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Prevalence and molecular characterization of methicillin resistant *Staphylococcus aureus* in pet dogs

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Abstract
Staphylococcus aureus is an opportunistic pathogen often carried asymptomatically in humans. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains have acquired a gene that makes them re-sistant to all beta-lactam antibiotics. The study was carried out to find out the prevalence of *Staphylococcus aureus* in pet dogs as well as Methicillin-resistant *Staphylococcus aureus* (MRSA) strains. A total of 155 nasal swab samples were collected from pet dogs attending Madras Veterinary teaching hospital, Chennai. The samples were processed by standard conventional procedures for isolation of the organism and molecular characterization of the isolates was done by using thermonuclease nuc gene for *Staphylococcus aureus* and mec A gene was used for methicillin resistant *staphylococcus aureus.* Out of 155 canine nasal swab samples screened 93(60.0%) showed colonies characteristic of staphylococcus species on Baird parker agar plates. Molecular characterization of these isolates by thermonuclease *nuc* gene showed that 88 (94.62%) isolates were PCR positive for *Staphylococcus aureus* and about 86 (92.47%) of the isolates were positive for *mec A* gene. The prevalence of *Staphylococcus aureus* and MRSA highlights the possibility of zoonotic transmission to humans who are in contact with pet dogs.

Keywords: Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, prevalence, zoonotic

Introduction
Staphylococcus aureus is a common human pathogen which can be capable of producing a wide variety of diseases, starting from skin and soft tissue infections to life threatening endocarditis, bacteraemia and necrotizing pneumonia (Gordon and Lowy, 2008) [5]. It can cause severe animal suppurative diseases such as mastitis, arthritis and urinary infections that are associated with various virulent factors. Antibiotic resistance is common phenomena encountered with *S. aureus* and MRSA is emerging as a pathogen of public health importance with zoonotic potential. Infections caused by *Staphylococcus aureus* have assumed public health significance due to development of multi drug resistant strains particularly MRSA and its epidemic colonies that are increasingly being found in hospitals and communities. The prevalence of MRSA in domestic animals like goats, sheep, cattle, horses and further in different companion animals such as dogs and cats (Walther *et al*, 2008) [23] (Saleha *et al*.,2010) [22] were reported and revealing the fact that MRSA has emerged as a potential zoonotic pathogen. Many reports worldwide suggests colonisation and transmission of *S. aureus*, including MRSA, between owners and their dogs (Kottler *et al*., 2008; Loeffler *et al*., 2005; Malik *et al*. 2006) [12, 14, 15]. These studies among the domestic and companion animals have raised the curtains for extensive further studies the issue of MRSA colonization and transmission to human beings particularly those who are in contact with the animals (Khanna *et al*., 2010) [9].

Methicillin is grouped under narrow spectrum beta-lactamase resistant penicillin. The mechanism of action is by interfering primarily with the synthesis of bacterial cell wall and will be responsible for binding of methicillin to penicillin binding proteins (PBPs) (Walther *et al*, 2008) [23]. *Staphylococcus aureus* has the ability to develop resistance to any antibiotic that comes in to clinical use (Pantosi *et al*., 2007) [18]. Methicillin resistance to *Staphylococcus aureus* is due to the acquisition of the mecA gene that encodes a new protein designated as PBP 2a which belongs to a family of enzymes necessary in building the bacterial cell wall. PBP 2a has very low affinity for beta –lactams (Pantosi *et al*., 2007) [18]. MRSA is developed by the introduction of a *mecA* carrying element in a methicillin susceptible *Staphylococcus aureus* (Enright *et al*., 2002) [4].

There have been a number of reports stating that animals may serve as reservoirs for MRSA infection of humans. In the last two decades, new generations of MRSA have emerged with the ability to transfer to human beings and food producing animals. There is a risk of transfer of MRSA to food animals to humans. The potential of MRSA to become a dangerous zoonotic pathogen could affect the epidemiology of MRSA in humans. As the prevalence of MRSA in animals is continuous to rise there is an inherent risk for new MRSA clones to evolve secondary to horizontal gene transfer and host selection pressure and then spread to human hosts. Thus the presence of MRSA in animals is a concern not only to veterinarians and animal health care workers but also to public health.

Methodology

Collection of Samples

Cotton tipped dry swab was inserted into the anterior nares of dogs and rubbed gently against the mucosa for approximately 5 seconds and it was placed in normal saline. A total of 155 nasal swabs were collected aseptically and were immediately brought to laboratory for further processing. The samples were collected from pet dogs attending to Madras Veterinary teaching hospital, Chennai.

Isolation and identification of *Staphylococcus aureus*

Nasal swabs were inoculated into sterile brain heart infusion (BHI) broth with 10% sodium chloride and incubated at 37 °C for overnight for propagation of Staphylococcus species. Selective plating was done by transferring a loopful of over-night grown inoculum on Baird parker agar media plates containing (5% egg yolk emulsion and 3.5% potassium tellurite) and was incubated at 37 °C for 24-48h to identify characteristic colonies as shown in (figure 1) BP agar medium (circular, smooth, convex, moist, grey black to jet black, frequently with light coloured margin, surrounded by opaque zone and frequently with outer clear zone.

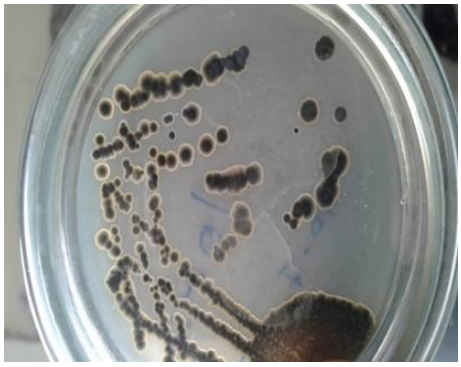

Fig 1: Circular, smooth, convex, moist, gray black to jet black, surrounded by opaque zone on Baird Parker Agar

Polymerase chain reaction

DNA extraction

Template DNA from Staphylococcus strains was carried out as per Lee (2003) [13]. Culture grown in brain heart infusion (BHI) broth was harvested and centrifuged at 12000 rpm for 10 minutes. The pellet was washed twice with 1ml sterile PBS and re-suspended in 100 µl nuclease free water and boiled for 15 min in a boiling water bath then it was subjected for snap chilling on ice for 20 min. The micro centrifuge tube was centrifuged at 12000 rpm for 10 min at 4 °C and the supernatant was used as the template for PCR assay for detection of *mec A* gene and *nuc* gene.

Standardization of PCR assays for detection of *nuc* and *mec A* gene

The various oligonucleotide primers and cyclic conditions used in the study are given in Table 1 and 2. All the 93 isolates were subjected to PCR targeting *nuc* gene and *mec A* gene to identify *S. aureus* and Methicillin resistant *staphylococcus aureus*. PCR was performed in a 25 µl reaction mixture which includes 12.5 µl master mix (AMPLI-QON),10pM concentration of each primer and 2.5 µl of DNA template and remaining volume was adjusted using nuclease free water. PCR Product was subjected to gel electrophoresis (1.5% agarose with 0.8µg/ml ethidium bromide) and the results were documented using gel documentation system (Biorad).

Table 1: Primer Used for Charterization of Isolates of Staphylococcus

S. No	Name	Sequence 5'-3'	BP	References
1	nuc A-F	GCGATTGATGGGTGATACGGTT	267	Brakstad *et al.*,
2	nuc A-R	AGCCAAGCCTTGACGAACTAAAGC		
3	mecA-F	GAAATGACTGAACGTCCGATAA	310	Kobayashi *et al.*,1994
4	mec A-R	CCAATTCCACATTGTTTCGGTCTAA		

Table 2: Cyclic Conditions Used For Primers

Cycling conditions					
Primers	**Initial denaturation**	**Denaturation**	**annealing**	**Extension**	**Final extension**
nuc-F	94 °C, 5min	94 °C, 30s	55 °C, 30s	72 °C, 1min	72 °C,5min
nuc -R	Repeated for 30 cycles				
mec A-F	94 °C, 5min	94 °C, 30s	50 °C, 40s	72 °C, 1min	72 °C, 5min
mec A-R	Repeated for 25 cycles				

Results and discussion

Out of 155 canine nasal swabs screened 93 showed colonies characteristic of staphylococcus species on Baird parker agar plates and the prevalence percentage of Staphylococcus spp. was found to be 60.0%. All the 93 isolates shown colony characteristic are then subjected to PCR assay for the molecular characterization of the isolates by using specific primers which showed 88 (98.62%) PCR positive for thermo nuclease *nuc* gene yielded DNA product size of 181bp and 86 (92.47%) showed that were PCR positives for (*mec A*) gene yielded DNA product size of 310bp (Fig 2 & 3).The positivity has been represented in graphical form (Fig.4).

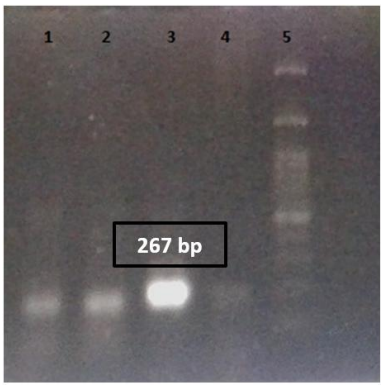

Fig 2: Agarose Gel Showing Amplicons Specific For *Nuc* Gene Of *S. aureus* From Canine Nasal Swabs Lane1,2,4: *nuc* specific amplicon Lane 5: 100 bp DNA ladder. Lane 3: +ve Control (181bp)

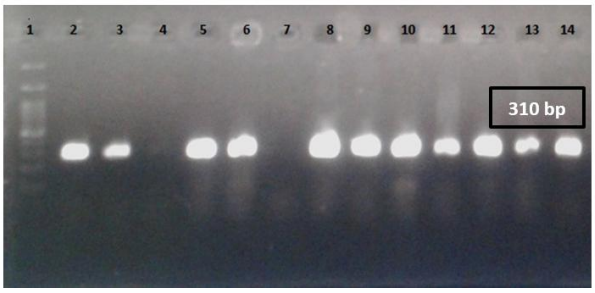

Fig 3: Agarose gel showing amplicons specific for *mecA* gene of *S. aureus* from canine nasal swabs Lane 2,4,5,6,7,8,9,10,11,12,13,14: *mec A* specific amplicon Lane 1: 100 bp DNA ladder. Lane 3: +ve CONTROL (310 bp)

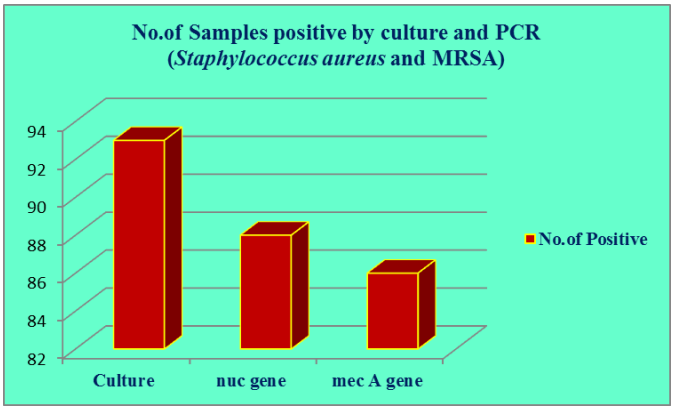

Fig 4: Number of samples positive by culture and PCR for *nuc* gene & *mec A* gene

The emergence of MRSA poses a serious public health threat and described as a cause of nosocomial infection in hospital settings, now MRSA has gained attention as community pathogen (Said-Salim *et al*., 2003) [20]. Brakstad *et al*., (1992) [2] used *nuc* gene for identification of *S. aureus* and for our study we have also used nuc gene as a marker to identify *S. aureus*. Accurate and early diagnosis of MRSA is crucial in effective control of spread of MRSA infections. PCR-based assays are considered as the gold standard for the detection of MRSA, due to the heterogeneous resistance by various phenotypic detection methods displayed by many clinical isolates (Sajith Khan *et al*., 2012) [21]. Cuny *et al*., 2010 [3] utilized the PCR technique for the detection of staphylococcal species from food and clinical samples and to detect *mec A* gene which encodes for methicillin resistance. Genotypic methods are more accurate in detecting methicillin resistant Staphylococci as compared to conventional susceptibility methods. In addition, several culture conditions can also influence methicillin resistance such as the temperature, pH and concentration of NaCl in the medium (Sabath, 1982) [19]. The *mec A* gene has been designated as the gene for methicillin resistance of *S. aureus* because MRSA produce a novel penicillin binding protein (PBP) in addition to the usual PBPs. This is the primary mechanism of Staphylococcal methicillin resistance and it is referred to as intrinsic resistance (Hartman and Thomas 1984) [6]. PBP2a has a low affinity for B Lactam antibiotics and is thought to function in their presence to confer resistance to the bacteria.

High incidence of MRSA in dogs has been reported by was about 31.60% were *mec A* positive. Methicillin resistant *S. aureus* strains, because of their high mortality have become a major concern worldwide (Hookey *et al*., 1998) [8]. Hata *et al* (2010) [7] reported that PCR based *mec A* gene amplification confirmed more than 99% of MRSA isolates. Several reports have documented an apparent increase in the number of MRSA infections in companion animals in recent years (Boag *et al*., 2004; O'Mahony *et al*., 2005) [1, 17]. Our results are in accordance with the results of various authors who has also reported the presence of methicillin resistance with Staphylococcus spp.

Human associated with canines are at great risk of *S. aureus* transmission in comparison with people associated with bovines and the possible causes for *S. aureus* transmission may be due to frequent contact with canines. The colonization of this antibiotic resistant organism in pet animals imposes major risk in both animal and humans. A relatively high percentage of MRSA was reported from humans by Islam *et al*., 2011 reported that 94 clinical strains of *S. aureus* from 255 of human isolates were *mec A* positive in Bangaladesh and Khulaifi Manal *et al*., 2009 [10] reported that 39(92.85%) out of 42 MRSA isolates were positive for *mec A* gene.

Transmission of bacterial strains between companion animals and their owners has demonstrated in several instances. Molecular analysis have shown the presence of indistinguishable MRSA strains in pets and humans living in the same household and have suggested the direction of transmission (Weese 2010) [9]. Both humans and animals are more often colonized than infected and both can act as reservoir of MRSA for recirculation of strains inside the household (Morgan 2008) [16].

Conclusion

This study report about the increase prevalence of MRSA isolates from canine nasal region warrants the need for appropriate control strategies for effective screening by molecular methods and containment of this pathogen which will aid in effective disease management. To conclude, PCR assay was found to be a rapid and accurate procedure for the

detection of MRSA infection compared to conventional methods, since the time taken for PCR assay is much less, prompt treatment can be initiated in view of medical and socio-economic costs.

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