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Antimicrobial Resistance Profile and Molecular Characterization of *Staphylococcus aureus* Isolated from Subclinical Mastitis of Dairy Cows in Mathura Region

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ABSTRACT

Background: Subclinical mastitis is the most underrated yet economically important disease of livestock. It goes undetected by clinical examinations, making routine surveillance and monitoring necessary for its detection. Among causative agents, *Staphylococcus aureus* is the most crucial one. The present study determines the prevalence, antimicrobial resistance profile and molecular characterization of *S. aureus* from subclinical mastitis of cattle in the Mathura region.

Methods: The present research was conducted during 2019-2021 and different gaushalas and dairies in and around the Mathura region were screened for mastitis by California mastitis test and somatic cell count. The samples positive were further tested by bacterial, biochemical *and* molecular tests along with an antimicrobial resistance profile.

Result: Our research found a significant amount of *S. aureus* in subclinical samples with the presence of *mecA* gene suggesting MRSA. The public health importance of *S. aureus* and emerging resistance against antibiotics demands regular monitoring and effective use of antimicrobial agents against the MRSA isolates.

Key words: Antimicrobial resistance, MRSA, *Staphylococcus aureus,* Subclinical mastitis.

INTRODUCTION

Subclinical mastitis (SCM) represents a significant proportion (20-25%) of the burden of mastitis in modern dairy management (Wilson *et al.*, 1997). It contributes to two-thirds of the economic losses in total milk production (Radostits and Arundel 2000; FAO, 2014). It affects milk quality and quantity causing a great economic loss for producers. In India, economic loss due to mastitis is reported as INR 6,053.21 crore, where the majority of the loss was found due to sub-clinical mastitis (70 to 80%) which accounted for around INR 4,365.32 crore (Kumari *et al.,* 2018).

In subclinical cases, animals are outwardly healthy and must be diagnosed using the california Mastitis Test (CMT) and Somatic Cell Count (SCC) (Pantoja *et al.*, 2009). SCC primarily comprises white blood cells (leucocytes), including macrophages, lymphocytes and polymorphonuclear leucocytes (essential neutrophils), which are produced by the cow's immune system in response to an infection. Upon bacterial invasion of the bovine mammary gland, leucocytes are recruited into the gland from the bloodstream, increasing the SCC, measured in the number of cells per ml of milk (Harmon, 1994). The most frequently used cutoff value to define subclinical mastitis is 2, 00, 000 cells/ml (Pyorala, 2003).

Staphylococcus aureus represents a major agent of contagious bovine subclinical mastitis and it commonly spreads from infected to non-infected cows at milking in absence of proper hygienic and managemental practices. Prevalence of subclinical mastitis in dairy animals caused by *S. aureus* has been reported in recent years from developing countries including Iran (Beheshti *et al.*, 2011), Brazil (Busanello *et al.*, 2017), Ethiopia (Birhanu *et al.*, 2017; Tegegne *et al.* 2021), Egypt (Algammal *et al.*, 2020; Moustafa *et al.* 2021) and Pakistan (Javed *et al.,* 2021). In India, subclinical mastitis in cattle and buffaloes has been reported in different states such as Karnataka, Punjab *and* others (Hegde *et al.,* 2013; Kaur *et al.,* 2015; Das *et al.,* 2017).

In recent years, extensive and indiscriminate use of antimicrobials in the treatment and control of mastitis has led to the emergence of resistant pathogens. Methicillin-Resistant *Staphylococcus aureus* (MRSA) carrying the *mec*A

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gene for a modified low-affinity penicillin-binding protein, conferring resistance to methicillin and most other β-lactam antibiotics pose a public health concern (Javed *et al.*, 2021; Moustafa*et al.,* 2021; Shrestha *et al.,* 2021). Likewise, some bovine MRSA isolates are multi-drug resistant against various antimicrobial classes (Abdeen *et al.*, 2021; Anter *et al.,* 2021; Khazaie and Ahmad, 2021).

Thus, subclinical mastitis is not only economically important to the farmers but also has public health significance associated with potential zoonotic risk and dissemination of livestock-acquired multi-drug resistant organisms. This highlights the importance of continuous surveillance and monitoring for antimicrobial resistance in veterinary and public health. The present study aimed to investigate the prevalence of subclinical mastitis in dairy animals in and around the Mathura region, the antimicrobial resistance profile *and* molecular characterization of *S. aureus* isolates.

MATERIALS AND METHODS

Sample

Milk samples (n=2135) of cows collected from different organized and unorganized dairy farms in and around the Braj region of Mathura were screened by California Mastitis Test (CMT) and samples positive in CMT were subjected to somatic cell count (SCC) for detection of SCM.

Bacterial isolation and identification

SCM samples were inoculated in BHI broth and incubated for 18-24 h at 37°C. They were streaked on nutrient agar and mannitol salt agar plates for 24 h at 37°C. Pure colonies were identified for morphological, cultural and biochemical characteristics.

Polymerase chain reaction (PCR)

The isolates were tested for the presence of *23S rRNA* gene of *S. aureus* isolates and *mecA* and *mecC* gene of MRSA. For this, bacterial DNA was extracted using a GenElute Bacterial Genomic DNA kit (Sigma -Aldrich) and the eluted DNA was stored at -20°C. Oligonucleotide primers were custom synthesized from Eurofins Genomics India Private Limited (Table 1).

The PCR was done in 25 µl reaction mixture containing 2.5 µl of 10 × KAPA Taq Buffer A with $MgCl_2$ (1.5 mM at 1 ×), 0. 5 µl of 10 mM dNTP Mix, 1 µl of each forward and reverse primers, 1 µl of DNA template and 19 µl of nuclease-free water in Thermocycler (ThermoFisher Scientific) with an initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation for 1 min at 94°C, annealing at 60°C for 1 min, extension at 72°C for 1 min and a cycle of final extension at 72°C for 10 min for *23S rRNA.* For *mecA* and *mecC* initial denaturation at 94°C for 5 min followed by 30 cycles each of denaturation for 30 sec at 94°C, annealing at 59°C for 1 min, extension at 72°C for 1 min *and* a cycle of final extension at 72°C was followed. The PCR products were electrophoresed with 6X loading dye (Sigma-Aldrich) in 1.5% agarose gel pre-mixed with 1% ethidium bromide (5 µg/100 ml) in 1× TAE at 80 V for 30 min. GeneRuler 50 and 100 bp DNA Ladder, ready-to-use (Thermo fisher) were run along with samples. The amplified products were visualized under the Gel documentation system (Bio-Rad).

Antimicrobial sensitivity test

Antimicrobial susceptibility of the bacterial isolates was determined by the disc diffusion (Bauer-Kirby) method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2018). A bacterial suspension of 0.5 McFarland standards were used on Muller Hinton Agar plates. The antibiotic disks were placed onto the agar surface and kept at 37°C for 24 h for incubation. The zone of inhibition by various antimicrobials was noted and the result was interpreted as susceptible, intermediate and resistant.

RESULTS AND DISCUSSION

The current study showed a 7.82% (167/2135) prevalence of subclinical mastitis detected by CMT and 3.27% (70/2135) by somatic cell count. Similar to this, subclinical mastitis detected by CMT was higher than by somatic cell count was also reported by Abed *et al.* (2021). In India, Preethirani *et al.* (2015) reported a prevalence of SCM upto 48.4% by SCC and 45.8% by CMT from South India. The combination of CMT and SCC was found ideal for determining the presence of SCM in large ruminantsand was supported by Preethirani *et al*. (2015) in South India.

The prevalence of subclinical mastitis in dairy cattle and buffalo was reported to be high in developing countries as reported to be 26.95% in Sindh, Pakistan (Baloch *et al.,* 2016), 45.97% in Faisalabad, Pakistan (Javed *et al.,* 2021), 49% in Srilanka (Rahularaj *et al.,* 2019), 35.9%, 28%, 46% and 47.16% in Egypt (Algammal *et al.,* 2020; Abdeen *et al.,* 2021; Abed *et al.,* 2021; Moustafa *et al.,* 2021) and 49% in Ethiopia (Tegegne *et al.,* 2021). In India, a higher prevalence has been reported in buffaloes by Preethirani *et al.* (2015) from South India. The lower prevalence in our study can be

Table 1: Primers for *23S rRNA, mec*A and *mec*C genes of *S. aureus.*

Gene	Oligo name	Sequence 5′-3′	Yield Nmol	Amplicon size (bp)	Reference
23S rRNA	23SrRNA F	AGCGAGTCTGAATAGGGCGTTT	35.40	894	SOP, INFAAR
	23SrRNA R	CCCATCACAGCTCAGCCTTAAC	38.20		
mecA	mecA F	TCCAGATTACAACTTCACCAGG	36.90	162	
	mecA R	CCACTTCATATCTTGTAACG	41.90		
mecC	mecC F	GAAAAAAAGGCTTAGAACGCCTC	31.30	138	
	mecC R	GAAGATCTTTTCCGTTTTCAGC	38.90		

due to the difference in the range of SCC taken into the consideration. In the present study, SCC at 2 lakhs/ml of milk was taken as positive for SCM and the rest of the other samples showing a higher range were ignored. Various geographical, topographical *and* climatic conditions also determine the prevalence rate while the breeds of the large ruminants also play a significant role in harbouring the disease. The exotic and mixed breeds are more susceptible to any kind of ailments while indigenous breeds are much more resistant and hardy (Hoffmann,2010).

Out of 56 *Staphylococcus* spp. isolates identified on basis of morphological, cultural *and* biochemical characteristics, 24 (42.85%) isolates confirmed the 894 bp amplicon in PCR for the presence of *23S rRNA* gene of *S. aureus* (Fig 1). Out of 56 *Staphylococcus* spp. isolates tested for the presence of methicillin-resistant *mecA* and *mecC* genes in PCR, 11(19.64 %) isolates showed 162 bp amplicon for the presence of *mecA* gene and no isolate was positive for *mecC* gene (Fig 2). Finally, out of 24 (42.85%) isolates positive for *23S rRNA* gene of *S. aureus,* six (26.08%) isolates showed the presence of*mecA* gene whereas, rest five (21.73%) isolates positive for *mecA* gene were negative in *23S rRNA* gene of *S. aureus.*

The prevalence of *S. aureus* in the present study was 34.28% (24/70) in the Mathura region determined genotypically by the presence of *23S rRNA* gene. A similar

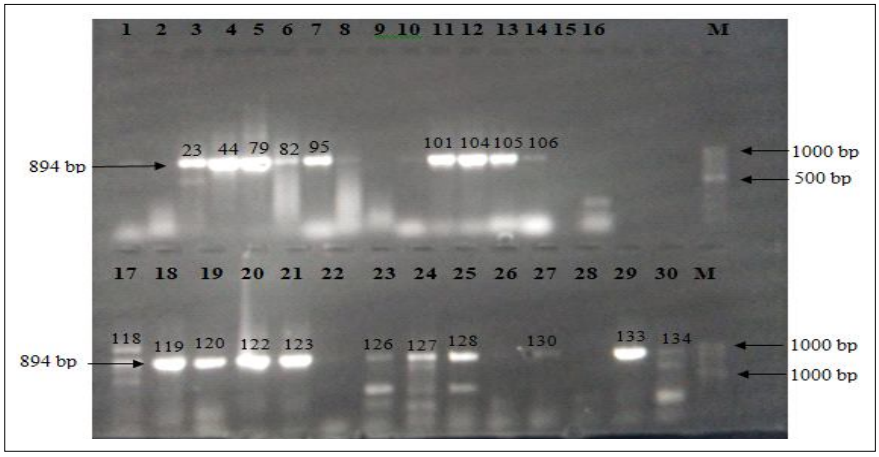

Fig 1: Agarose gel electrophoresis showing amplification of 23*SrRNA* gene of *Staphylococcus aureus.*
Lane M: GeneRular 50 bp DNA ladder (Tharmo Scientific SM0373).
Lane 3-7, 11.14, 17-21, 23-25, 27.29 and 30: Bacterial islotes positive for 23*SrRNA* gene (894 bp).

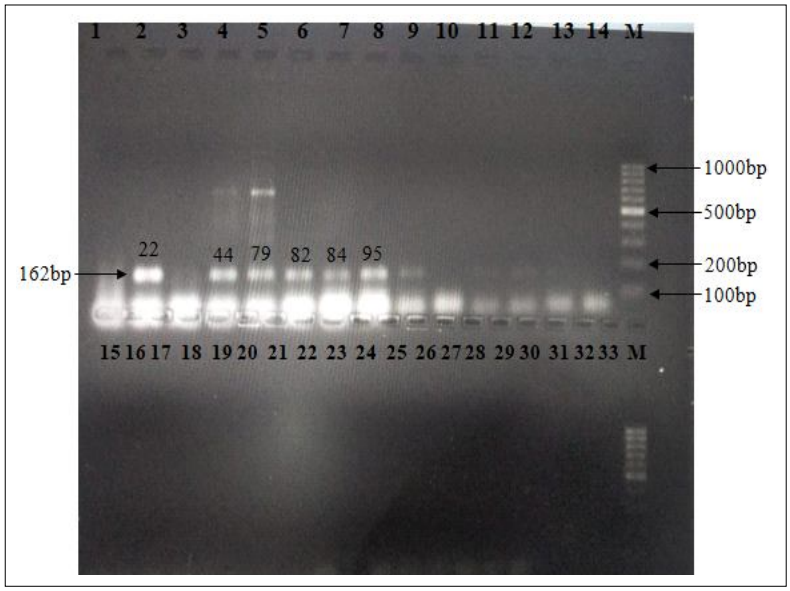

Fig 2: Agarose gel electrophoresis showing amplification of *mecA* gene of MRSA.
Lane M: 100 bp DNA Ladder (Thermo Scientific SM0241).
Lane 2, 4-9: Bacterial islolates positive for *mecA* gene (162bp).
Lane 1, 3, 10-33: Bacterial islotes negative for *mecA* gene.

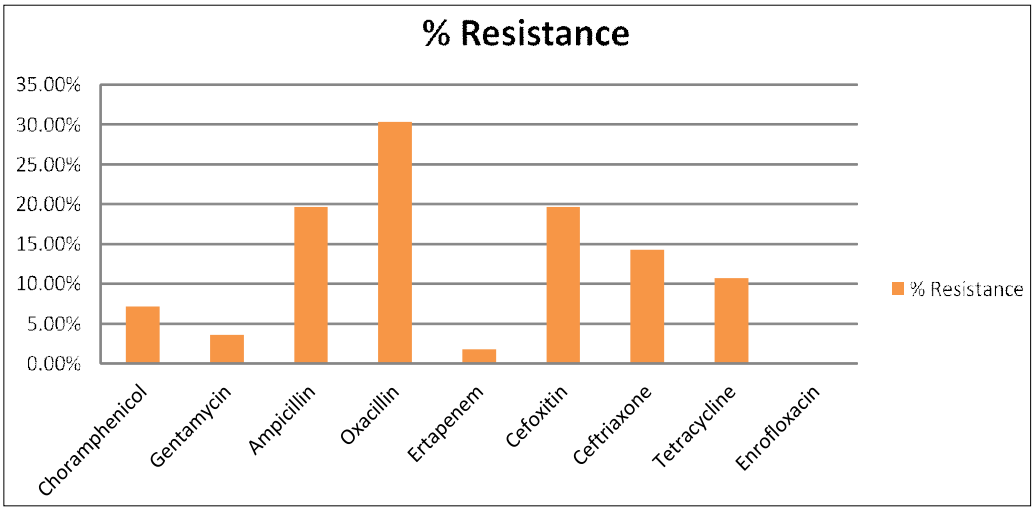

Fig 3: Drug resistant pattern of *Staphylococcus* isolates.

prevalence rate of *S. aureus* was reported in studies conducted in Pakistan at 37.14% (Javed *et al.,* 2021) and 44.9%, (Abed *et al.,* 2021). A lower prevalence of *S. aureus* (7.3%) was reported by Preethirani *et al.* (2015) in buffaloes in South India and Egypt by Moustafa *et al.* (2021) as 24.4% *S. aureus*. *S. aureus* is considered to be the predominant causative agent for causing subclinical mastitis.

Antimicrobial therapy is the chief component of modern clinical practice but due to the excessive use of antibiotics, the incidence of antibiotic-resistant strains of *S. aureus* has direfully increased and made the treatment process very complicated (Altaf *et al.,* 2020). The development of antibiotic resistance in pathogens has emerged as a serious public health concern as this pathogen can be transferred to human beings through improper handling or consumption of infected milk or meat products (Caruso *et al.,* 2016).

In the present study, the prevalence of MRSA was 19.64% (11/56) for the presence of *mecA*gene possessing *S.aureus* isolates. It was reported to be slightly higher than previous studies reporting 13.68% in Iran (Khazaie and Ahmad, 2021), 14.12% in Pakistan (Javed *et al.,* 2021), 10.71% in Egypt (Abdeen *et al.,* 2021) and 6.9% in Nepal (Shrestha *et al.,* 2021). However, a very high percentage was reported from Egypt 90.78% by Moustafa *et al.* (2021) and 100% by Algammal *et al.* (2020). Both of them reported the prevalence based only on phenotypic characters as compared to genotypic characterization for the same.

The percentage of resistance found for *Staphylococcus* spp., for chloramphenicol, gentamicin, ampicillin, oxacillin, ertapenem, cefoxitin, ceftriaxone and tetracycline were 7.14%, 3.57%, 19.64%, 30.35%, 1.78%, 19.64%, 14.28%, 10.71%, respectively while Enrofloxacin being completely susceptible for all the isolates in the present study (Fig 3). Our study showed 12.5% (7/56) of multidrug resistance in *Staphylococcus* spp. for 3 or more than 3 classes of antimicrobial drugs used in common veterinary practice. Anter *et al.* (2021) reported a prevalence of 25 % MDR in *S. aureus* from mastitic milk in dairy cows. The public health importance of *S. aureus* and emerging resistance against antibiotics like oxacillin, ampicillin and cefoxitin drugs having more resistance, demands regular monitoring and effective use of antimicrobial agents against MRSA isolates.

CONCLUSION

In the present study, the detection of MRSA in subclinical mastitis suggested regular screening of subclinical mastitis at the farm level as it may go undetected due to a lack of knowledge and necessary diagnostic facilities. The prevalence report and detection of multi-drug resistant bacteria are very meagre in Braj region. Therefore, the screening of cows in gaushalas for subclinical mastitis and detection of multi-drug-resistant bacteria is important not only for effective treatment but also to prevent the transfer of the multi-drug-resistant bacteria to humans.

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Conflict of interest: None.

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