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Molecular Characterization of Methicillin-resistant *Staphylococcus aureus* (MRSA) Isolates from Ruminants

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ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is one of the leading causes of infections in animals as well as human beings and is associated with significant morbidity, mortality, length of stay and cost burden.

Methods: In this study, 725 samples of nasal swabs and milk were collected randomly from cattle, buffalo, sheep and goats and these samples were inoculated on Mannitol salt agar mixed with Oxacillin Resistance Selective Supplement for the molecular characterization of MRSA isolates through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and polymerase chain reaction (PCR) test.

Result: Out of 725 samples, 171 were found positive for Oxacillin resistant *S. aureus*. The SDS-PAGE showed different bands of molecular weight 13, 17, 20, 24, 26, 28, 33, 36, 39, 43, 47, 59, 64, 72, 86, 97 and 121 kDa. An amplified *mec A* DNA fragment (137) of 533 base pairs (bp) and PCR product of *fem A* (133 isolates) of 510 bp were detected in isolates.

Key words: *fem A*, *mec A* genes, MRSA, PCR, Ruminants, SDS-PAGE.

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen that causes severe morbidity and mortality worldwide. *Staphylococcus aureus*, a unique bacterium, has adaptive power for antibiotics that lead to the emergence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in the 1960s. Recent studies have documented the increased costs associated with MRSA infection. Pneumonia and bacteremia account for the majority of MRSA serious clinical infections, but intra-abdominal infections, osteomyelitis, toxic shock syndrome, food poisoning and deep tissue infections are also important clinical. It is a frequent cause of both subclinical and clinical mastitis in dairy cattle, which causes significant financial losses for dairy farmers all over the world (Seeger *et al*., 2003). It results in both subclinical and clinical mammary gland infections. Moreover, enterotoxigenic *S. aureus* is the cause of almost 10% of food-borne illnesses linked to dairy products (Zigo *et al*., 2021).

Staphylococcus species showed that whole-cell and extracellular protein profiles differed in several protein bands in *Staphylococcus aureus*, *S. epidermidis*, *S. simulans* and other species of Staphylococcus; however, the differences were not sufficient for reliable differentiation of Staphylococcus species by the SDS-PAGE method Berber *et al.* (2003). Abid *et al.* (2019) compared two novel *S. aureus* surface protein extraction methods with the biotinylation method and evaluated the immune-reactivity of extracted proteins. The presence of different band patterns among MRSA isolates has been shown by SDS-PAGE and its importance in epidemiology has also been implied (Gaston *et al*.,1998; Costas *et al*.,1990). The conventional methods are time consuming and difficult so molecular diagnosis of MRSA is increasingly important for rapid detection. During PCR the number and size of the fragments generated are the basis for typing an isolate (Hussain *et al*., 2018; Xia and Wolz, 2014). The polymerase chain reaction is a molecular technique that can rapidly detect the *mec A* gene (Jonas *et al*., 2002; Oliveira *et al*.,

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2002; Rocchetti *et al*., 2018; van Belkum and Rochas, 2018; Abed *et al*., 2020). Developing it for MRSA has been hampered because *mec A* is highly conserved in all staphylococcal species.

MATERIALS AND METHODS

Collection of samples

A total of 725 samples of nasal swab and milk samples were collected for the study. The samples from nasal passage (375) were collected from cattle (100), buffalo (100), sheep (100) goat (75) and The composite milk samples (350) were collected from cattle (100), buffalos (100), sheep (75) and goats (75) maintained at the Livestock Farm Complex (LFC), from the animals presented at Veterinary Clinical Complex (VCC) of College of Veterinary Science and Animal Husbandry and animals maintained by local farmers around the university campus. Isolation and identification of *S. aureus* was done as per the recommendations of the National Mastitis Council (NMC, 1990) and the National Committee for Clinical Laboratory Standards (NCCLS, 1997). Mannitol salt agar mixed with oxacillin resistance selective supplement was used for isolation of MRSA from nasal swab and milk samples. MRSA standard culture obtained from Veterinary Type Culture Collection (VTCC) ICAR-NRC on Equines, *Hisar* was used as standard. The organism on slants was subjected to Gram staining and biochemical tests *viz*. catalase test, coagulase test, methyl red test (MR Test), Voges-Proskaur test (VP-Test), sugar fermentation test, haemolysis on blood agar, DNase test and latex agglutination test.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Genomic DNA was extracted by the procedure described by Ausubel *et al.* (1989). Denatured whole cell proteins were analyzed by SDS-PAGE according to the method described by Laemmli (1970).

Preparation of samples

Isolates of MRSA were plated out on nutrient-agar plates and incubated at 37°C for 24 h. A sweep of colonies from these plates sufficient to give approximately 2 µg bacterial dry weight/ml was inoculated into 150 ml volumes of nutrient broth and incubated at 37°C overnight in an orbital incubator for 3 min at 12100 rpm. The collected cells were washed three times with sterile distilled water and stirred after adding 25 µl SDS sample buffer (0.06 M Tris, 2.5% glycerol, 0.5% SDS, 1.25% β-mercaptoethanol and 0.001% bromophenol blue). The proteins were denatured in boiling water for 5 min. The supernatant was then centrifuged again for 3 min at 12100 rpm, collected in an eppendorf tube and kept at -50°C until the electrophoresis was carried out.

Denatured proteins were analyzed by SDS-PAGE according to Laemmli (1970). This method used a 2 cm layer of 4% acrylamide stacking gel and a 10 cm layer of 10% acrylamide separating gel. Sigma wide range marker was used as molecular weight standard in SDS-PAGE. Electrophoresis was performed with a discontinuous buffer system in a BRL gel apparatus model V16-2BRL Gaithersburg MD, USA. The gel was run at a constant current of 35 mA until the bromophenol blue had reached the bottom. Gels were then stained with Coomassie Brilliant Blue R 250 (Sigma).

Polymerase chain reaction

Subsequent to biochemical characterization, the *S. aureus* isolates that appeared methicillin resistant in diffusion test were subjected to amplification of *mec A* gene and *fem A* gene using protocol with specific primers sets. The amplified products were imaged by running them in 1.5% agarose containing 0.5% µg/ml ethidium bromide.

Preparation of DNA template by heating

All the clinical samples that appeared methicillin resistant in disc diffusion test were cultured in the Brain heart infusion (BHI) broth culture by incubating overnight at 37°C and subsequently, subjected for confirmation of *Staph. aureus* by targeting the *mec A* and *fem A* gene based specific assay. Briefly, 1 ml of overnight broth was centrifuged at 4000 rpm for 15 min. the supernatant was discarded and the pellet was resuspended in 200 µl of nuclease free water (NFW). The Suspension was heated to 94°C for 10 min and immediately chilled on ice to release the DNA after the breakage of the cell. After centrifugation at 8000 rpm, the supernatant was used as a DNA template.

Primers

The following oligonucleotide primers specific for *mec A* and fem A gene sequences of methicillin resistant *S. aureus* from published primer sets were synthesized by Merck India and were used in the present study (Table 1).

Reaction mixture for PCR

The Amplification reaction was performed in a total volume of 25 µl for one PCR reaction as given below (Table 2). The reaction mixture was performed by two different ways as follows:

Gene amplification conditions

The amplification of target sequences was carried out under the following conditions using a thermal cycler (ProFlex PCR machine) (Table 3).

Confirmation of PCR products

The amplified PCR products was confirmed for its excepted size in 1.5% agarose gel prepared in 0.5X TBE buffer as per the method of Sambrook *et al*. (1989) using horizontal submarine electrophoresis apparatus (Torson). A 1.5% agarose gel prepared in 0.5X TBE buffer was boiled for 2 min and allowed to cool down to 50°C. Ethidium bromide was then added in the gel to a final concentration of 0.5 µg/ml and mixed thoroughly. The gel casting platform was then

placed on a leveled surface and the open sides were sealed with adhesive tape was removed.

Confirmation of PCR products

The amplified PCR products were confirmed for their excepted size in 1.5% agarose gel prepared in 0.5X TBE buffer as per the method of Sambrook *et al*. (1989) using horizontal submarine electrophoresis apparatus (Bio-Rad). A 1.5% agarose gel prepared in 0.5X TBE buffer was boiled for 2 min and allowed to cool down to 50°C. Ethidium bromide was then added in the gel to a final concentration of 0.5 µg/ml and mixed thoroughly. The gel casting platform was then placed on a leveled surface and the open sides were sealed with adhesive tape was removed. The set gel with gel casting platform was then submerged in a sufficient quantity (about 1 mm above the gel level) of electrophoresis buffer (TBE) in the electrophoresis tank keeping the wells towards cathode end.

10 ul of PCR product was loaded into respective wells. The molecular weight marker (100bp DNA ladder, Thermo scientific) was also mixed with bromophenol blue dye in similar quantities and loaded into the first well of gel. Electrophoresis was carried out at 5 volt/cm current and the progress of motility was monitored by migration of the dye. At the end of electrophoresis, the gel was visualized and analyzed using a Gel documentation system (Bio-Rad) for an amplified product of the desired length.

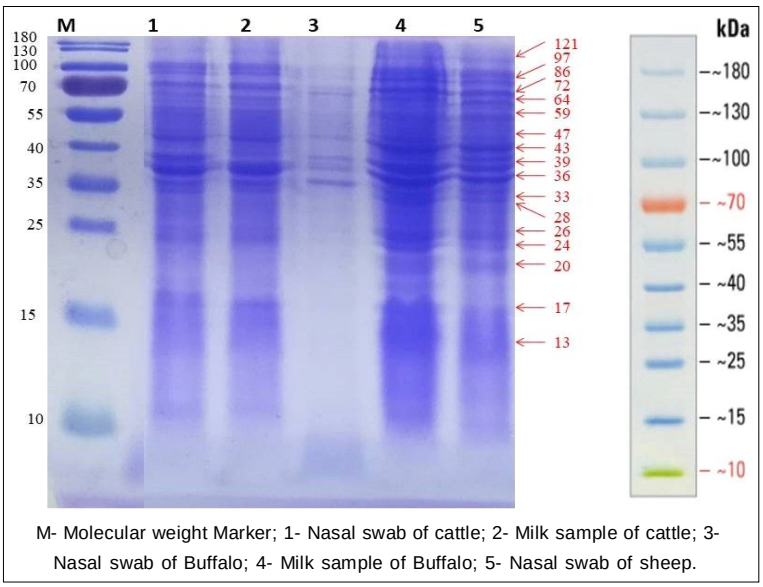
RESULTS AND DISCUSSION

In the present study, samples which included nasal swabs (375) and milk samples (350) were collected randomly from cattle, buffalo, sheep and goats. Out of 725 samples, 96 nasal and 75 milk samples (total 171 samples) were found positive for Oxacillin resistant *S. aureus*. These 171 MRSA isolates were confirmed by Gram's Staining and biochemical tests. All the positive isolates were subjected to Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for the identification of major polypeptides in whole cell protein. All the isolates irrespective of animal species and site of collected sample revealed almost similar molecular weight polypeptides in size and number. The gel showed polypeptides ranging between 20 and 200 kDA. Depending upon the intensity of bands 17 major polypeptides of molecular weight 13 kDA, 17 kDA, 20 kDA, 24 kDA, 26 kDA, 28 kDA, 33 kDA, 36 kDA, 39 kDA, 43 kDA, 47 kDA, 59 kDA, 64 kDA, 72 kDA, 86 kDA, 97 kDA and 121 kDA were observed in whole cell protein of all the *S. aureus* isolates (Fig 1and 2).

The DNA was extracted from all positive *S. aureus* isolates that exhibited methicillin resistance in disc diffusion method and extracted DNA was subjected to Polymerase Chain Reaction PCR for the amplification of *mec A* and *fem A* genes which are considered to be responsible for methicillin resistance. Out of 171 methicillin

Table 1: The oligonucleotide primers used for PCR.

Target gene	Primer name	Nucleotide sequence (5′-3′)	Product length (base pair)	Reference
mec A	Mec -A1	(+) AAAATCGATGGTAAAGGTTGGC	533	Rocchetti *et al*., 2018
	Mec-A2	(-) AGTTCTGCAGTACCGGATTTGC		
fem A	Fem-A1	(+) AAAAAAGCACATAACAAGCG	510	Manikandan *et al*., 2011
	Fem-A2	(-) GATAAAGAAGAAACGAGCAG		



M- Molecular weight Marker; 1- Nasal swab of cattle; 2- Milk sample of cattle; 3- Nasal swab of Buffalo; 4- Milk sample of Buffalo; 5- Nasal swab of sheep.

Fig 1: Whole cell protein profile of MRSA in SDS-PAGE.

resistance *S. aureus* isolates recovered from cattle, buffalo, sheep and goat during this study, *mec A* and *fem A* genes were amplified in 137 and 133 isolates with an amplicon 533 kb (Fig 3) and 510 kb (Fig 4) respectively. The MRSA isolates were positive for *mec A* gene, 19 out of 23 (82.61%), 27 out of 39 (69.23%), 19 out of 23 (82.61%), 18 out of 20 (90.00%), 25 out of 29 (89.66%), 10 out of 11 (90.91%), 9 out of 14 (64.28%), 10 out of 12 (83.33%) in cattle nasal swab sample, cattle milk, buffalo nasal swab, buffalo milk, sheep nasal swab, sheep milk, goat nasal swab and goat milk respectively (Table 4).

Table 2: Composition of reaction mixture using readymade master mix.

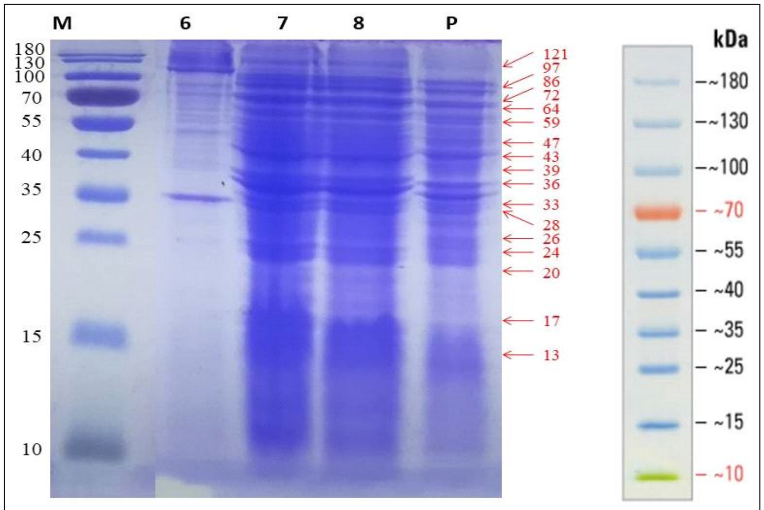
Components	Volume
Master mix (2× Dream taq buffer)	12.5 µl
Forward primer (10.0 pmol)	1.25 µl
Reverse primer (10.0 pmol)	1.25 µl
Nuclease free water	7.5 µl
DNA template	2.5 µl
Total volume	25.0 µl

The MRSA isolates were positive for *fem A* gene, 19 out of 23 (82.61%), 25 out of 39 (64.10), 18 out of 23 (78.26%), 17 out of 20 (85.00%), 25 out of 29 (89.66%), 10 out of 11 (90.91%), 9 out of 14 (64.28%), 10 out of 12 (83.33%) in cattle nasal swab sample, cattle milk, buffalo nasal swab, buffalo milk, sheep nasal swab, sheep milk, goat nasal swab and goat milk respectively (Table 4).

The presence of different band patterns among MRSA isolates has been shown by SDS-PAGE and its importance in epidemiology has also been implied (Gaston *et al*., 1998). In this study depending upon the intensity of bands 17 major polypeptides were of molecular weight 13 kDA, 17 kDA, 20 kDA, 24 kDA, 26 kDA, 28 kDA, 33 kDA, 36 kDA, 39 kDA, 43 kDA, 47 kDA, 59 kDA, 64 kDA, 72 kDA, 86 kDA, 97 kDA and 121 kDA observed in whole cell protein of all the *S. aureus* isolates from cattle, buffalo, sheep and goat. It is supported by the finding of Jayshree, (2018) that analysed human and animal isolates were subjected to SDS-PAGE for the identification of major polypeptides in whole cell protein of isolates with the ranged from 20-200 kDA. Depending upon the intensity of bands out of 23

Table 3: Amplification programme used.

Parameters	*mec A* gene		*fem A* gene	
	Temperature	Time	Temperature	Time
Heated lid	105°C		105°C	
Initial denaturation	94°C	5 min	94°C	5 min
No. of cycles	35		30	
Denaturation	94°C	30 sec	95°C	30 sec
Annealing	55°C	30 sec	55°C	30 sec
Extension	72°C	1 min	72°C	30 sec
Final extension	72°C	5 min	72°C	10 min
Final hold	04°C		04°C	

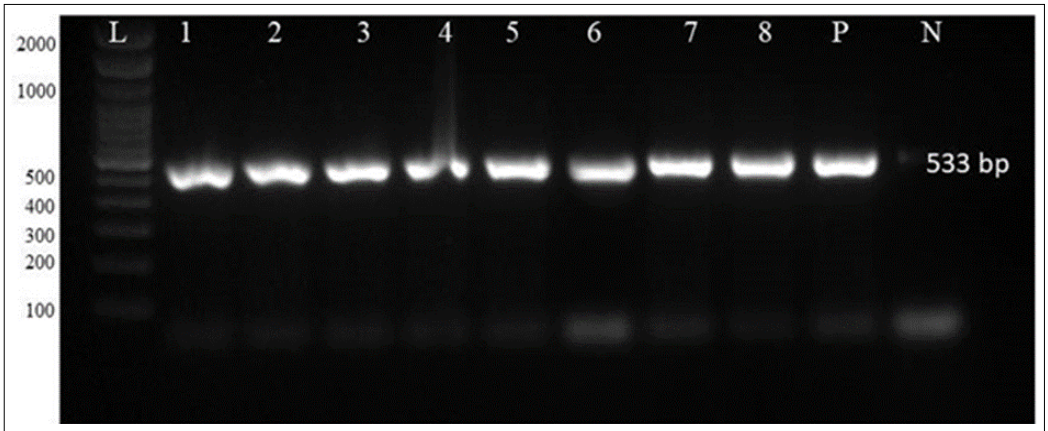


M- Molecular weight marker; 6- Milk sample of sheep; 7- Nasal swab sample of goat; 8- Milk sample of goat and P- Positive control *Staphylococcus aureus.*

Fig 2: Whole cell protein profile of MRSA in SDS-PAGE.

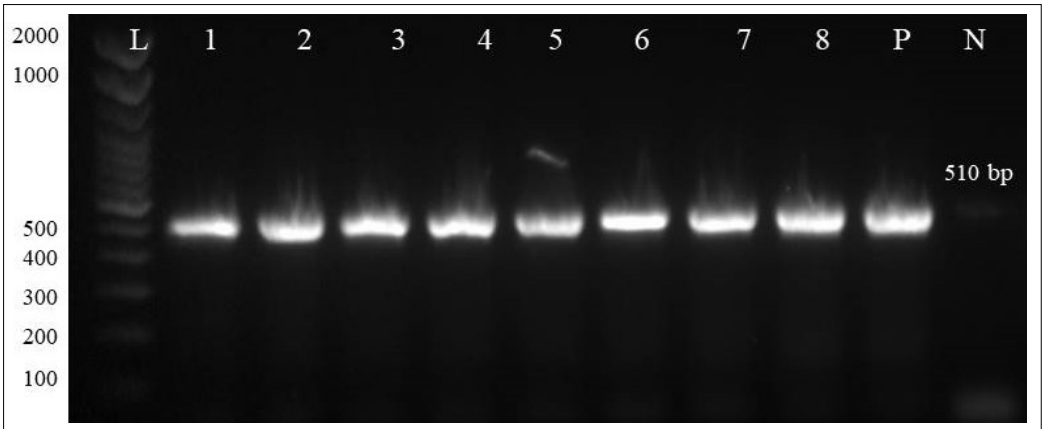
polypeptides 12 major polypeptides were of molecular weight 20 kDA, 28 kDA, 33 kDA, 39 kDA, 43 kDA, 59 kDA, 64 kDA, 72 kDA, 86 kDA, 97 kDA, 121 kDA and 200 KDA. Additionly 11 minor polypeptides were also observed in whole cell protein of all the *S. aureus* isolates. It has been used for taxonomic and typing tools analysis (Manikandan *et al*., 2009).

The DNA was extracted from all *S. aureus* isolates that exhibited methicillin resistance in disc diffusion method and extracted DNA was subjected to PCR for the



L- Ladder marker; 1- Nasal swab of cattle; 2- Milk sample of cattle; 3- Nasal swab of Buffalo; 4- Milk sample of Buffalo; 5- Nasal swab of sheep; 6- milk sample of sheep; 7- Nasal swab sample of goat; 8- Milk sample of goat and P- Positive control *Staphylococcus aureus*; N- Negative control.

Fig 3: Agarose gel electrophoresis of PCR products of amplified *mec A* genes.



L- Ladder marker; 1- Nasal swab of cattle; 2- Milk sample of cattle; 3- Nasal swab of Buffalo; 4- Milk sample of Buffalo; 5- Nasal swab of sheep; 6- Milk sample of sheep; 7- Nasal swab sample of goat; 8- Milk sample of goat and P- Positive control *Staphylococcus aureus*; N- Negative control.

Fig 4: Agarose gel electrophoresis of PCR products of amplified *fem A* genes.

Table 4: Molecular characterization of MRSA isolates by *mec A* and *fem A* gene PCR assay.

Samples	Cattle nasal swab	Cattle milk	Buffalo nasal swab	Buffalo milk	Sheep nasal swab	Sheep milk	Goat nasal swab	Goat milk	Total
MRSA incidence	23	39	23	20	29	11	14	12	171
mec A	19	27	19	18	25	10	9	10	137
Percentage (%)	82.61	69.23	82.61	90.00	89.66	90.91	64.28	83.33	80.11
fem A	19	25	18	17	25	10	9	10	133
Percentage (%)	82.61	64.10	78.26	85.00	86.21	90.91	64.28	83.33	77.78

amplification of *mec A* and *fem A* genes which are considered to be responsible for methicillin resistance. Out of 171 methicillin resistance *S. aureus* isolates recovered during this study, *mec A* and *fem A* genes could be amplified in 137 and 133 isolates with an amplicon of 533 kb and 510 kb respectively. The data indicated a high prevalence of MRSA in cattle. (80.11% for *mec A* and 77.78% for *mec A*).

Singh *et al.* (2015) reported that 18 (24%) isolates from 75 samples of mastitic milk and 10 (28.57%) isolates from 35 samples of nasal swab that appeared to be resistant to methicillin in disc diffusion method showed amplification of *mec A* gene. Similarly, Shanehbandi *et al*., (2014) also reported that all 110 MRSA isolates that screened positive for coagulase, also positive for *mec A* gene. In the present study, *fem A* gene was exhibited by 15 out of 18 MRSA isolates from mastitic milk and 8 out of 10 MRSA isolates from nasal swabs. Maniknandan *et al*. (2011) reported all MRSA isolates from clinical pus samples to be positive for *fem A* gene.

Singh *et al*. (2016) used the disc diffusion method to determine the methicillin resistance of 57 isolates from 240 milk samples. The PCR amplification process revealed that 53 (92.98%) of the isolates were positive for *mec A* and 42 (73.68%) for fem A genes, with amplicon sizes of 510 bp and 533 bp, respectively.

CONCLUSION

In the SDS-PAGE examination, 17 major polypeptides ranging between 20 and 200 kDA were observed in the whole cell protein of all the *S. aureus* isolates. All the MRSA isolates irrespective of animal species and site of collected sample revealed almost similar molecular weight polypeptides in size and number. Both *mec A* and *fem A* genes are responsible for methicillin resistance in methicillin resistance *Staphylococus aureus* (MRSA).

Conflict of interest statement

All authors declared that there is no conflict of interest.

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