

Original Research Article

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Identification of *Staphylococcus pseudintermedius* and MRSP in Dogs with Pyoderma by PCR

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ABSTRACT

Pyoderma is one of the most common dermatological disorders encountered in small animal practice. Almost ninety per cent of the cases of pyoderma in dogs is caused by bacteria belonging to the genus *Staphylococcus* and now *Staphylococcus pseudintermedius* is recognized as the main etiological agent responsible for pyoderma. Further, Methicillin Resistant *S. pseudintermedius* is being reported worldwide and represents a serious threat to the health of dogs. However not much work on *S. pseudintermedius* has been done in India. Hence the study, with the primary objective being isolation, identification and genotypic characterization of staphylococcal organisms responsible for pyoderma in dogs along with detection of genotypic resistance to methicillin in these organisms. Dogs presented to the Veterinary College Hospital, Bangalore with clinical signs suggestive of pyoderma were chosen as subjects for the study. Materials from lesions from these dogs were collected, processed and cultured in Mannitol Salt Agar. DNA extracted from these isolates were subjected to multiplex PCR using species specific primers and were also tested for methicillin resistance genotypically by PCR targeting the *mecA* gene. All the twenty isolates were staphylococci and eleven of the isolates were found to be *S. pseudintermedius* and nine were *S. aureus*. Sixteen of the twenty isolates were resistant to methicillin genotypically on PCR. The study conclusively proved the occurrence of *S. pseudintermedius* in dogs with pyoderma which probably has not been reported from India. Further, it also proved the occurrence of methicillin resistant *S. pseudintermedius* and *S. aureus* in dogs with pyoderma which could be important from the perspective of therapeutic management of pyoderma.

Keywords

Pyoderma,
Staphylococci,
S. pseudintermedius,
Methicillin Resistant
S. pseudintermedius
(MRSP),
mecA gene

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Introduction

Dermatological disorders in dogs are of major concern due to its multiple etiological factors, higher cost of treatment and the long duration of therapy and management required. Therefore appropriate diagnosis and

identification of exact etiology is the key for successful therapeutic management (Scott and Paradis, 1990). Pyoderma is one of the most common dermatological disorders encountered in small animal practice. Almost ninety per cent of the cases of pyoderma in dogs are caused by coagulase positive

Staphylococci. In the the early 70s and 80s, *Staphylococcus aureus* was believed to be the main organism responsible for pyoderma. More recently, biotyping methodology for coagulase positive staphylococcal species indicated that the pathogenic coagulase positive staphylococcus in the dog was *Staphylococcus intermedius* (Berg *et al.*, 1984). Of the staphylococcal species, *Staphylococcus intermedius* has been implicated in approximately 90% of cases of pyoderma in dogs. However, in actuality, isolates based on phenotypical characteristics originally identified as *S. intermedius* have been found to be from three different species, *Staphylococcus intermedius*, *Staphylococcus pseudintermedius* and *Staphylococcus delphini*. For definitive identification of these species, molecular diagnostic methods, such as polymerase chain reaction (PCR) techniques are required (Bannoehr *et al.*, 2009) and the term *Staphylococcus intermedius* Group (SIG) is now used to refer to the three previously mentioned isolates (*S. intermedius*, *S. pseudintermedius* and *S. delphini*) as a group (Bannoehr *et al.*, 2007 and Sasaki *et al.*, 2007).

Consequently, since the reclassification of the species, it has been proposed that all canine isolates belonging to the SIG should be considered as *S. pseudintermedius* unless proven otherwise by genetic typing methods (Devriese *et al.*, 2008). *Staphylococcus pseudintermedius* is a canine commensal and opportunistic pathogen, which is analogous to *S. aureus* in human beings. Antibiotic resistance in staphylococci is of great concern due to increasing incidence of methicillin resistance among staphylococci. Of late, several reports are emerging of methicillin resistance in SIG. Also, a high rate of multidrug resistance is being reported among Methicillin Resistant *S. pseudintermedius* (MRSP) strains in dogs (Schwarz *et al.*, 2008).

Methicillin resistance in *S. pseudintermedius* similar to *S. aureus* is mediated by the *mecA* gene which encodes the penicillin binding protein 2a (PBP 2a) which has low affinity for beta lactam antimicrobials and therefore confers resistance to staphylococcus (Moon, 2012). In 2013, Videla stated that methicillin resistance and resistance to other antimicrobials regularly used by veterinarians is common among *S. intermedius* which can complicate treatment. The first report of *mecA* gene responsible for methicillin resistance in *S. intermedius* was in 1999 and MRSP was first reported in 2005. Since then, resistance to methicillin and to other antimicrobials has become increasingly common, making this bacterium a possible reservoir for antimicrobial resistance genes.

Thus, taking into consideration the fact that *Staphylococcus pseudintermedius* is recognized as the main etiological agent responsible for pyoderma and that not much work has been done in India to detect the presence of this bacteria, this study was taken up. Further, Methicillin Resistant *S. pseudintermedius* (MRSP) is being reported worldwide and represents a serious threat to the health of dogs and yet not much work on MRSP has been done in India.

The primary objectives of the study were to detect the occurrence of different species of staphylococci with emphasis on *S. pseudintermedius* and to detect methicillin resistance in different species of staphylococci causing pyoderma in dogs.

Materials and Methods

Culture, Coagulase, Catalase testing and Gram's staining

Twenty animals presented to Veterinary College, Bangalore with clinical signs suggestive of pyoderma such as papules,

pustules, erythema, alopecia, pruritus and epidermal collarettes were selected as subjects for bacterial culture, coagulase and catalase testing, Grams staining and molecular studies. Samples were collected from the lesions using sterile cotton swabs and subjected to bacterial culture, primarily using nutrient broth or brain heart infusion broth and subcultured using Mannitol Salt Agar. All plates were incubated aerobically at 37°C for 18-24 hrs for observation of characteristic growth and tentative identification was done based on the morphology of colonies. Individual colonies were selected and catalase and coagulase testing (tube coagulase) was done as per standard procedure (Elmer *et al*, 1988) and Gram's staining was done and biochemical characterization was done using a commercially available kit (HiStaph kit, Himedia, Mumbai, India)

DNA extraction and PCR

The isolates from culture were subjected to DNA extraction as per the standard protocol prescribed by the manufacturer using a kit procured commercially (AMpurE Bacterial gDNA Mini Spin, Amnion Biosciences Pvt Ltd, Bangalore, India) and Multiplex PCR (m-PCR) was carried out for identification of staphylococcus to the species level targeting the *nuc* gene loci for differentiation of species using the primer sequences given in Table 1 (Sasaki *et al*, 2010).

Multiplex PCR was carried out using a programmable master cycler with a reaction mixture volume of 25 ul. The DNA samples were subjected to thermal cycling conditions as given below

1. Initial denaturation at 95° C for 10 min followed by
2. Denaturation at 95° C for 30 sec
3. Annealing at 56° C for 1 min
4. Extension at 72° C for 1 min

5. Final extension at 72°C for 10 min
6. Hold at 4° C

After completion of PCR, 3 ul of the amplified products along with 100 bp DNA ladders mixed with 6X gel loading dye were subjected to electrophoresis on 2 % agarose gel (prepared using TAE buffer, agarose and Ethidium Bromide). One positive control and one negative control were used with the test samples. The PCR product size was determined by comparing with a standard molecular marker (DNA ladder) and the images were captured using Gel Documentation system.

Detection of methicillin resistance by PCR

The extracted DNA from all the twenty isolates were utilized for molecular studies on methicillin resistance targeting the *mecA* gene. PCR was carried out using a programmable master cycler (Eppendorf, Hamburg, Germany).The reaction mixture volume was 25 *ul*. (using 10X Taq buffer, Taq polymerase, dNTPs, Primers, Template and Nuclease Free Water) PCR was carried out with the following thermal cycler conditions

1. Initial denaturation at 94° C for 1 min followed by
2. Denaturation at 94° C for 30 sec
3. Annealing at 50° C for 1 min
4. Extension at 72° C for 2 min
5. Final extension at 72°C for 10 min
6. Hold at 4° C

The steps 2, 3, 4 were repeated (programmed) for 30 cycles. After completion of PCR, 3 *ul* of the amplified products along with 100 bp DNA ladders mixed with 6X gel loading dye were subjected to electrophoresis on 2 % agarose gel (prepared using TAE buffer, agarose and Ethidium Bromide).One positive control and one negative control were used

with the test samples. The primers used for the study i.e *Staph mecA* gene (F-TGGCTATCGTGTCAACAATCG and R-CTGGAACCTTGTTGAGCAGAG) were designed at the Department of Microbiology, Veterinary College, Bangalore for an earlier study (Sundareshan, 2012). The PCR product size was determined by comparing with a standard molecular marker (DNA ladder) and the images were captured using Gel Documentation system.

Results and Discussion

Culture, Coagulase, Catalase testing and Gram’s staining

All the twenty isolates showed growth on Mannitol Salt Agar, were catalase and coagulase positive and showed the presence of Gram positive cocci on Gram’s staining.

Identification of species by Multiplex PCR

Eleven (55%) of the twenty isolates were found to be *S.pseudintermedius* which corresponded to 926 bp and nine (45%) were *S.aureus* that corresponded to 359 bp in the DNA ladder. The results are depicted in Table 2, Fig I

Detection of Methicillin Resistance by PCR

All the twenty Staphylococci isolates were subjected to PCR. Sixteen out of 20 isolates yielded 304 bp corresponding to *mecA* gene specific for methicillin resistance. Of the sixteen *mecA* positive isolates, 8 (50%) were *S. aureus* and 8 (50%) were *S. pseudintermedius* as depicted in Table 3, Fig II

Table.1 Oligonucleotide Primers used for M-PCR for Identification of Coagulase Positive Staphylococci Species

Species to be identified	Primer	Sequence (5’-3’)	Product Size (bp)	Reference
<i>S. aureus</i>	au-F3 au-nucR	TCGCTTGCTATGATTGTGG GCCAATGTTCTACCATAGC	359	Sasaki <i>et al</i> (2010)
<i>S. intermedius</i>	in-F in-R3	CATGTCATATTATTGCGAATGA AGGACCATCACCATTGACATATTGAAACC	430	Sasaki <i>et al</i> (2010)
<i>S. schleiferi</i> subsp. <i>Coagulans</i> and <i>S. schleiferi</i> subsp. <i>Schleiferi</i>	sch-F sch-R	AATGGCTACAATGATAATCACTAA CATATCTGTCTTTCGGCGCG	526	Sasaki <i>et al</i> (2010)
<i>S. pseudintermedius</i>	pse-F2 pse-R5	TRGGCAGTAGGATTCGTTAA CTTTTGTGCTYCMTTTTGG	926	Sasaki <i>et al</i> (2010)

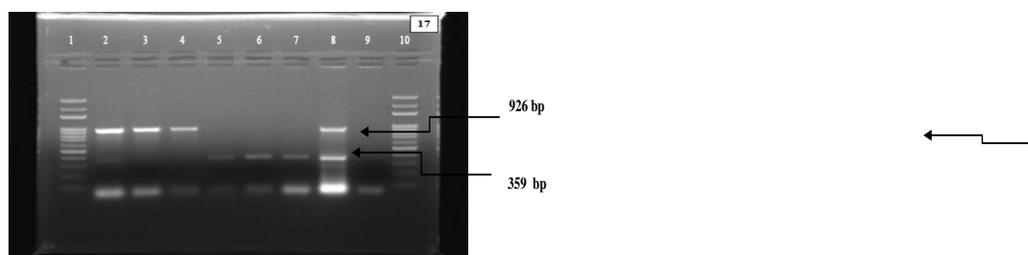
Table.2 Species-wise Identification of Staphylococci through M-PCR in Dogs with Pyoderma (n=20)

Sl. No.	Method of identification	<i>S. aureus</i>		<i>S. pseudintermedius</i>	
		Positive		Positive	
		Numbers	Per cent	Numbers	Per cent
	Multiplex PCR	9	45	11	55

Table.3 Species-wise Resistance of Staphylococci Isolated from Dogs with Pyoderma to Methicillin by PCR (n=20)

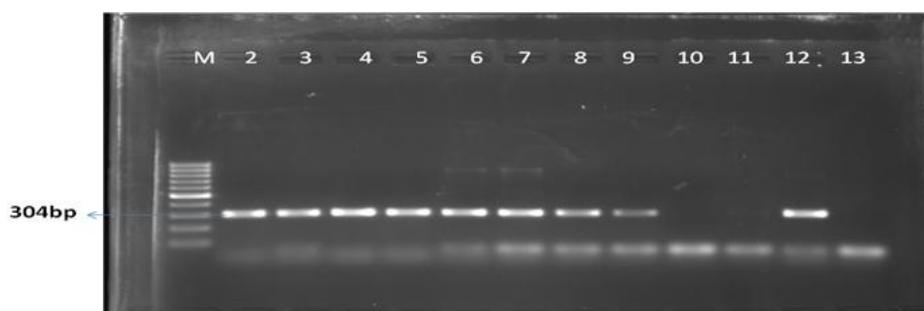
Method of evaluation	<i>S. aureus</i>		<i>S. pseudintermedius</i>	
	Number	Percent	Number	Percent
PCR (Genotypic- <i>mecA</i> gene)	8	50	8	50

Fig.1 Gel Electrophoresis of Multiplex -PCR Depicting Different Staphylococci Species



Lane 1 & 10: 100bp DNA ladder
 Lane 2, 3 & 4: *S. pseudintermedius* which corresponded to 926 bp
 Lane 5, 6 & 7 : *S. aureus* which corresponded to 359 bp
 Lane 8 : Positive control
 Lane 9 : Negative control

Fig.2 Gel Electrophoresis of PCR Depicting Different Staphylococci Species Coding for *Meca* Gene



Lane 2-9 & 12: *mec A* positive isolates which corresponded to 304 bp amplicon
 Lane 13: Negative control
 Lane M: 100bp DNA ladder
 Lane 10-11: *mec A* negative isolates

Staphylococcal pyoderma is a common dermatological disorder in dogs which frequently occurs as a result of an underlying cause. Thus, the results of the present study discusses the etiology of pyoderma and corroborates the reports of various workers (Devriese *et al*, 2006., Bannoehr *et al*, 2009.,

Fitzgerald, 2009., Kadlec *et al*, 2010., Bannoehr & Guardabassi, 2012) who have reported *Staphylococcus pseudintermedius* and Methicillin Resistant *Staphylococcus pseudintermedius* (MRSP) as the major pathogens for pyoderma in dogs. *S aureus* usually occurs as a secondary pathogen. To

our knowledge this is probably the first report on the occurrence of *S pseudintermedius* and MRSP in India.

In the present study, of the 20 isolates examined, 55% (11) proved to be *S. pseudintermedius* and 45% (9) were *S. aureus* on PCR. It is now well established that *S. pseudintermedius* is the major staphylococci causing pyoderma in dogs. Devriese *et al* (2005) following necropsy examination of dogs and cats demonstrated the occurrence of a new species of staphylococci with a distinct taxonomy closely related to *S. intermedius* and *S. delphini*. Fitzgerald (2009) in a review on *Staphylococcus intermedius* Group (SIG) of organisms discussed about how *S. intermedius* has long been regarded as the cause of pyoderma in dogs and how genetic diversity of *S. intermedius* has resulted in the reclassification of *S. intermedius* and identification of *S. pseudintermedius* as the common canine pathogen. Bannoehr *et al* (2009) reported that a diagnostic technique involving PCR-RFLP which allows for differentiation of *S. pseudintermedius* from closely related members of SIG which may not be possible by biochemical methods. Kadlec *et al* (2010) stated that *S. pseudintermedius* was the most frequent causative agent of canine pyoderma and that it may also be associated with wound infections, urinary tract infections and otitis externa in dogs. Bannoehr & Guardabassi (2012) in their review on *S. pseudintermedius* stated that dog is the natural host for *S. pseudintermedius* and that definitive identification of this organism relies on molecular methods. This is primarily because *S. pseudintermedius* cannot be distinguished from *S. intermedius* by phenotypic methods. Further, due to the lack of standardized and specific phenotypic tests, the routine presumptive identification of *S. pseudintermedius* is based on the fact that it is the only member of the SIG that has been isolated from dogs. The results of the present

study was based on multiplex PCR with species specific primers for various coagulase positive staphylococcal species i.e *S. aureus*, *S. intermedius*, *S. pseudintermedius* and *S. schleiferi* all of which have been reported in dogs with pyoderma by other workers. Sasaki *et al.*, 2007 and Van Hoovels *et al.*, 2006 have stated that conventional microbiological diagnostic tests often fail to distinguish between *S. pseudintermedius* and *S. intermedius*, leading to *S. pseudintermedius* being frequently misidentified as *S. intermedius* or *S. aureus*. This has also been corroborated by other workers (Sasaki *et al.*, 2010 and Videla 2013).

Antibacterials represent one of our most effective therapeutic defenses against infectious diseases. However, the continuous use of antibacterials is under enormous threat due to bacterial resistance. The development of antibacterial resistance is a major issue that can compromise the treatment of infectious diseases as well as other advanced therapeutic procedures (Videla, 2013). Methicillin resistant staphylococci strains have emerged as serious pathogens over the last decade. These strains are usually multidrug resistant thus making successful therapy difficult. Besides they are now a major cause of hospital and community acquired infections associated with high morbidity and mortality. Further, because of the close association between man and animals especially dogs, the threat of transmission of diseases from man to animals and animals to man cannot be over emphasized.

One of the primary causes of resistance to beta-lactamase resistant penicillins (methicillin being the prototype) in staphylococcal isolates is presence of the *mecA* gene, which encodes a supernumerary penicillin binding protein (PBP2a) with reduced affinity for beta-lactams. Resident PBPs play important roles in the formation of

the bacterial cell wall peptidoglycans. These PBPs can be inactivated by the presence of beta-lactam antimicrobials, leading to abnormal cell wall synthesis and bacterial death. However, the poor affinity for beta-lactams associated with the carriage of the *mecA* gene, serves as a mechanism of protection for the bacteria, evading disruption of the peptidoglycan layer and preventing bacterial death.

In present investigation, 16 out of 20 isolates yielded 304 bp corresponding to *mecA* gene specific for methicillin resistance. In the 16 *mecA* positive isolates, 8 (50%) isolates were *S. aureus* and 8 (50%) isolates were *S. pseudintermedius*. This is in agreement with Feng, *et al.*, (2012) who recorded 144 methicillin resistant *S. pseudintermedius* isolates (50%) from a total of 288 MRS positive dogs and cats. On the contrary, Videla in 2013 classified 194 isolates as methicillin susceptible or resistant based on *mecA* PCR out of which he reported forty-six (23.7 %) isolates as methicillin susceptible and 148 (76.2 %) as methicillin-resistant. In another study, published in 2006 by Morris *et al.*, it was reported that as many as 17% of the *Staphylococcus pseudintermedius* isolates were methicillin resistant.

The study has detected the presence of Methicillin sensitive *Staphylococcus aureus*, *Staphylococcus pseudintermedius* and, MRSA, MRSP mediated by *mecA* gene. The occurrence of *Staphylococcus schleiferi* and MRSS as well investigations into some of the other genes mediating methicillin resistance can be looked into in the future as a continuation of the present study.

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