

## Original Research Article

<https://doi.org/10.20546/ijcmas.2020.909.265>

## Identification of Methicillin Resistant *Staphylococcus intermedius* and *Staphylococcus aureus* in Dogs with Pyoderma

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### ABSTRACT

Clinical cases presented to Veterinary College Hospital, Bangalore, Karnataka were used as subjects for the study. Samples were collected from lesions of dogs showing clinical signs typical of pyoderma and were subjected to bacterial isolation and culture with mannitol salt agar, coagulase and catalase testing and biochemical characterization for species differentiation as per standard procedures. Of the 91 samples examined, 88 were coagulase positive staphylococci, which following biochemical characterization were classified as *S.aureus* (16 isolates) and *S intermedius* (72 isolates). These were further subjected to sensitivity testing for Methicillin / Oxacillin by disc diffusion as per standard procedures. Fourteen of the 88 isolates were found to be methicillin resistant of which 5 isolates were *S aureus* and 9 were *S intermedius*. Six of the methicillin resistant isolates (Four *S intermedius* and two *S aureus* as per biochemical characterization) were further subjected to DNA extraction and PCR targeting the *mecA* gene specific for methicillin resistance. Four of the 6 isolates (3 *S intermedius* and one *S aureus*) yielded 304 bp corresponding to *mecA* gene, one of the primary genes responsible for methicillin resistance. The variation between the results observed by disc diffusion and PCR in this study may be due to the fact that there are additional genetic material responsible for methicillin resistance including genes encoding production of Pantone Valentine Leucocidin (PVL). Thus the negative results of 2 of the isolates for *mecA* gene in the present study could be attributed to either overestimation of methicillin resistance by disc diffusion or it could be due to some other gene mediating resistance.

#### Keywords

*S. intermedius*, *S aureus*, Pyoderma, *mecA*, Methicillin, Resistance

#### Article Info

Accepted:  
17 August 2020  
Available Online:  
10 September 2020

### Introduction

Dermatological disorders are one of the primary disorders encountered in small animal practice. Of these, pyoderma is one of the most common dermatological disorders diagnosed. It is also one of the most frustrating problems to cope with both for the

pet owner as well as the clinician primarily because of the long duration of therapy required, frequent recurrence and also due to the fact that every major or minor dermatological disorder would invariably result in secondary pyoderma. Though several causes have been implicated as precipitating factors, it is essentially a bacterial infection of

the skin. Until very recently *S aureus* was believed to be the primary pathogen responsible for pyoderma. However later on i.e. in the 80's it was found that another coagulase positive Staphylococci, *S intermedius* was the main etiological agent implicated in pyoderma (Ihrke, 1983; Hoskin *et al.*, 1985; Kunkle, 1987; DeBoer, 1995; Mason, 1997; Craig, 2003). More recently, however *S intemedius* has been reclassified and it now belongs to a group of organisms referred to as SIG comprising of *S intermedius*, *S pseudintermedius* and *S delphini*. Of these species, it is now known that *S pseudintermedius* is the organism responsible for causing pyoderma in dogs (Fitzgerald, 2009; Zakour *et al.*, 2012) and it was in 2005 that it was first identified as the primary etiological agent for canine pyoderma ((Devriese *et al.*, 2005) However, it is often reported as *S intermedius* as it cannot be differentiated from *S intermedius* by any of the routine biochemical diagnostic tests available for differentiating different species of staphylococci (Devriese *et al.*, 2005).

The increased incidence of staphylococcal infections in humans and the indiscriminate use of antibiotics has now resulted in the emergence of Methicillin Resistant Staphylococci (MRS). Methicillin Resistant *Staphylococcus aureus* (MRSA) is being reported worldwide over the last several years in humans as well as animals. Of late, however Methicillin Resistant *Staphylococcus intermedius* (MRSI) and Methicillin Resistant *Staphylococcus pseudintermedius* (MRSP) are also being reported (Frank and Loeffler, 2012). *S intermedius* was normally believed to be pathogenic only in dogs. However now there are several reports wherein they are implicated in human infections. Thus due to the close association between human beings and dogs as pets presence of Methicillin Resistant *Staphylococcus intermedius* could have far reaching zoonotic implications,

making early detection and diagnosis of paramount importance. The present study was a part of a larger study on pyoderma in dogs.

## **Materials and Methods**

Cases of dermatological disorders were selected non randomly for the study based on detailed history, clinical examination and skin scraping examination. Lesions such as pustules, masses, nodules were first cleaned with sterile cotton and punctured using 2.5 ml sterile dispovan syringes with 21 Guage needles. Materials were then collected aseptically from these lesions by aspiration of the contents. The material so collected using sterile swabs from lesions was inoculated into Brain Heart Infusion broth and incubated at 37°C for 12-24 hrs. A loopful of the inoculum was then streaked on to pre-prepared Mannitol Salt Agar petriplates and incubated for 24 hrs at 37°C and the morphology examined as per the standard procedure described by Cruickshank *et al.*, (1975). Following the study of morphology and plate reading, the isolates identified as staphylococci were subjected to sub-inoculation onto Brain Heart Infusion agar petriplates and incubated at 37°C for 12- 24 hrs. They were further subjected to Grams staining, followed by catalase testing and coagulase testing (tube coagulase test) as per the procedure described by Elmer *et al.*, (1988) and further subjected to biochemical characterization to differentiate the different species using the Histaph Kit (Hi Media, India)

## **Methicillin sensitivity / resistance testing and detection of MRSA and MRSI**

Antibiotic sensitivity testing for methicillin was carried out using Methicillin (MET 5 mg) as well as Oxacillin (OX 1 mcg) discs by disc diffusion method as per the procedure described by Bauer *et al.*, (1966). The

materials from isolates which were positive for staphylococci were streaked uniformly onto sterile petriplates containing Mueller Hinton Agar and antibiotic discs placed on them and examined after incubation as per the procedure described. The zone of inhibition was then measured using the standard chart provided with the discs and expressed in millimetres. Sensitivity / resistance was assessed based on comparison with the standard chart provided with the discs.

A few of the isolates (6) found resistant to methicillin by disc diffusion method were further subjected to DNA extraction as per the standard protocol prescribed by the manufacturer using the kit procured from Real Genomics (Hi Yield Genomic DNA mini kit) and PCR for detection of *mecA* gene responsible for methicillin resistance

PCR was carried out using a programmable master cycler (Eppendorf, Germany) as per the procedure described. The reaction mixture of 25 ul (using 10X Taq buffer, Taq polymerase, dNTPs, Primers, Template and Nuclease Free Water) was prepared in a step wise manner as per procedure in 0.2 ml thin walled PCR tubes placed in mini cooler. After mixing the contents, the tubes were centrifuged and fitted with caps and placed firmly inside the thermal cycler and the thermal cycler conditions were set as detailed below.

1. Initial denaturation at 94° C for 5 min followed by
2. Denaturation at 94° C for 30 sec
3. Annealing at 50° C for 30 sec
4. Extension at 72° C for 1 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 added 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 (*Staph mecA* gene) (F-T G G C T A T C G T G T C A C A A T C G and R-C T G G A A C T T G T T G A G C A G A G) which were designed at the Department of Microbiology, Veterinary College, Bangalore for the Indo-American project on “Rational development of an effective vaccine for prevention of staphylococcal mastitis in bovines” were used for the study (Sundareshan, 2012). The images were captured using Gel Documentation system.

## Results and Discussion

### Identification of Staphylococcal organisms responsible for causing pyoderma in dogs

All 91 samples subjected to bacterial culture showed growth on MSA (Fig. 1& 2). On coagulase testing 88 of the isolates were found to be coagulase positive (Fig. 3) and 3 were coagulase negative (Table 1). All the 88 isolates were catalase positive. Grams staining revealed the presence of Gram positive cocci (Fig. 4 and 5). The samples included cases of primary pyoderma as well as pyoderma secondary to other dermatological disorders such as demodicosis, atopy and flea allergy dermatitis. After observing the colony morphology, and subculturing, the isolates were further classified species-wise based on the results of biochemical tests using a commercial biochemical assay Kit (Fig 6). Seventy two (81.81%) of the 88 coagulase positive isolates were found to be *S. intermedius* and Sixteen (18.18%) of the isolates were *S.aureus*.

Generally the bacteria associated with pyoderma includes *S. aureus*, *S. intermedius* /

*S pseudintermedius*, *S. schleiferi*, *Proteus* spp and *E coli*. However reports from several workers including Ihrke (1983), Berg *et al.*(1984), Hoskin *et al.*, (1985), Kunkle (1987) have indicated that Staphylococci and especially *S. intermedius* is the primary pathogen in pyoderma and the others are usually secondary invaders. In view of this, emphasis was laid on identifying different species of staphylococci causing pyoderma in the cases presented at the Veterinary College Hospital, Bangalore, Karnataka

The occurrence of 81.82% of *S. intermedius* and 18.18% *S. aureus* in the clinical cases of pyoderma in the study (Table 1) is in accordance with the report of Berg *et al.*, (1984) who reported an occurrence of 85.37% of *S. intermedius* and Raus and Love (1983) who reported 75.68% of *S. intermedius* and

17.73% of *S. aureus* following a study of coagulase positive isolates. However, Medleau *et al.*, (1986) reported the occurrence of *S. intermedius* in 98% of the coagulase positive isolates examined and Philips and Williams (1984) reported the presence of *S. intermedius* in 83% of coagulase positive isolates examined. Woldehiwet and Jones (1990) also confirmed observations of other workers that *S. intermedius* was the dominant coagulase positive species in dogs. However, there are also reports of occurrence of other species of staphylococci such as *S. schleiferi* (Frank *et al.*, 2003) and *S. delphini* (Lewis, 2010) in dogs with pyoderma but these are most commonly found in recurrent pyoderma and not as primary pathogens as reported by Frank *et al.*, (2003).

**Table.1** Results of Staphylococcal culture of material from lesions of dogs with pyoderma (n=91)

	Coagulase negative	Coagulase positive		
		Total	<i>S.aureus</i>	<i>S. intermedius</i>
<b>Total No. of isolates</b>	3	88	16	72
<b>Per cent</b>	3.30	<b>96.70</b>	<b>18.18</b>	<b>81.81</b>

**Table.2** Results of antibiotic sensitivity and resistance patterns with Methicillin and Oxacillin in dogs with pyoderma by disc diffusion method

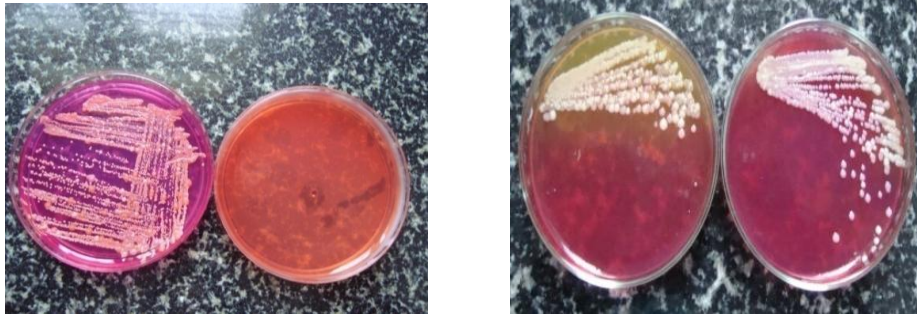
Antibiotic disc	Sensitive	Resistant	Per cent resistance
<b>Methicillin (n=88)</b>	74	14	15.91
<b>Oxacillin (n=88)</b>	74	14	15.91

**Table.3** Comparison of Methicillin resistance of *S aureus* and *S intermedius* in dogs with pyoderma

s				Comparison with only <i>S. aureus</i> isolates n=16		Comparison with only <i>S. intermedius</i> isolates n=72	
<i>S aureus</i>		<i>S intermedius</i>		Numbers resistant	Per cent	Numbers resistant	Per cent
Numbers resistant	Per cent	Numbers resistant	Per cent	Numbers resistant	Per cent	Numbers resistant	Per cent
5	5.68	9	10.23	5	31.25	9	12.5

\* 6 of the 14 Methicillin resistant isolates were subjected to DNA isolation and PCR and 4 were found positive for *mecA* gene, which is one of the important genes responsible for methicillin resistance

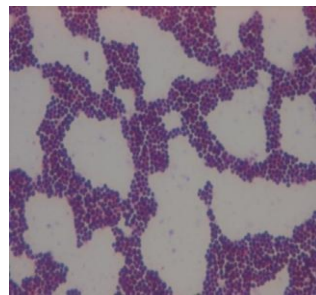
**Fig.1 and 2** Colonies of staphylococcus spp in Mannitol salt Agar isolated from a case of pyoderma in dog. Note the change in colour and the mixed type of growth



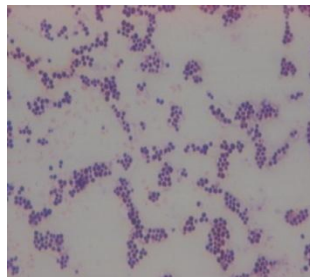
**Fig.3** Coagulase Test on isolates from lesions of pyoderma in dogs



**Fig.4** Gram staining of culture of purulent material from skin showing *S. intermedius* Grams x 1000



**Fig.5** Gram staining of culture of purulent material from skin showing *S. intermedius* Grams x 1000



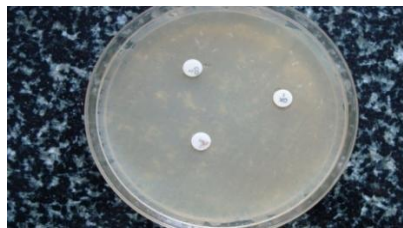
**Fig.6** Biochemical tests for identification of staphylococcal species with a commercial kit- Hi staph kit (Himedia)



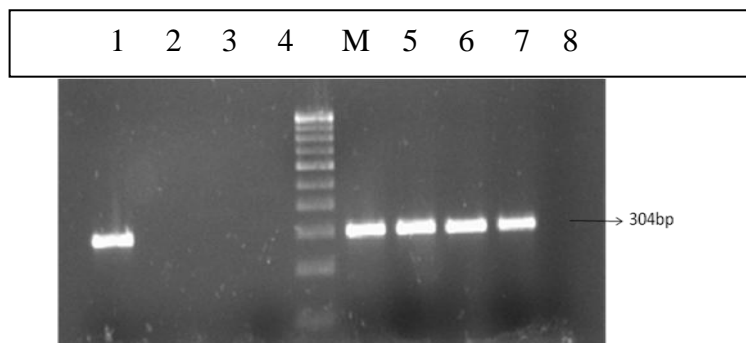
**Fig.7** Antibiotic sensitivity test for Methicillin showing zone of inhibition



**Fig.8** Colony of staphylococcus sp showing resistance to Methicillin



**Fig.9** Photograph of PCR with 4 of isolates showing 304 bp corresponding to *mecA* gene indicating methicillin resistance



Lane 1: Positive control (phenotypically and genotypically methicillin resistant *S.aureus* )

Lane 2: Negative control (phenotypically methicillin sensitive *S. aureus*)

Lane M: 100bp DNA ladder

Lane 3-8- samples (3, 4-negative: 5 ,6, 7, 8-Positive)

### Detection of methicillin resistance

The results of Methicillin and Oxacillin sensitivity/resistance testing are depicted in Table 2 and 3 and Fig 7,8 and 9. All the 88 coagulase positive isolates were subjected to sensitivity testing using methicillin and oxacillin discs. A total of 14 isolates i.e. 15.91% were found to be phenotypically methicillin resistant and the remaining 74 isolates were methicillin sensitive. Of the 14 methicillin resistant isolates, 5 of the isolates (5.68%) were *S. aureus* and 9 of the isolates (10.23%) were *S. intermedius*.

The results of species-wise methicillin resistance are depicted in Table 2. On species wise assessment of the result of culture examination Methicillin Resistant *S. aureus* (MRSA) was 31.25% (i.e. 5 out of 16) and Methicillin Resistant *S. intermedius* (MRSI) was 12.5% (9 out of 72).

Six of the methicillin resistant staphylococci isolates (four *S. intermedius* and two *S. aureus* as per biochemical characterization) were further subjected to genotyping. Four of the six isolates (Three *S. intermedius* and one *S. aureus*) corresponded to 304 bp in the DNA ladder corresponding to *mecA* gene specific for methicillin resistance. The results are illustrated in Fig 9.

Methicillin resistant Staphylococci 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 the threat of transmission of diseases from man to animals and animals to man cannot be overemphasized.

In the present investigation, of the 88 coagulase positive isolates, methicillin resistance (phenotypically) was found in 14 (15.91%) which included 5 (5.68%) *S. aureus* and 9 (10.23%) *S. intermedius*. On a cursory glance it would appear that occurrence of methicillin resistance is more in *S. intermedius*. However when one compares the total number of positive isolates of *S. intermedius* (72) and *S. aureus* (16) to the methicillin resistant isolates i.e. five *S. aureus* and nine *S. intermedius*, it is evident that the incidence of methicillin resistant *S. aureus* (MRSA) is far more (31.25%) than methicillin resistant *S. intermedius* (12.5%) The occurrence of methicillin resistant *S. aureus* in humans as well as in animals is being reported since the early 1970s. However, it is the emergence of methicillin resistant *S. intermedius* that is a cause for concern especially because of its zoonotic potential, which is slowly being realized, as a result of the close association between dogs and their owners. Thus it could have far reaching implications not only for pets but for humans as well. A similar report had been made by Bemis *et al.*, (2006) who however reported occurrence of methicillin resistant *S. intermedius* following a comparative study to be as high as 30%. Similarly, Duquette and Nuttall (2004) reported 23 of the 25 methicillin resistant isolates from canine wounds and skin lesions to be *mecA* gene positive, of which nine were MRSA, one was MRSI and fifteen were coagulase negative species of staphylococci.

DNA isolation and PCR analysis of six of the isolates showing methicillin resistance by disc diffusion method confirmed four isolates to be positive for *mecA* gene. Methicillin resistance is due to a modified Penicillin Binding Protein, PBP2a encoded by the *mecA* gene located on one of the six types of Staphylococcal Chromosomal Cassettes (SCCS) which vary in size and the smallest of

these containing the *mecA* gene is SCC *mec* type IV according to Morgan (2008). Loeffler *et al.*, (2007) in fact, first reported the emergence of multidrug resistant and methicillin resistant *S. intermedius* in Europe which was identified by the presence of *mecA* gene. Gradelski *et al.*, (2001) also reported a positive correlation between methicillin and oxacillin resistance and presence of *mecA* gene. However they also stated that at least 50% of the *mecA* negative strains were falsely classified as methicillin and oxacillin resistant. This may be due to the fact that there are additional genetic materials responsible for methicillin resistance including genes encoding production of *Panton Valentine Leucocidin (PVL)* which are comparatively less common in animals as reported by Morgan, (2008), Duquette and Nuttall, (2004). Thus the negative results of 2 of the isolates for *mecA* gene in the present study could be attributed to either overestimation of methicillin resistance by disc diffusion as reported by Duquette and Nuttall (2004) who opined that traditional disc diffusion methods overestimate methicillin resistance or it could be due to some other gene mediating resistance as indicated by Morgan (*loc.cit*).

However, it should be emphasized that in the present study, *S. intermedius* was identified phenotypically by morphology and biochemical characterization and it is now being reported that the isolates being identified as *S. intermedius* could very well be *S. pseudintermedius* as the two species cannot be differentiated phenotypically by biochemical characterization. However methicillin resistance of these organisms was detected both phenotypically by antibacterial sensitivity testing as well as genotypically by PCR for the presence of *mecA* gene. Hence the importance of occurrence of Methicillin Resistant Staphylococci be it *S. aureus*, *S. intermedius* or *S. pseudintermedius* cannot be

overemphasised considering the zoonotic threat these organisms represent and also the difficulty in treating such infections.

### Acknowledgements

The authors would like to gratefully acknowledge the Vice Chancellor, KVAFSU, Bidar and The Dean, Veterinary College Bangalore, Karnataka, for providing the facilities to undertake this study.

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**How to cite this article:**

Kshama, M. A., S. Yathiraj, Srikrishna Isloor, S. Sundaresh and Veeresh, B. H. 2020. Identification of Methicillin Resistant *Staphylococcus intermedius* and *Staphylococcus aureus* in Dogs with Pyoderma. *Int.J.Curr.Microbiol.App.Sci.* 9(09): 2122-2131.  
doi: <https://doi.org/10.20546/ijcmas.2020.909.265>