (75.0)
CL (30 µg)	21 (61.8)	9 (75.0)
DA (2 µg)	24 (70.6)	10 (83.3)
FD (300 µg)	33 (97.1)	10 (83.3)
CIP (5 µg)	20 (58.8)	–
ENR (5 µg)	–	6 (50.0)
N (30 µg)	26 (76.5)	10 (83.3)
DO (30 µg)	27 (79.4)	9 (75.0)
Amoxicillin/clavulanic acid (20/10 µg)	21 (61.8)	9 (75.0)

may be because Muñoz *et al*. (2012) used the BBL ™ Crystal GP diagnostic kit (Becton Dickinson®) instead of the molecular diagnostic method used in this study.

Staphylococcus pseudintermedius is the most reported CoPS in canine otitis externa and pyoderma, with frequencies around 87.9% (Zur *et al*., 2016; Alvarez *et al*., 2020), so many veterinarians assume that it is this bacterium. In this study, it has been observed that at least for the cases of external otitis, 30.34% of the *Staphylococcus* species were *S. schleiferi* subsp. *coagulans*, a value to take into account for the cases reported in Peru.

Yamashita *et al*. (2005) and Swe *et al*. (2016) mention the coagulase and urease tests as beneficial for the differentiation of *S. schleiferi* subspecies, but the urease test was not considered in this study because there is a percentage of variability in terms of interpretation, as earlier reported by Zdovc *et al*. (2004).

This study shows a relatively low susceptibility of 58.8% (20/34) for CIP and 50% (6/12) for ENR, antibiotics belonging to the group of fluoroquinolones used empirically as the first option for treatment of otitis externa and pyoderma. These results are similar to those found by Kunder *et al*. (2015) and Palomino-Farfán *et al*. (2020), where the susceptibility to CIP and ENR was 47.7% and 51.3%, respectively. *Staphylococcus* species have been reported to possess genes that are resistant to fluoroquinolones; this could explain why the susceptibility is so low (Muñoz *et al*., 2012; Schwarz *et al*., 2018).

Another group of antibiotics used as first-line treatment for skin and ear diseases is CL and amoxicillin. In the present study, a susceptibility of 61.8% (21/34) of the ear isolates and 91.67% (11/12) of the skin was found, being higher than those found by Kunder *et al*. (2015) of 38.5%, but lower than those reported by Penna *et al*. (2010) of 77.5% and Muñoz *et al*. (2012) where they found 100% sensitivity to this group of β-lactams. This could be due to the empirical CL treatment that many veterinarians perform when observing coccoid forms on cytology of ear or skin discharge without really knowing their antibiotic response (Wiebe, 2015).

Strains of *Staphylococcus* that phenotypically show resistance to OX are known as Methicillin-Resistant *Staphylococcus*, the best-known species being *Staphylococcus aureus* (MRSA). MRSAs are of great importance in public health because they cause most nosocomial infections in human medicine (Sakoulas *et al*., 2001).

This study demonstrated that there is a resistance to OX in 38.2% (13/34) of the otitis externa isolates, being almost identical to that shown by Cain *et al*. (2011), where they found the resistance of 39% (20/51) of isolates of *S. schleiferi* coagulase positive. These percentages are lower than those found by Kunder *et al*. (2015), where they evaluated the resistance of *S. schleiferi* isolated from dogs in two regions of the United States, finding values of 62% (72/116) and 73% (74/101) of strains resistant to methicillin.

Kunder *et al*. (2015) reported that exposure to recurrent infections and constant antibiotic treatments favor the colonization of resistant *Staphylococcus* methicillin. The consequences of this are reflected in not only their resistance to penicillin (penicillin, amoxicillin, and ampicillin) and first and second-generation cephalosporins (CL, cephalothin, and cefazolin), but it may also be related to resistance to macrolides, aminoglycosides, tetracyclines, and fluoroquinolones (Morris *et al*., 2006; Kawakami *et al*., 2010). Considering the relationship between owners and pets, many of these resistant bacteria could pose a risk of zoonosis or reverse zoonosis (Frank *et al*., 2009).

In conclusion, the detection of *S. schleiferi* subsp. *coagulans* are higher in cases of otitis externa than in canine pyoderma. Antimicrobial resistance is increasing, as shown in this study, antibiotics commonly used to treat otitis externa and pyodermas such as fluoroquinolones are not effective, so it is crucial to perform a bacteriological culture with its respective antibiogram to know exactly its resistance profiles. Likewise, the presence of methicillin-resistant *S. schleiferi* subsp. *coagulans* have been detected, 38.24% (13/34) in cases of external otitis and 8.3% (1/12) in cases of pyoderma, which in some cases have been shown to be multidrug-resistant and possess the *mec*A gene.

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Conflict of interest

The authors declare that there is no conflict of interest.

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