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Identification and Characterization of Methicillin-Resistant *Staphylococcus aureus* Isolated from Bovine Mastitis

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A B S T R A C T

Staphylococcus aureus is a main cause of bovine mastitis and a major pathogen affecting human health. The emergence and spread of methicillin-resistant *Staphylococcus aureus* (MRSA) has become a significant concern for both animal health and public health. This study investigated the incidence of MRSA in milk samples collected from bovine mastitis and characterised the MRSA isolates using genotypic methods. A total of 55 *Staphylococcus aureus* isolates were identified from bovine mastitis by phenotypic methods such as colony morphology, Gram's staining and biochemical tests. Genotypic confirmation was made by using *Sa442* gene based PCR. For the phenotypic detection of methicillin resistant *Staphylococcus*, CHROM agar was found superior to the oxacillin screen agar. This provides an alternative for the detection of MRSA in clinical laboratories, especially when PCR is unavailable. Out of 11 MRSA isolates, only 4 isolates were found to be positive for methicillin resistance targeting a 533 bp fragment, which has led the scientists to think about possible mechanism rendering *Staphylococcus* resistant to beta lactamase other than presence of *mecA* gene. In the phylogenetic analysis, *mecA* gene sequence of all the four MRSA showed maximum nucleotide identity with the Human MRSA isolates which indicated that cross species spilling of MRSA had rendered it as one of the important zoonotic bacteria.

Introduction

Mastitis in dairy animals is considered as one of the most important economic diseases resulting into huge economic loss to the country. In India, the overall economic loss due to mastitis is estimated to be Rs. 7165.51 crores (Bansal and Gupta, 2009). A plethora of bacteria has been isolated and designated as etiological agents of mastitis in dairy animals, but *Staphylococcus aureus* has been reported as the major pathogen that produces both sub-clinical and clinical intramammary infection (Sharma and Sindhu, 2007).

Staphylococci have a reputation of rapidly developing resistance to virtually any antibiotic drugs (Pantosti *et al.*, 2007 and Lentino *et al.*, 2008). The resistance of *Staphylococcus aureus* to antimicrobial agents has been extensively documented and it contributed significantly to the treatment failure (Sudhakar *et al.*, 2009 and Kumar *et al.*, 2010). Resistance to methicillin that indicates resistance to all beta-lactam agents was first reported in 1961, the date that marks the appearance of Methicillin-resistant

Staphylococcus aureus. For many years, Methicillin resistant *Staphylococcus aureus* was considered only a human pathogen, until a report of a MRSA infection in a dairy cow suffered in 1972 (Devriese *et al.,* 1972). Methicillin resistance is due to the acquisition of the *mecA* gene, that encodes a new protein designated PBP2a, belonging to a family of enzymes necessary in building the bacterial cell wall. PBP2a has a very low affinity for β-lactam antibiotics and confers resistance to methicillin and the other β-lactams (Pantosti *et al.,* 2007).

The *mecA* gene is located on a mobile genetic element, named staphylococcal cassette chromosome *mec* (SCC *mec*) inserted in the *Staphylococcus* chromosome up stream to the orf X (Katayama *et al.,* 2000).

The transmission of bovine MRSA to humans is possible and may contribute to outbreaks in human populations (Lee, 2003). Hence, it is necessary to determine the endemic strains of *Staphylococcus* in dairy animals which are highly pathogenic and methicillin-resistant. Sequencing of *mecA* gene of MRS is used to determine the genetic relationship between the animal and human isolates of MRS, to understand the role of animals as reservoir for MRS infection to humans and vice-versa (Vishnupriya *et al.,* 2014).

The objective of the present study was to gain insight into the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) among *Staphylococcus aureus* isolated from bovine mastitis in Gujarat. The MRSA found were characterized by sequencing in order to gain more insight in their epidemiology. In addition, we compared sequence of MRSA isolated from animals with the sequence of humans MRSA isolates available on Genebank in order to investigate whether these strains are related and to examine their zoonotic potential.

Materials and Methods

Isolation and Identification of *Staphylococcus aureus*

A total of 165 milk samples from suspected cases of clinical mastitis in cows (n = 94) and buffaloes (n = 71) belonging to North Gujarat were collected aseptically in sterilised vials. In addition, a total of 34 samples were detected positive for SCM, which included 25 samples from cows and 9 samples from buffaloes were also collected aseptically. Milk samples were collected after cleaning the teats, discarding a few streams of milk and scrubbing the teat ends with cotton balls moistened with 70% alcohol. In all, 199 individual quarter milk samples were obtained. Milk samples were inoculated on the plates of Nutrient Agar by spreading heavy inoculums of thoroughly mixed milk. The plates were incubated at 37°C for 24 hours. Thereafter, colonies showing golden yellow pigmented or white colony colour indicative of presumptive *Staphylococcus aureus* were transferred to Mannitol Salt Agar which is considered as selective medium for *Staphylococcus.* The colonies forming yellow colouration indicative of mannitol fermentation were assumed to be *S. aureus*. Further identification of these presumptive staphylococcal colonies was first based on conventional methods including Gram stain staining, colony morphology, catalase test, oxidase test and coagulase test with rabbit plasma.

Genotypic confirmation of *Staphylococcus aureus*

All the probable *Staphylococcus aureus* isolates were subjected for Species specific sa442 gene based PCR. The primers used were sa442 F AATCTTTGTCGG TACACG ATATTCTTCACG and sa442 R CGTAATG AGATTTCAGTAGATAATACAACA (Martineau *et al.,* 1998).

Phenotypic identification of methicillin resistant *Staphylococcus* isolates

Oxacillin screen agar test

A bacterial inoculum of each strain was made and turbidity was adjusted to 0.5 McFarland. One drop of this suspension was inoculated on Mueller-Hinton agar containing 4.00 per cent of NaCl and 6 µg of oxacillin ml^{-1} (Hi-Media). Plates were incubated at 35^{o}C for 24 hrs. The strain showing growth on the plate containing oxacillin was considered to be resistant to methicillin.

CHROM agar

CHROMagar (Hi-Media) is a new chromogenic medium for the identification of MRSA. For each strain, a bacterial suspension adjusted to 0.5 McFarland was used. Subsequently, a swab was dipped in the suspension and streaked onto a CHROMagar plate. The growth of any pink to mauve colony was considered to be positive, indicating MRSA.

Detection of mecA

The presence of the mecA gene was detected by PCR as described before (Fluit *et al.*, 2001). MecA DNA was amplified with the primers mecA F AAAATCGATGGTAAAG GTTGGC and mecA R AGTTCTGCAGT AC CGGATTTGC. Samples were subjected to 35 cycles consisting of 30 sec. at 94^{o}C, 30 sec. at 55^{o}C an annealing temperature and 1 min at 72^{0}C in a thermocycler. The PCR product was visualized on a 1.5% agarose gel using ethidium bromide and a UV transilluminator.

Sequencing and phylogenetic analysis of Methicillin resistant *Staphylococcus* isolates

Genomic DNA was extracted and then submitted for partial sequencing of *mecA* gene at the Eurofins Genomics (Bangalore, India). The DNA sequencing was performed by using Applied Biosystems BigDye® Terminator v3.1 and v1.1 Cycle Sequencing Kits with Applied Biosystems 3730*xl* 96 capillary DNA Analysers instrument.

The specificity of the sequences obtained, the nucleotide variations and amino acid variations with respect to the *mecA* gene sequence of MRSA strains were determined using BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted for the four MRSA isolates (SKN-4, SKN-5, SKN-6 and SKN-7) along with Human MRSA isolates from Tamil Nadu (KC243783), Pondicherry (JF710611), Portugal (JF946505) and Denmark (KF169798) retrieved from the Gene Bank.

Nucleotide sequences were aligned using the Clustal w alogarithm implemented in the Mega 6.06 program package (Center for Evolutionary Medicine and Informatics, the Biodesign Institute, USA) and Neighbor Joining tree (NJ tree) was constructed.

Results and Discussion

A total of 55 *Staphylococcus aureus* isolates were recovered from bovine mastitis. In Gram's stained culture smears under microscope, all the 55 isolates revealed spherical and irregular clusters like bunch of grapes. Bio-chemical characterization revealed that 100.00, 100.00 and 89.09 per cent isolates were Catalase positive, Oxidase negative and coagulase positive, respectively.

Genotypic confirmation of *Staphylococcus aureus*

All the 55 isolates yielded 108 bp amplicon in sa442 gene amplification which confirmed them as a *Staphylococcus aureus* (Fig. 1)

Phenotypic identification of MRSA isolates

The conventional detection assays are simple and relatively cheap methods for detecting methicillin resistance. Out of 55 *Staphylococcus aureus* isolates, 7 and 11 isolates were detected as methicillin resistant by Oxacillin screen agar and CHROM agar, respectively. Among the two methods used for detection of methicillin resistant isolates, the sensitivity of CHROM agar and oxacillin screen agar methods in comparison to genotypic method was 100.00 and 71.42 57.14 per cent, respectively. Thus, CHROM agar was found superior to the oxacillin screen agar method.

Table.1 Per cent Nucleotide Identity in MRSA Isolates

	KF169798	**JF946505**	**SKN-5**	**SKN-7**	**KC243783**	**JF710611**	**SKN-4**	**SKN-6**
KF169798	100.00	42.99	45.31	47.03	48.39	48.44	44.44	46.46
JF946505		100.00	54.64	58.73	57.17	57.53	56.08	56.88
SKN5			100.00	91.80	**86.27**	85.89	86.92	92.73
SKN7				100.00	**96.10**	95.70	94.35	95.48
KC243783					100.00	99.81	**86.63**	**94.90**
JF710611						100.00	86.27	94.50
SKN4							100.00	95.49
SKN6								100.00

Fig.1 & 2

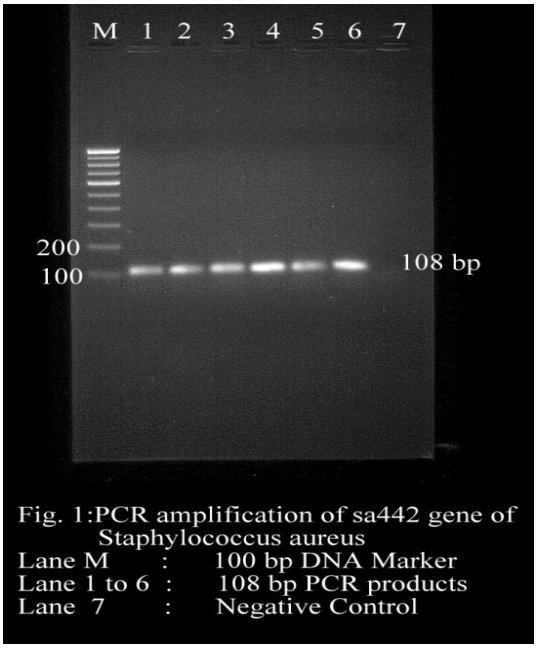


Fig. 1:PCR amplification of sa442 gene of Staphylococcus aureus
Lane M : 100 bp DNA Marker
Lane 1 to 6 : 108 bp PCR products
Lane 7 : Negative Control

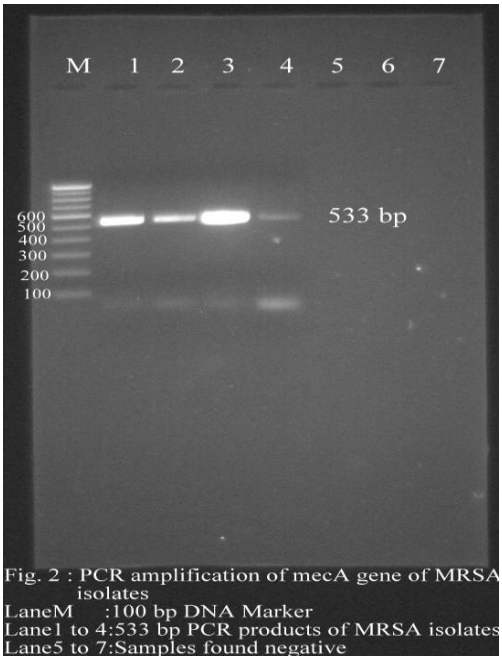
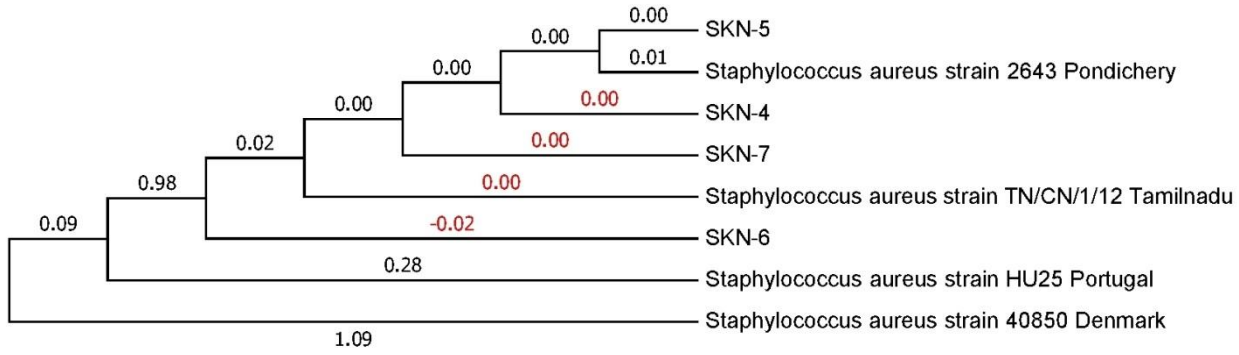


Fig. 2 : PCR amplification of mecA gene of MRSA isolates
LaneM :100 bp DNA Marker
Lane1 to 4:533 bp PCR products of MRSA isolates
Lane5 to 7:Samples found negative

Fig.3



Similar findings were also observed by Taguchi *et al.,* (2004) and Karthy *et al.,* (2009). This provides an alternative for the detection of MRSA in clinical laboratories, especially when PCR is unavailable.

Higher sensitivity of CHROMagar in this study might be attributed to the fact that CHROMagar with cefoxitin supplement was potent inducer of the *mecA* gene; this could explain why heterogeneous MRS populations that variably express the *mecA* gene are better detected by CHROMagar with cefoxitin than medium with oxacillin, which is a weak inductor of PBP2a production. Furthermore, it was less affected by the hyper production of penicillinases, it required no special incubation temperature as was required when the testing was done with oxacillin screen agar (Mallick and Basak, 2010 and Mathews *et al.,* 2010).

Genotypic identification of MRSA isolates

Accurate detection of methicillin resistance in *Staphylococcus aureus* by routine methods is difficult due to the presence of two subpopulation of *Staphylococcus aureus* (*i.e.*, one susceptible and other resistant), which may coexist within a culture. All the cells in culture may carry the genetic information for resistance, but a small numbers can express this kind of resistance in routine susceptibility testing performed in the laboratory. This phenomenon is termed as heterogeneous resistance and occurs in *Staphylococcus aureus* resistant to penicillinase stable penicillin such as methicillin and oxacillin (Cavassini *et al.,* 1999). The basis of most methicillin resistance is the production of an additional penicillin-binding protein, PBP2' or PBP2a, mediated by the *mecA* gene which is an additional gene found in methicillin resistant *Staphylococcus* and with no allelic equivalent in methicillin susceptible *Staphylococcus*. Therefore, the earlier worker (Krishnan *et al.,* 2002 and Brown *et al.,* 2005) considered test based on detection of *mecA* gene using PCR as rapid, accurate, commercially available and the gold standard for the detection of methicillin resistant *Staphylococcus* and found even correctly identify the most heterogeneous and borderline strains. However, in the present study, 11 phenotypical MRSA isolates from bovine mastitis were analysed by PCR for the presence of *mecA* gene. The *mecA* gene was detected only in 4 of 11. Thus, seven *Staphylococcus aureus* isolates phenotypically resistant to methicillin in this study did not carry *mecA* gene. This type of discrepancy in correlation between the *mecA* gene and phenotypically methicillin resistance has been reported earlier by Schnellmann *et al.,* (2006) and Moon *et al.,* (2007). The possible reasons for this type of discrepancy

are that the isolates appeared to show poor expression of *mecA* genes or production of methicillinase (alteration of PBP subtypes) or seem to overproducing β-lactamase. The phenotypic expression of resistance could vary due to growth conditions which are also involved in the expression of methicillin resistance (Zmantar *et al.,* 2008; Turutoglu *et al.,* 2009 and Turkylmaz *et al.,* 2010).

Sequencing and phylogenetic analysis of MRSA isolates

There are increasing reports on MRSA infection or colonization in animals and their zoonotic potential (vanDuijkeren *et al.,* 2004; Malik *et al.,* 2006; Schnellmann *et al.,* 2006; Weese *et al.,* 2006 and Kaszanyitzky *et al.,* 2007). However, a few veterinary reports have compared the *mecA* gene from animal isolates with human MRS strains (Malik *et al.,* 2006 and Schnellmann *et al.,* 2006). Hence, the present study was also intended for comparison of the *mecA* gene from animal MRSA isolates with the human MRSA strains available on Gene Bank.

The same results were also stated for the presence of *mecA* genes from bovine mastitis (Turutoglu *et al.,* 2009 and Vishnupuriya *et al.,* 2014) dogs, cats (Malik *et al.,* 2006) and horses (Schnellmann *et al.,* 2006). Based on the results from the present study and the other studies (vanDuijkeren *et al.,* 2004 and Malik *et al.,* 2006), it can be construed that these isolates might have originally come from humans, considering that the methicillin resistance among human *Staphylococcus* isolates is common. However, in the recent years, it has been suggested that humans in close contact with MRSA infected or colonized animals may be infected (Weese *et al.,* 2006 and Kaszanyitzky *et al.,* 2007) or human infections may be originated by consumption of food-stuff made from infected animals (Lee, 2003).

In conclusion, mecA-positive *S. aureus* isolated from animals in Gujarat, but their prevalence appears to be low. Both phenotypic and genotypic analysis showed that the four MRSA isolates of bovine origin were indistinguishable. It could be concluded that the bovine MRSA strains described in this study were genetically related to the human strain, which may indicates the transmission between bovine and humans. Periodic surveillance for antimicrobial resistance patterns of MRSA isolated from dairy cows with mastitis could be an important measure in detecting the emergence and spreading of such resistance.

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