The Pharma Innovation Journal 2021; SP-10(11): 2215-2218

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(11): 2215-2218

www.thepharmajournal.com
Received: 08-09-2021
Accepted: 09-10-2021

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Detection of methicillin resistant *Staphylococcus aureus* from milk of bovines suffering from mastitis

Jasleen Kaur, Pawanjit Singh Cheema, Naresh Kakkar, Jeenu Manoj and Rajesh Chhabra

Abstract
Methicillin resistant *Staphylococcus aureus* (MRSA) a common 'superbug' poses a clinical threat in human beings as well as animals and is a serious pathogen leading to variety of infections. The present study was conducted to determine the occurrence of MRSA in milk of mastitis buffaloes. A total of 232 quarter milk samples received in College Central Laboratory for routine examination were processed for identification and isolation of *Staphlococcus aureus*. Preliminary identification of *Staphylococcus aureus* was done on the basis of growth characteristics on blood agar and mannitol salt agar, catalase test, oxidase test and HiStaph latex agglutination kit. One hundred and two isolates were identified as *Staphylococcus aureus* with yellowish discoloration around the colonies in manitol salt agar and typical small yellow colonies on blood agar. For detection of MRSA, growth on MeReSa agar and PCR assay targeting *mecA* gene was used. On the basis of growth on MeReSa agar, 11 (10.78%) isolates were identified as MRSA showing bluish green colonies whereas PCR assay revealed 33 (32.35%) were found to possess the *mecA* gene with 162 bp product size. The presence of such a large proportion of MRSA in milk from mastitis animal is a significant challenge for food industry and dairy industry.

Keywords: MRSA, *mecA* gene, MeReSa agar, Mastitis, PCR assay

Introduction
Mastitis is a multifactorial disease imparting huge economic loss to the dairy sector worldwide (Constable *et al*., 2017). In India, losses have been estimated to the tune of Rs. 7165.51 crores per annum (Bansal and Gupta, 2009) [4]. There are over 250 known causative agents of mastitis, including bacteria, fungi, algae and viruses (Bhuvana and Shome, 2013) [5]. Among different causative agents, *Staphylococcus aureus* (*S. aureus*) is the most common cause leading to both clinical and subclinical mastitis in bovines (Charaya *et al*., 2014; Sharma *et al*., 2018, Chhabra *et al*., 2020) [7, 14, 8]. *S. aureus* can produce more than 30 virulence factors, including surface-associated factors, exotoxins and degradative enzymes that contribute to establishing and maintaining infection (Marechal *et al*., 2011). These factors include fibrinogen-, fibronectin-, collagen-binding proteins, exotoxins, hemolysins (alpha, beta, delta, and gamma), leukocidins, toxic shock syndrome toxin, epidermolysins, antibiotic resistance genes (*mec*A, *bla*Z) and, biofilm formation. These factors allow it to colonize, invade and multiply in bovine mammary epithelial cells which in turn make antimicrobial agents poorly effective (Marechal*et al*., 2011; Spoor *et al*., 2013) [15]. Among different virulence factor, methicillin resistant property makes the bacteria resistant to multiple antibiotics particularly β-lactam antibiotics (Etinosa *et al*., 2016) [9]. Methicillin resistant property of bacteria is mediated by *mecA* gene located on the mobile element of the staphylococcal chromosome cassette *mec* (*SCCmec*). Because of the significance of MRSA infection in humans, and the common use of cloxacillin, an antimicrobial similar to methicillin/oxacillin, for mastitis treatment or prevention, pressure on the dairy industry to monitor MRSA infections is increasing (Barkema *et al*., 2009) [3]. Therefore, the present study was conducted to determine the presence of methicillin resistant property in *Staphylococcus aureus* isolated from bovine milk samples.

Material and methods
Sample processed and preliminary identification of *S.aureus*
A total of 232 quarter milk samples received in college central laboratory for routine examination were processed for identification and isolation of *Staphlococcus aureus*. Preliminary identification of *Staphylococcus aureus* was done on the basis of growth characterisitics on blood agar and mannitol salt agar, catalase test, oxidase test and HiStaph latex agglutination kit.

Phenotypic identification of methicillin resistant *S. aureus*
Colonies were inoculated on Hi Chrome Me Re Sa Hi Veg agar for 24 hrs at 37°C for methicillin resistance. Bluish green colonies on Hi Chrome Me Re Sa Hi Veg agar were considered as methicillin resistant.

Genotypic identification of *S. aureus* for methicillin resistance

Primers used for PCR
Published oligonucleotide primers of interest for methicillin resistance targeting *mecA* gene F: TCCAGATTACAACTTCACCAGG and R: CCACTTCATATCTTGTAACG 9 (Oliveira, D.C. and deLencastre, H., 2002) were purchased from Integrated DNA Technology (IDT) in purified and lyophilized form. Stock solution (100 µM) of primers was prepared according to the instructions supplied by the manufacturer. For preparation of working solution (10 µM), 10 µl of stock solution was added to 90 µl of Nuclease Free Water (NFW).

Positive and negative control for PCR assay
DNA extracted from MRSA ATCC strain no. 700699 and MSSA ATCC strain no. 25923 was used as positive and negative control for PCR assay for detection of *mecA* gene for methicillin resistance. NFW was taken as negative control.

DNA extraction
Genomic DNA of *S. aureus* was extracted using QIA amp Blood Kit (Qiagen, Germany) following the manufacturer's protocol.

Amplification of DNA by PCR assay
DNA extracted from pure colonies were used for amplification by PCR assay. A total of 25 µl reaction mixture was prepared in 200 µl thin walled PCR amplification tubes. The reaction contained 12.5 µl GoTaq Green Master Mix (2X) (Promega), 1 µl of each forward and reverse primer of 10 µM concentration and 3 µl template DNA of sample to be tested. Volume of NFW was calculated and added to complete the reaction volume to 25 µl. PCR tube was vortexed and spinned for 10 sec. PCR amplification was performed in thermo cycler (Bio-Rad, USA).

Thermo cycler conditions
Same conditions were used in PCR assay as Oliveira, D. C., & de Lencastre, H., 2002 with initial denaturation at 94°C-5 min, 30 cycles of denaturation at 94°C-30 sec, annealing at 59°C-60 sec and extension at 72°C-60 sec followed by final extension at 72°C-10 min.

Agarose gel electrophoresis for PCR products
PCR products were run on a 1% w/v agarose gel containing ethidium bromide (0.5 µg/ml in 1x TAE buffer (Tris-acetate-EDTA) at 75 V for 1 hr, after loading 10 µl of PCR product per well. A 100 bp plus ladder (Molecular marker) was included for estimation of size of amplicon for 23S rRNA gene. After the completion of electrophoresis, the gels were viewed on a UV transilluminator and photographed for record.

Results
A total of one hundred and two isolates were identified as *Staphylococcus aureus* with yellowish discoloration around the colonies in manitol salt agar as shown in fig.1 and typical small yellow colonies on blood agar as shown. Phenotypic characterization of 102 isolates was done to determine methicillin resistant property by growing on MeReSa agar and 11 isolates (10.78%) manifested characteristic bluish green colonies on MeReSa agar as shown in fig. 2. Similarly, all the isolates identified as *S. aureus* by molecular assay, were subjected to identification of *mecA* gene. Amplification of *mecA* gene was seen in 32.35% isolates showing characteristics 162 bp amplicon as shown in fig. 3.

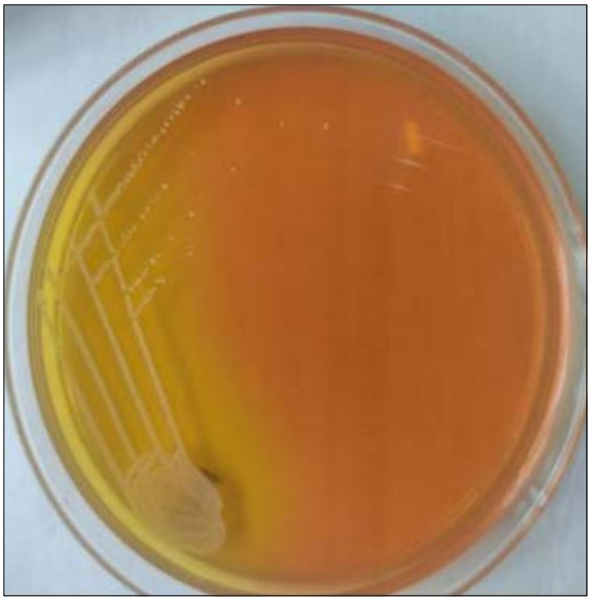

Fig 1: Growth of *S.aureus* on MSA showing bright yellow color discoloration around colonies

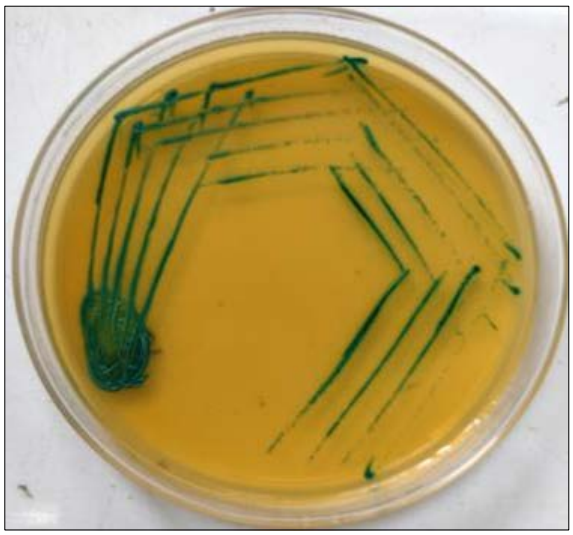

Fig 2: Growth of MRSA on MeReSa agar showing bluish green color colonies

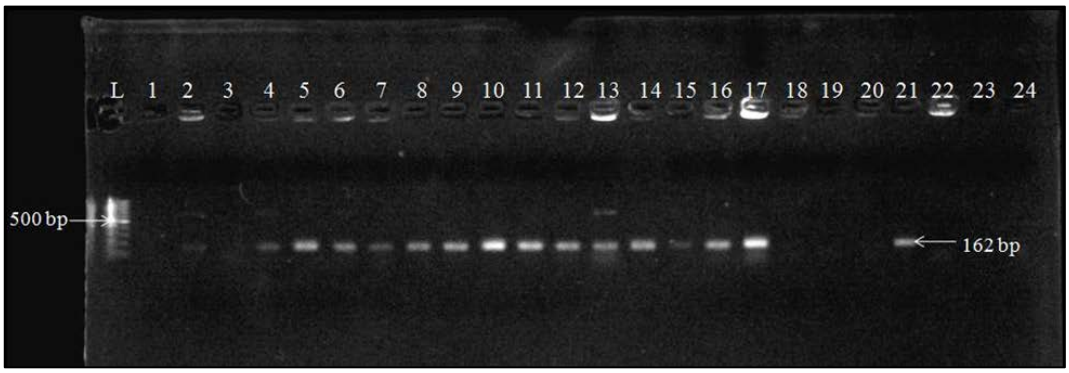

Fig 3: Agarose (1%) gel electrophoresis result of PCR for molecular identification of MRSA showing 162 bp amplicons of *mecA*gene. L: 100bp ladder; 2, 4-17: Positive test samples; 1, 3, 18-20, 23, 24: Negative test samples; 21: *S. aureus* ATCC 700699; 22: *S. aureus* ATCC 25923 (Negative control).

Discussion

Resistance to methicillin is attributed to the existence of *mecA*gene on the *S. aureus* chromosome, which is encoded for the synthesis of PBP2a. As compare to present study, lower percentage of MRSA was reported by Hamid *et al*., 2017 [12]; Biswas *et al*., 2018 [6]; Girmay *et al*., 2020 [10] whereas higher percentage by Guimaraes *et al*., 2017; Algammal *et al*., 2020 [2]; Ahmed *et al*., 2020 [10]. Variation in different researches can be attributed to many factors *viz*. different antibiotics being used in different area for treatment purposes and difference in managemental factors. Higher percentage of MRSA isolated in present study from clinical mastitis cases is an alarming situation for most of dairy herds and dairy sector as it can lead to huge economic losses and also possess risk of zoonotic spread of bacteria to human civilization. The variation in results may also be due to the fact that MRSA carried many resistant determinants in chromosome and plasmids. Mastitis caused by MRSA are difficult to treat because of resistant to antibiotic and can spread to human beings causing numerous type of infection involving different parts of body. Phenotypic determination of methicillin resistant property by growth on MeReSa agar is based on the principle of cleavage of chromogenic mixture present in the composition, which leads to its conversion to bluish green mixture by MRSA. Only 10.78 % of isolates showed growth on MeReSa agar, as compared to 32.35 % by *mecA* gene. This may be related to non-expression of gene phenotypically. This can be attributed to possible involvement of other genes in the process of beta lactam resistance which can affect the expression of *mecA* gene or some strains may produce low level of PBP2a thus escaping classical detection and phenotypically misidentified as methicillin sensitive despite carrying *mecA* gene. Moreover, the detection of a gene does not necessarily means that it is expressed. The presence of such a large proportion of MRSA in milk from mastitis animal is a significant challenge for food industry and dairy industry.

Acknowledgment: The authors acknowledge the financial support rendered by Dean, College of Veterinary Sciences and Prof.& Head, Department of Veterinary Microbiology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar.

Ethical matters: Approval for research was not required as the clinical milk samples received for examination of mastitis in College Central Laboratory were processed.

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