RESEARCH ARTICLE

Isolation, Molecular Detection and Multidrug-Resistant Major Coagulase-Positive Staphylococci from Recurrent Canine Pyoderma

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ABSTRACT

Bacterial pyoderma is one of the most frequent skin diseases in dogs. Recurrent pyoderma is often secondary to an underlying skin disease and is most commonly caused by coagulase-positive staphylococci. The main objectives of this study was to assess the level of resistance acquired by the most common bacteria responsible for causing recurrent pyoderma in dogs. The methodologies used were molecular methods, minimum inhibitory concentration, and antibiotic sensitivity testing to study resistance among the isolated bacteria. A total of 80 samples were collected from the dogs affected with different types of canine pyoderma, presented to the Veterinary Clinical Complex of the College in Junagadh (Gujarat, India). Of these, 9 (11.25%) dogs were diagnosed with recurrent canine pyoderma. From these 9 cases, 7 (77.78%) isolates were confirmed as coagulase-positive Staphylococcus comprising, 5 (71.42%) *S. pseudintermedius* and 2 (28.58%) *S. schleiferi subsp. coagulans*. Among the 7 major CoPS isolates, a higher percentage of antibiotic resistance was shown against penicillin-G, followed by amoxyclav and amikacin, and 3 (42.86%) isolates showed multidrug resistance among selected antibiotics of different classes, and one (14.28%) isolate of *S. schleiferi subsp. coagulans* showed methicillin-resistant (MRSS) phenotypically by disc diffusion method. These findings highlight the need for periodic assessment of pathogens and drug susceptibility patterns in different areas to ensure judicious use of antibiotics.

Key words: Antibiotic resistance, Multidrug resistance, Recurrent canine pyoderma, Staphylococcus schleiferi subsp. coagulans.

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INTRODUCTION

Pyoderma in dogs is most commonly caused by the coagulase-positive *Staphylococcus pseudintermedius* (Lynch and Helbig, 2021), but it can also be brought on by *Staphylococcus aureus* and *Staphylococcus schleiferi* subsp. *coagulans* (Loeffler and Lloyd, 2018). Other bacteria include certain anaerobes, aerobic coryneforms, coagulase-negative staphylococci, *Micrococcus* spp. and α-haemolytic streptococci. Dogs with deep pyoderma may have Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Proteus* spp. and *Escherichia coli* (Jane *et al.,* 2014). Pyoderma's pathophysiology occurs in two stages. The pathogenic commensal first colonizes the areas of the skin's surface, causing surface pyoderma, such as skin fold pyodermas (intertrigo) and pyotraumatic dermatitis. The second phase starts with the bacterial invasion of the skin's stratum corneum, resulting in impetigo, folliculitis with hair follicle invasion, and cellulitis (Prior, 2021).

Pyoderma refers to bacterial infections in dogs that show complete response to appropriate systemic and topical antibacterial therapy, resulting in dogs appearing normal between episodes of infection. The majority of the dogs with pyoderma respond well to initial treatment and do not experience a recurrence of the infection. However, there are an unknown percentage of dogs that may relapse after appearing to have fully recovered. Recurrent superficial pyoderma is more common than recurrent deep pyoderma, and it is worth noting that some cases initially diagnosed as recurrent deep pyoderma may actually be instances where a complete cure was never achieved. In cases of idiopathic recurrent pyoderma, diagnostic tests have been unable to reveal an underlying cause (Weese, 2007). The underlying causes of recurrent pyoderma may be a persistent underlying skin disease, bacterial hypersensitivity, immunodeficiency, resistant strains of *Staphylococcus intermedius,* other *Staphylococcus* species like *S. pseudintermedius*, *S. schleiferi, S. aureus* and non-staphylococcal species (DeBoer, 1995).

In the present scenario, recurrent canine pyoderma can indeed pose significant challenges, especially when caused by multidrug-resistant and methicillin-resistant coagulase-positive *Staphylococcus*. These bacteria have developed resistance to many antibiotics that are commonly used to treat infections, making them difficult to eradicate.

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Additionally, managing recurrent pyoderma requires a comprehensive approach that includes addressing underlying factors contributing to the dog's susceptibility to infections, such as allergies, hormonal imbalances, or skin conditions (Morris *et al.,* 2006). This study was undertaken to identify the most common coagulase-positive staphylococci isolated from recurrent cases of skin infection in dogs and to demonstrate their multiple antimicrobial drug-resistance against routinely used antibiotics.

MATERIALS AND METHODS

A total of 80 skin swabs were collected aseptically from clinical cases of canine pyoderma **(**August 2022 to April 2023**)** by gentle rolling of the sterile cotton swab moistened with sterile 0.9% saline solution, across the border of the skin lesion (pustular and crusty lesion) and were transported to the laboratory by placing it in the sterile container in ice on the same day of sampling.

Identification and Biochemical Characterization of *Staphylococcus* spp.

The skin swabs collected from the affected dogs were inoculated in Brain Heart Infusion (BHI) broth for 6 to 8 h, followed by streaking on the BHI agar and incubated at 37°C for 24 h. The Gram-positive *Staphylococci* organisms were tentatively identified using Gram's staining method. The isolated organisms were streaked on Mannitol Salt Agar (MSA) and incubated at 37°C for 24 h. Coagulase-positive staphylococci (e.g., *Staphylococcus aureus*) produced yellow colonies with yellowish discoloration of the surrounding medium, while coagulase-negative staphylococci produced pink or colorless colonies with no discoloration of the surrounding medium. Biochemically, for confirmation of coagulase-positive *Staphylococcus*, catalase-positive, oxidase-negative, and coagulase-positive reaction, and mannitol fermentation were considered as the basis for identification. The haemolysis patterns of all the isolates were studied using 5% sheep blood agar, as per Quinn *et al.* (2011).

Isolation of Bacterial Genomic DNA

Isolation of bacterial genomic DNA from pure staphylococcal cultures was carried out using the conventional method (Proteinase K-SDS method) as per Sambrook and Russell (2001), with minor modifications. The purity and concentration of isolated DNA were assessed using µDrop™ Plate in a µDrop plate reader (Thermo Scientific, USA.)

Molecular Detection of *Staphylococcus* spp. and its Resistant Genes

For species identification, a set of primers, cycling conditions, and their product size were studied as described by different authors. The 16S rRNA gene (Martineau *et al.,* 2001) was amplified for the detection of *Staphylococcus* spp., whereas *S. aureus, S. pseudintermedius* and *S. schleiferi* subsp. *coagulans* were identified by amplification of the *Au-nuc, Pse-nuc* and *Sch-nuc* genes, respectively, as described by Gonzalez-Dominguez *et al. (2020)*.

The resistant genes from the isolates recovered from recurrent canine pyoderma were studied. The details of the oligonucleotide primers, PCR cycling conditions and product size were studied for methicillin (*mecA*), mupirocin (*mupA),* mupirocin (*mupLL*) and vancomycin (*vanA*), as described by Tamakan and Gocmen, (2022), Sum *et al*. (2020), Abdulgader *et al*. (2020) and Mahmood and Flayyih, (2014), respectively. The composition of the reaction mixture for all the sets of primers was as follows: The PCR reaction was carried out in a total 25 µL reaction mixture composed of 12.5 µL 2X master mix (Thermo Scientific, Lithuania), 1 µL each of 10 pmol forward and reverse primer (Eurofins Genomics India Pvt. Ltd., Bangalore), 3 µL genomic DNA and 7.5 µL Nuclease free water.

The amplification reactions were carried out using a programmable thermal cycler (Verity, Applied Biosystems by Life Technology, Singapore). To identify the amplicon of the targeted sequence, 10 µL PCR product was loaded with gel loading dye in 1.5% w⁄v agarose gel containing 0.5 µg/mL ethidium bromide with a DNA ladder and electrophoresis was done in 1x Tris-acetic acid-EDTA (TAE) buffer at 80 V for 60 min. The amplified product was visualized using a gel documentation system (Bio-PrintST4® VilberLourmat).

Antibiotic Sensitivity Testing

All the coagulase-positive staphylococci (CoPS) isolates obtained from the recurrent canine pyoderma cases were studied by *in vitro* antibiotic sensitivity testing as per the Kirby-Bauer (Bauer *et al.,* 1966) method on Mueller Hinton agar (MHA) medium (HiMedia Laboratory Private Limited, Mumbai) by disc diffusion method. Total 19 antibiotic discs with different potencies were used during the study. The isolates showing resistance against ≥ 3 antimicrobial classes were defined as multidrug-resistant, as described by Magiorakos *et al.* (2012).

Determination of Minimum Inhibitory Concentration (MIC) by the E-Test

The MIC of all CoPS isolates was determined by E-test using commercial MIC determination paper strips (Ezy MIC™ strips), obtained from HiMedia Laboratories, Mumbai. These strips contain pre-coated antibacterials in a concentration gradient manner capable of showing MICs upon testing against the test organism. The following MIC strips were used to determine resistance patterns of antibiotics: Mupirocin (EMO87): 0.064-1024 µg/mL (Mupirocin low-level resistance: 8-256 µg/mL, Mupirocin high-level resistance: ≥ 512 µg/mL) and Vancomycin-Cefoxitin (EM0771): VAN: 0.19-16.0 µg/mL; CX: 0.5-64 µg/mL.

RESULTS AND DISCUSSION

Identification and Biochemical Characterization of *Staphylococcus* spp.

A total of 80 dogs were diagnosed with canine pyoderma clinically. Out of these, 9 (11.25%) cases of dogs were diagnosed with recurrent canine pyoderma (Fig. 1), including 3 (33.33%) male and 6 (66.67%) female from the history of the owner, and clinical records at VCC about repeated visits of dogs with the same ailment. Out of these 9 cases of canine recurrent pyoderma, 9 isolates were confirmed as *Staphylococcus* spp. based on staining, morphology, growth characteristics, haemolysin production and various biochemical tests (Table 1). Out of total 9 *Staphylococcus* spp. isolates, 7 (77.78%) were confirmed as CoPS (tube coagulation test positive) and 2 (22.22%) were confirmed as CoNS (tube coagulation test negative). The prevalence of recurring canine pyoderma is progressively rising in dogs and it frequently correlates with the presence of methicillin and multidrug-resistant bacteria. When confronted with such instances, veterinarians frequently recommend alternative treatment approaches, such as combination therapies or newer antibiotics that the bacteria may still respond to. Nevertheless, these alternatives can be costlier and may not consistently yield the desired outcomes.

In the current study of canine recurrent pyoderma cases, a total of 77.78% of isolates were identified as major CoPS, which includes *Staphylococcus pseudintermedius* (71.42%) and *Staphylococcus schleiferi* subsp. *coagulans* (28.58%). Similar results were obtained by Abusleme *et al.* (2022), who reported 84.48% *S. pseudintermedius* and 15.52% *S. aureus*. Costa *et al.* (2021) reported 32% isolates of *Staphylococcus schleiferi* subsp. *coagulans,* corresponding to all canine pyoderma related isolations in the last 2 decades. Rana *et al.* (2022) reported 78.16% *S. pseudintermedius*, 19.71% *S. aureus* and 2.11% other *Staphylococcus* spp., whereas a higher percent of incidence was obtained by Chaudhary *et al.* (2019), who reported 87% *S. intermedius,* 8% *S. aureus* subsp. *aureus*, 3% other *Staphylococcus* spp. and 2% *S. schleiferi* subsp. *Coagulans*. Tamakan and Gocmen, (2022) reported 87.5% *S. pseudintermedius*, 9.37% *S. aureus* and 3.12% *S. schleiferi*. However, a lower percentage was reported by Shah *et al.* (2017) with 69.49% *S. pseudintermedius*, 25.42% *S. aureus* and 5.08% other *Staphylococcus* spp.

Molecular Detection of *Staphylococcus* spp. and its Resistant Genes

A total of 9 isolates were identified as *Staphylococcus* species by PCR using a *Staphylococcus* genus specific 16S rRNA gene primer (Table 1; Fig. 2). Of these, 7 (77.78%) isolates were confirmed as CoPS (tube coagulation test). Out of these 7 confirmed coagulase-positive staphylococcal isolates, 5 (71.42%) isolates yielded the desired fragment of the 99 bp amplicon of the *Pse-nuc* gene, specific for *S. pseudintermedius* (Table 1; Fig. 3) and 2 (28.58%) isolates yielded the desired fragment of 115 bp amplicon of the *Sch-nuc* gene, specific for *S. schleiferi* subsp. *coagulans* (Table 1; Fig. 4). None of the isolates were amplified for the presence of resistant genes studied.

Table 1: Phenotypic and genotypic detection of various species of *Staphylococcus* from recurrent canine pyoderma

Sr. No.	Sample No.	Phenotypic methods				Genotypic methods				
		KOH and Oxidase	Catalase and Coagulase	MSA	Pattern of haemolysis	16S rRNA	*Pse-nuc*	*Sch-nuc*	*Au-nuc*	*MecA, mupA, mupLL, vanA*
1	CP 1	-	+	NF	β	+	+	-	-	-
2	CP 6	-	+	NF	β	+	+	-	-	-
3	CP 7	-	+	NF	γ	+	+	-	-	-
4	CP 8	-	+	NF	γ	+	-	+	-	-
5	CP 26	-	+	NF	α	+	+	-	-	-
6	CP 33a	-	+	NF	α	+	+	-	-	-
7	CP 80a	-	+	NF	β	+	-	+	-	-

Fig. 1: Recurrent surface pyoderma in dog

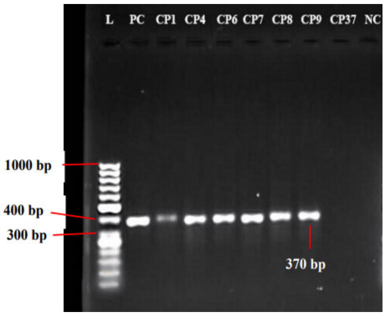

Fig. 2: Genus specific PCR of *Staphylococcus* spp. for 16S rRNA gene (370 bp). L: 50 bp plus ladder, PC: Positive control (*S. aureus* ATCC 43300), CP1, CP4, CP6, CP7, CP8, CP9: Samples positive for *Staphylococcus* spp. isolated from canine pyoderma, CP37: Negative sample, and NC: Negative control (*E. coli* MTCC 722)

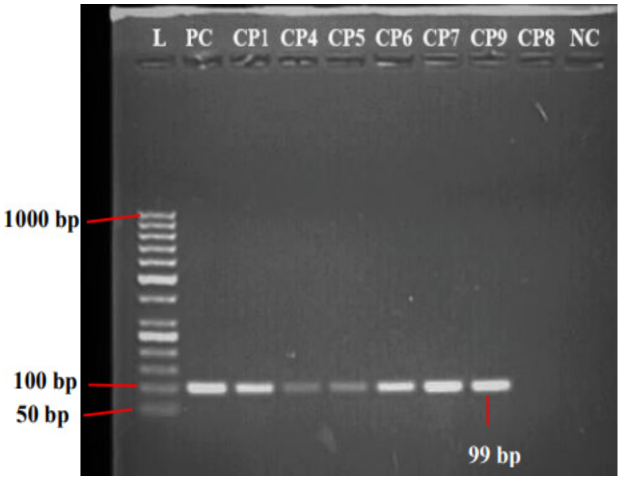

Fig. 3: Species specific PCR of *Staphylococcus pseudintermedius* for *Pse-nuc* gene (99 bp). L: 50 bp plus ladder, PC: Positive control (*Staphylococcus pseudintermedius* ATCC 49444), CP1, CP4, CP5, CP6, CP7, CP9: Samples positive for *Staphylococcus pseudintermedius*. CP 8: Negative sample, and NC: Negative control (*E. coli* MTCC 722)

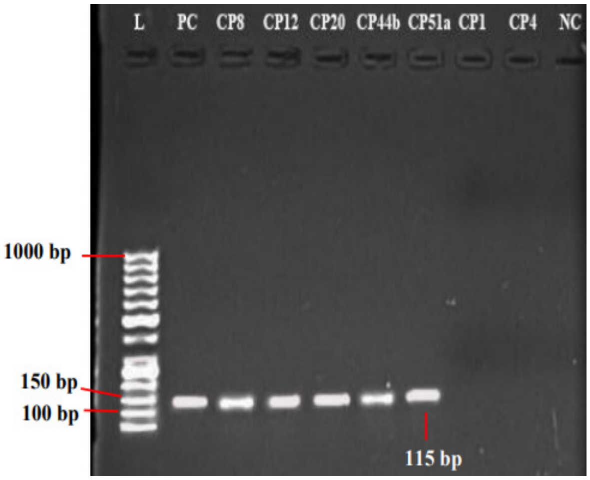

Fig. 4: Species specific PCR of *Staphylococcus schleiferi* subsp. *coagulans* for *Sch-nuc* gene (115 bp). L: 50 bp plus ladder, PC: Positive control (*Staphylococcus schleiferi* subsp. *coagulans* ATCC 49545), CP8, CP12, CP20, CP44b, CP51a: Samples positive for *Staphylococcus schleiferi* subsp. *coagulans***,** CP1, CP4: Negative samples, NC: Negative control (*E. coli* MTCC 722)

Antibiotic Sensitivity Testing and MIC

The antibiogram pattern of seven isolates recovered from recurrent cases of canine pyoderma was studied. Among major CoPS isolates, a higher percentage of antibiotic resistance was shown against penicillin-G (42.86%), followed by amoxyclav and amikacin (28.57% each), whereas a lower percentage of antibiotic resistance was observed against methicillin, cefpodoxime, gentimicin, levofloxacin, enrofloxacin, oxytetracycline, chloramphenicol, co-trimoxazole and clindamycin (14.29% each).

Similarly, Gonzalez-Dominguez *et al.* (2020) found that 6% of *S. pseudintermedius, S. aureus* and *S. schleiferi* isolates were resistant to ampicillin/sulbactam, and 74% isolates were resistant to trimethoprim/sulphamethoxazole. Prior (2021) reported 84.2% and 70.2% of *S. pseudintermedius* isolates were resistant to penicillin and amoxicillin/clavulanic acid, respectively. Lai *et al*. (2022) observed 77.83% of *Staphylococcus* isolates resistant to penicillin-G. Dziva *et al*. (2015) noted 36.9%, 33.8%, and 16.9% of CoPS isolates resistant to tetracycline, trimethoprim/ sulphamethoxazole and gentamicin, respectively. Abusleme *et al.* (2022) indicated that 15.6%, 2.2%, and 0% of CoPS isolates were resistant to tetracycline, gentamicin and amikacin, respectively. Conversely, Hariharan *et al.* (2014) reported all *Staphylococcus* isolates susceptible to amoxicillin/clavulanic acid and cefpodoxime. Additionally, Lai *et al.* (2022) found 45.21%, 32.8%, and 20.12% of *Staphylococcus* isolates resistant to clindamycin, gentamicin and enrofloxacin, respectively.

Out of these 7 isolates, 1 (14.28%) isolate showed methicillin resistance phenotypically by the disc diffusion method but could not yield the desired fragment of the *mecA* gene and was considered as methicillin-resistant *Staphylococcus schleiferi* subsp. *coagulans* (MRSS).

The rise in methicillin-resistant *Staphylococcus* spp. in recent years, posed a threat not only to animals but also to humans. This is because domesticated animals can contribute to the spread of these highly resistant strains within households (Morris *et al.,* 2012). Over the past years, numerous studies have revealed the emergence and increasing prevalence of methicillin and multidrug resistance in staphylococci isolated from not only humans but also various veterinary species, including horses and dogs (Magiorakos *et al.,* 2012). Similar to the current investigation, Abusleme *et al.* (2022) found that 24.4% of CoPS isolates carried the *mecA* gene. Ananda Chitra *et al*. (2018) and Lai *et al*. (2022) reported 28% and 34% MRSP isolates, respectively. However, Gonzalez-Dominguez *et al.* (2020) and Prior, (2021) reported a higher percentage of MRSP isolates at 73.6% and 85.96%, respectively. Conversely, Dziva *et al.* (2015), Rana *et al.* (2022) and Tamakan and Gocmen, (2022) reported lower percentages of MRSP isolates at 8.69%, 8.1% and 7.14%, respectively.

Out of 7 isolates, 3 (42.86%) isolates showed multidrug resistance among 19 selected antibiotics of different classes. None of the isolates amplified the desired fragment of resistant genes studied and none of these showed resistance by the E-test, which was conducted using a commercial MIC determination paper strip. The most common species isolated from recurrent canine pyoderma was *S. pseudintermedius,* followed by *S. schleiferi* subsp. *coagulans*, unidentified *Staphylococcus* spp., and *E. coli* (mixed infection) and the most effective antibiotics were ampicillin/sulbactam, cefepime, ceftriaxone and ceftizoxime. Therefore, clinicians should consider incorporating these antibiotics into the treatment regimen for repeated skin infections in dogs.

In a similar way, Mikaela and Domrnico (2023) reported that 44.9% of the isolates were MDR-SS and showed methicillin resistance (OXA), which was associated with multidrug resistance in *S. schleiferi*. These findings aligned with previous research, suggesting that MR strains can acquire multidrug resistance genes through alternative genetic mechanisms (Cain, 2013). Furthermore, exposure to systemic antibiotics prior to culture was identified as a significant risk factor for multidrug resistance in both *S. pseudintermedius* and *S. schleiferi* (Mikaela and Domenico, 2023). In a study by Perez-Sancho *et al.* (2020), it was reported that 66 (47.48%) isolates consisting of 60 *S. pseudintermedius* and 6 *S. aureus* exhibited multidrug resistance (MDR) across various clinical conditions in animals.

The incidence of MRSP and MRSS infections has been increasing in recent times. MRSP has been identified as a significant One Health concern (Prior, 2021). Since humans are not the natural hosts for *S. pseudintermedius* and *S. schleiferi*, there is evidence suggesting that these bacteria, acting as reservoirs, can transfer antimicrobial-resistant genes to the skin flora of humans due to the close interaction between humans and animals. Given that MRSP can persist in the environment for extended periods, there is a risk of bacteria spreading from dogs to humans and vice versa. The risk factors for acquiring MRSP are similar to those for human MRSA, including repeated antibiotic treatments, frequent hospital visits, and invasive procedures, posing a threat to dog owners with weakened immune systems or households with multiple pets. With the global increase in the prevalence of MRSP-related infections in humans and dogs, it is now considered an emerging zoonotic agent (Prior, 2021).

CONCLUSIONS

This research was conducted to identify the bacteria responsible for recurrent canine pyoderma and their resistance to various antibiotics. The study has outlined the prevalence and origin of the main *Staphylococcus* species causing canine pyoderma. The findings showed a high occurrence of methicillin resistance and multi-drug resistance among the coagulase-positive *Staphylococci* species, with a notable increase in resistance to certain β-lactam and non-β-lactam antibiotics compared to previous research. These results prompt clinicians to reconsider their approach to treating canine skin infections. The study conclude that the precise execution of bacterial culture and antimicrobial profiling, along with the molecular identification of resistance genes in Staphylococcus isolates, establishes a comprehensive methodology that significantly benefits the clinical diagnosis, treatment, and overall prognosis of dogs suffering from pyoderma.

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