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Characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) from dogs using SCC*mec* typing

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Abstract
Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have now gained importance leading to increased diagnosis and detection in companion animals. They act as potential reservoir of infection to humans. The study was undertaken to know the occurence of MRSA in dogs in and around Puducherry region. Out of 105 nasal swabs collected from dogs, 50 CPS and 54 CONS could be isolated and were characterized up to species level based on the biochemical reactions. Only the CPS isolates were taken up for further study. Antimicrobial susceptibility testing employing 17 antimicrobial agents indicated that 90% of these isolates were sensitive to cloxacillin and enrofloxacin. PCR targeting *mecA* gene could detect methicillin resistance in 10% of the CPS isolated from the nasal swabs of dogs. All the *mecA* positive isolates from nasal swabs of dogs belonged to SCC*mec* Type III.

Keywords: Dogs, PCR, *mecA*, MRSA, SCC*mec* type

1. Introduction
Staphylococcus aureus represents a colonizer and a pathogen for humans as well as for various animal species. The prevalence of methicillin-resistant *S. aureus* (MRSA) in human medicine has constantly increased in many parts of the world. The first MRSA was reported in 1961 in UK [1]. Since then, there is an increase in the number of reported MRSA clinical infections also in veterinary practice worldwide which suggests that there is an awareness about MRSA infection in veterinary practice leading to more number of diagnosis and detection in companion animals such as dogs, cats and other pets as well. Moreover domestic animals have been increasingly exposed and susceptible to MRSA infections and are now a potential reservoir of infection to humans [2, 5].
The transfer of MRSA between humans and animals has been corroborated by typing studies which showed that MRSA from dogs and cats were typically identical to hospital-associated lineages dominant in the particular countries [6, 8]. The *mecA* genes from animal isolates of Staphylococci were identical to those found in human MRSA strains and therefore were suggestive of possible zoonotic transfer [9, 10]. The Methicillin resistance in *S. aureus* is mediated by the penicillin binding protein (PBP2a) that have low affinity for beta-lactam antibiotics. It is encoded by the gene *mecA*, residing on a large mobile genetic element designated Staphylococcal cassette chromosome mec (SCC*mec*).
The infections are generally caused by MRSA isolates that harbor SCC*mec* types I, II, and III [11]. Community-associated MRSA (CA-MRSA) isolates, which carry SCC*mec* type IV or V, are now prevalent and exceeded methicillin-susceptible *S. aureus* (MSSA) in skin and soft tissue infections [12, 14]. These isolates were reported to cause severe, often necrotizing, soft tissue infections and pneumonia [12, 16]. Detection of *mecA* gene by genotypic method is considered as gold standard for detecting methicillin resistance [17, 19].
A thorough understanding of the molecular epidemiology and evolution of MRSA is required to help detect, track, control and prevent disease due to this organism. SCC*mec* typing is one of the most important molecular tools available for understanding the epidemiology and clonal strain relatedness of MRSA, particularly with the emerging outbreaks of community-acquired MRSA occurring on a worldwide basis [20, 23].

No comprehensive study of MRSA in companion animals like dogs have been carried out in India.

Therefore the aim of the present study was to analyze the occurrence of MRSA in dogs from Puducherry region and to understand whether dog could act as reservoir for MRSA infection to humans.

2. Materials and Methods

2.1 Collection and processing of samples

The study included 105 dogs from both genders, from different breeds presented to Teaching Veterinary Clinical Complex with different clinical conditions, Rajiv Gandhi Institute of Veterinary Education and Research (RIVER), Puducherry. Nasal swabs were collected aseptically and transported to the Department of Veterinary Microbiology, RIVER, Kurumbapet, Puducherry.

2.2 Isolation and Identification of Coagulase Positive Staphylococci (CPS)

The nasal swabs were inoculated onto 7.5% NaCl-Luria broth and incubated at 37ºC for 24-48 h. After overnight incubation, the inoculums were streaked onto Mannitol Salt agar plates and incubated at 37°C overnight for the selective isolation of *Staphylococcus*. The Gram positive cocci in clusters were subjected to coagulase test. Only the coagulase positive Staphylococci (CPS) were taken up for further study. All the CPS isolates were identified up to species level based on the methods described by Barrow and Feltham [24] and Bergey's Manual of Systematic Bacteriology [25].

2.3 Antimicrobial sensitivity test

All the CPS positive isolates were subjected to antimicrobial susceptibility testing. Antimicrobial susceptibility test was done as per the standard disc diffusion method described by Bauer *et al*. [26] using 17 different antimicrobial agents namely A-Ampicillin (10 µg), Am- Amoxycillin (10 µg), Av-Amoxycillin/Clavulanic acid (30 µg), Cn-Cefoxitin, Ci-Ceftriaxone (30 µg), Ce-Cephotaxime (30 µg), Cf-Ciprofloxacin (5 µg), C-Chloramphenicol (30 µg), Cx-Cloxacillin (5 µg), Co-trimoxazole (25 µg), Ex-Enrofloxacin (10 µg), G-Gentamicin (10 µg), M-Methicillin (5 µg), Ox-Oxacillin (1 µg), P-PenicillinG (10 U), S-Streptomycin (10 µg), T-Tetracycline (30 µg). The interpretation of zone diameter was carried out according to Clinical Laboratory Standard Institute [27]. The organisms were reported as susceptible, intermediate and resistant to the antimicrobial agents tested.

2.4 Reference strains used in the study

The reference strains of *Staphylococcus aureus* MTCC 87 were obtained from Institute of Microbial Technology, Chandigarh, India. The reference strain for Methicillin-resistant *Staphylococcus aureus* N-315 and the reference strain for SCC*mec* typing were generous gifts from Dr. Teruyo Ito of Juntendo University (Tokyo, Japan).

2.5 Detection of Methicillin-resistance and SCC*mec* typing using Polymerase Chain Reaction

The preparation of template DNA from Staphylococcus strains was carried out as described by Zhang *et al*. [28]. The primers used in the study were given in Table 1. The PCR amplification was carried out as follows: 5 µl of DNA was added to 45 µl of PCR mixture of 1xTaq polymerase buffer, 4mM $MgCl_2$, 1U Taq polymerase, 400µM of each deoxynucleoside triphosphate and 300 nM of primers. The PCR amplification was done in an automated thermal cycler (Eppendorf Mastercycler, Germany) according to the running conditions as elaborated by Zhang *et al*. [28], Brakstad *et al*. [29] and Mo and Wang, [30] for Staphylococci, *S. aureus* and *mecA* gene, respectively. The PCR assay for SCC*mec* typing was carried out with each set of SCC*mec* primers as uniplex assay as described by Zhang *et al*. [31]. The PCR products were analyzed by electrophoresis in 1.5% agarose gel having ethidium bromide at a final concentration of 0.5 µg/ml. The gel was visualized under UV transilluminator and the images were documented in a gel documentation system (Bio-Rad Laboratories, USA).

3. Results and Discussion

Out of 105 nasal swabs collected from dogs in and around Puducherry, A total of 104 gram positive cocci were obtained. Out of which, 50 CPS and 54 CONS could be isolated. Of the 50 CPS, 32 were characterized as *S. aureus* and the rest 18 as *S. intermedius*. The detailed identification of all these organisms were given in Table 2. These results were in accordance with Loeffler *et al*., [32]. As per their study, Staphylococci were isolated from 41/45 dogs sampled (91%). Among 105 Staphylococcal isolates from dogs, CPS accounted for 48.07% (50/104).

The results of antimicrobial susceptibility testing of CPS isolates from nasal swabs of dogs showed more than 90% sensitivity to cloxacillin. More than 85% of CPS was sensitive to enrofloxacin and gentamicin. Among 50 CPS from nasal swabs of dogs, two isolates were completely sensitive to all the antimicrobials tested. Forty four isolates were resistant to more than two or more antimicrobials tested. More than 90% of the CPS were resistant to penicillin G and ampicillin (Table 3). The results were in accordance with Bruno *et al*., [33] who showed that multidrug resistant staphylococci was detected in 26/36 of CPS (72.2%).

The CPS isolates which showed resistance to methicillin by disc diffusion method were also 100% resistant to penicillin G, ampicillin and amoxycillin/clavulanic acid and 65% were resistant to oxacillin. In another study, thirty multiple drug resistant *S. aureus* isolates were recovered from 147 humans and dogs, who were clinically ill [34].

Further in the present study, Of the 104 isolates, subjected to PCR with Staphylococcus genus specific primers, 78 isolates were confirmed to carry genes specific for Staphylococci and thirty two out of 50 isolates (64%) were found to carry the *nuc* gene specific for *S. aureus* and five out of 50 CPS (10%) were found positive for *mecA* gene (Fig. 1). According to Martineau *et al*., [35] phenotypic tests for methicillin resistance may provide unsatisfactory results, since the microorganism may carry a gene for a resistance factor and expression of this gene may be influenced by environmental conditions and culture factors. The detection of *mecA*, a gene encoding Penicillin Binding Protein that have low affinity for β-lactam antibiotics, by the PCR, is considered a gold-standard technique for detection of methicillin resistance [36].

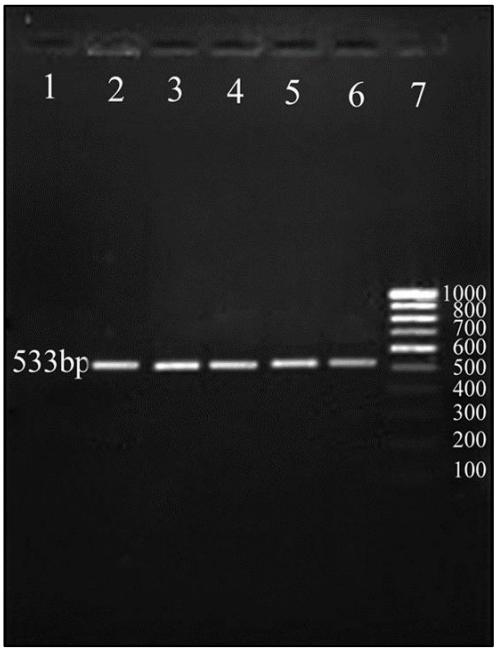
Loeffler *et al*. [32] reported nine per cent prevalence of methicillin resistance in Staphylococci isolated from nasal swabs of dogs in a small animal referral hospital, United Kingdom. In Japan, a single case of methicillin resistant Staphylococci was reported in an inpatient dog but none

could be detected in any of the 30 outpatient dog screened for methicillin-resistance [37]. Methicillin resistant Staphylococci were isolated from two out of 132 (1.5%) nasal swab samples of dogs from Guelp, Hamilton, Burlington, Toronto and Ontario Veterinary Colleges [38]. Aklilu *et al*. [39] studied the methicillin resistance in Staphylococci from nasal swab samples of dogs and cats in Malaysia and found that 10% of the isolates from dogs and six per cent of the isolates from cats carried the *mecA* gene. They concluded that dogs and cats in Malaysia are potential reservoirs for methicillin resistance in Staphylococci.

In this study, Among the five *mecA* positive isolates, four isolates were *SCCmec* type III and one isolate carried both *SCCmec* type III and type V by PCR assay. The details of identification of SCC*mec* typing in methicillin resistance in Staphylococcus isolates, are presented in Table 4 and Fig. 2. Tessie *et al*. [40] reported that out of four *mecA* positive strains that were isolated from dogs, two of them contained SCC*mec* type III while one isolate contained a type V genetic element. The fourth isolate was non typable using the primers designed by Zhang *et al*. [31]. In another study Zhiyong *et al*. [41] reported SCC*mec* type III from a veterinary staff and two dogs from a small animal clinic in China.

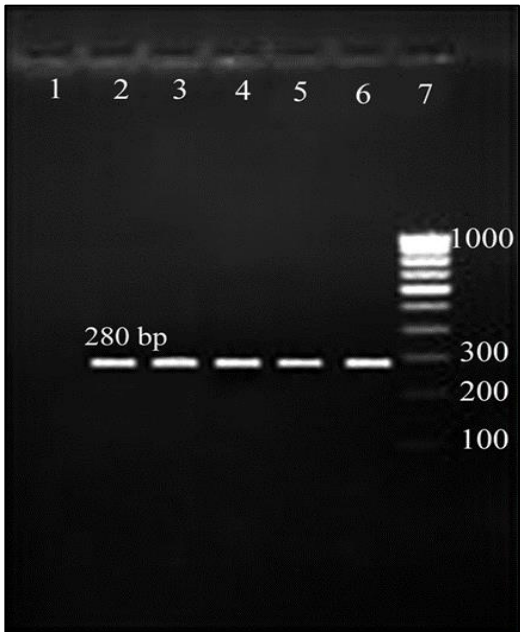
The prevalence of MRSA among dog population in Pondicherry region was recorded as 10%. However further studies using DNA based methods such as PFGE, MLST and spa tying of these MRSA isolates will improve our understanding of the mobile genetic element carrying the *mecA* gene and the clonal relationship of these MRSA isolates from humans and dogs. This will aid in the understanding the transmission of MRSA between dogs and persons in contact with pets. Further it may also help in formulating strategies for the control of spread of MRSA infections.

Periodic surveillance of antimicrobial resistance patterns of MRSA isolated from pets could be an important measure in detecting the emergence and spreading of such resistance. Veterinary hospitals should establish guidelines to minimize cross-contamination by MRSA and other methicillin-resistant staphylococci [42]. Barrier precautions should also be practiced in treating animals having recognized MRSA infections and these animals should be further isolated. To the best of our knowledge this is the first report of identification and characterization of MRSA in dogs from India.



Lane 1 - Negative control
Lane 2, 3, 4, & 5 - Test Isolates D3, H3, I1l, 67VE
Lane 6 - Positive control
Lane 7 - 100bp ladder

Fig 1: Screening of Staphylococci for detection of *mecA gene*



Lane 1 - Negative control
Lane 2, 3, 4 & 5 - Isolate No. D3, D73, D89, D90
Lane 6 - Positive control
Lane 7 - 100bp ladder

Fig 2: Screening of Staphylococci for detection of SCCmec type III

Table 1: Details of the Primers used in the study

Organism	Target	Primer sequence (5'-3')	Size
Staphylococci(Zhang *et al*., 2004)	Genus specific 16S rRNA	AAC TCT GTT ATT AGC GAA GAA CA CCA CCT TCC TCC GGT TTG TCA CC	756 bp
Staphylococcus aureus (Brakstad *et al*., 1992)	*nuc* gene	GCG ATT GAT GGT GAT ACG GT AGC CAA GCC TTG ACG AAC TAA AGC	270 bp
Methicillin resistance in Staphylococci (Mo and Wang,1997)	*MecA* gene	AAA ATC GAT GGT AAA GGT TGG C AGT TCT GCA GTA CCG GAT TTG C	533 bp
Staphylococcus aureus (Zhang *et al*., 2005)	SCC*mec* I	GCT TTA AAG AGT GTC GTT ACA GG GTT CTC TCA TAG TAT GAC GTC C	613 bp
Staphylococcus aureus (Zhang *et al*., 2005)	SCC*mec* II	CGT TGA AGA TGA TGA AGC G CGA AAT CAA TGG TTA ATG GAC C	398 bp
Staphylococcus aureus (Zhang *et al*., 2005)	SCC*mec* III	CCA TAT TGT GTA CGA TGC G CCT TAG TTG TCG TAA CAG ATC G	280 bp
Staphylococcus aureus (Zhang *et al*., 2005)	SCC*mec* IVa	GCC TTA TTC GAA GAA ACC G CTA CTC TTC TGA AAA GCG TCG	776 bp
Staphylococcus aureus (Zhang *et al.*, 2005)	SCC*mec* IVb	TCT GGA ATT ACT TCA GCT GC AAA CAA TAT TGC TCT CCC TC	493 bp
Staphylococcus aureus (Zhang *et al*., 2005)	SCC*mec* IVc	ACA ATA TTT GTA TTA TCG GAG AGC TTG GTA TGA GGT ATT GCT GG	200 bp
Staphylococcus aureus (Zhang *et al*., 2005)	SCC*mec* IVd	CTC AAA ATA CGG ACC CCA ATA CA TGC TCC AGT AAT TGC TAA AG	881 bp
Staphylococcus aureus (Zhang *et al*., 2005)	SCC*mec* V	GAA CAT TGT TAC TTA AAT GAG CG TGA AAG TTG TAC CCT TGA CAC C	325 bp

Table 2: Staphylococci isolated from nasal swabs of dogs

Group	Gram Positive Cocci	Number of Isolates	Percent
	Staphylococcus spp	**104**	**99.04**
Coagulase positive Staphylococci	*Staphylococcus aureus*	32	64
	Staphylococcus intermedius	18	36
Coagulase negative Staphylococci	*Staphylococcus hyicus*	19	35.18
	Staphylococcus lentus	9	16.66
	Staphylococcus saprophyticus	9	16.66
	Staphylococcus arlettae	4	7.40
	Staphylococcus simulans	3	5.55
	Staphylococcus epidermidis	2	3.70
	Staphylococcus hominis	2	3.70
	Staphylococcus xylosus	2	3.70
	Staphylococcus sciuri	1	1.85
	Staphylococcus caprae	1	1.85
	Staphylococcus scheliferi	1	1.85
	Staphylococcus saccharolyticus	1	1.85

A-Ampicillin, Am- Amoxycillin, Av-Amoxycillin/Clavulanic acid, Cn- Cefoxitin, Ci-Ceftriaxone, Ce-Cephotaxime, Cf-Ciprofloxacin, C-Chloramphenicol, Cx-Cloxacillin, Co-trimoxazole, Ex-Enrofloxacin, G-Gentamicin, M-Methicillin, Ox-Oxacillin, P- PenicillinG, S-Streptomycin, T-Tetracycline.

Table 3: Antimicrobial sensitivity pattern of coagulase-positive Staphylococci isolated from nasal swabs of dogs

Sensitive	Intermediate	Resistant
96% to Cx	64.44% to Ci	96% to P
86% to Ex, G		94% to A
72% to T		74% to Av
68% to C	58% to Ce	62% to Am
66% to Co,Cf		52% to Ox,Cn
60% to S		34% to M

Table 4: Identification of SCC*mec* types and sub types in *mec*A positive Staphylococcus isolates from dogs

Isolate Number	MEC A	Type I	Type II	Type III	Type IV A	Type IV B	Type IV C	Type IV D	Type V
D3	+	-	-	+	-	-	-	-	-
D73	+	-	-	+	-	-	-	-	-
D80	+	-	-	+	-	-	-	-	-
D89	+	-	-	+	-	-	-	-	+
D90	+	-	-	+	-	-	-	-	-

4. Conclusion

In conclusion, The prevalence of MRSA among the dog population in Pondicherry region was recorded as 10%. Antimicrobial susceptibility testing indicated that 90% of these isolates were sensitive to cloxacillin and enrofloxacin and 95% were resistant to penicillin G and ampicillin. All

the *mecA* positive isolates from nasal swabs of dogs belonged to SCC*mec* Type III except one isolate (D89) which carried both type III and V. However further studies using DNA based methods will improve our understanding of the clonal relationship of these MRSA isolates from humans and dogs. It may also help in formulating strategies for the control of spread of MRSA infections.

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