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Phenotypic and genotypic detection of MRSA isolated from bovine clinical and sub clinical mastitis

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Abstract
Methicillin-resistant *Staphylococcus aureus* is a clinically prevailing bacterium resistant to numerous antibiotics. The genetic factor *mec* genes are known to responsible for methicillin resistance. In this study, a total of 68 *S. aureus* isolates from clinical and sub clinical bovine mastitis were screened for cefoxitin disk diffusion assay and 52 isolates were identified as MRSA. Out of these MRSA, 11 isolates were either habouring *mec*A or *mec*C genes with higher incidence of *mec*A detection than *mec*C gene. The molecular detection of *mec*A and *mec*C genes among MRSA further add in molecular epidemiology with understanding the rise of multidrug resistance.

Keywords: *mec*A, *mec*C, MRSA, clinical and subclinical mastitis

Introduction
Mastitis is an altered condition of the mammary gland and characterized by pathologic changes in the glandular tissue as well as physical, chemical, and bacterial changes in milk. Subclinical mastitis in particular is one of the most prevalent types of disease in highly productive dairy animals (Lakshmi and Jayavardhanan, 2016) [1]. Mastitis in animals leads to decline in milk quality and yield, have a significant economic impact on the dairy industry (Saluja *et al*., 2005) [2].
Staphylococcus aureus as the most significant mastitis-causing agent accounts for about one-third of the cases of clinical and subclinical mastitis (Topuzoglu *et al*., 2015) [3]. Penicillin (a β-lactam antibiotic) was one of the antimicrobials to which *S. aureus* developed resistance, and penicillin-resistant strains were isolated from different clinical and non-clinical samples (David and Daum, 2010) [4]. It has remarkable capabilities to develop resistance against multiple antibiotics as methicillin-resistant *Staphylococcus aureus* (MRSA) strains identified quickly after its widely use. Before its discovery in mastitic cow in 1972, MRSA was initially believed to be associated with disease in human (Devriese *et al*., 1972) [5]. After emergence of MRSA, its infection in humans, domestic and wild animals were reported from Asia, Europe, Australia, North and South America (Rich and Roberts, 2004 [6]; Girmay *et al*., 2020) [7]. The staphylococcal cassette chromosome mec-SCCmec, a 21-67 kbp mobile genetic element habours the *mec*A gene and other antibiotic resistance determinants has acquired and integrated into MRSA genome (Ito *et al*., 2001[8]; Ito *et al*., 2004) [9]. Penicillin-Binding Proteins 2a (PBP2a), an alternative trans-peptidase with low binding affinity for the majority of β-lactam antibiotics, is also encoded by the *mec*A gene that leads to its resistance not only to methicillin but also to all members of the extended-spectrum β-lactam antibiotics (Kim *et al*., 2012) [10]. The *mec*C is a novel genetic determinant produced from *mec*A mutations that only shares 63% identity with PBP2a encoded by *mec*A. The *mec*C containing MRSA isolates associated with livestock can spread to other Staphylococci or human associated MRSA (Vandendriessche *et al*., 2013) [11]. Thus, with this information, the present study was performed for detection of the phenotypic MRSA with its genetic determinants *i.e*., *mecA* and *mecC* genes in *S. aureus* isolates from bovine clinical and subclinical mastitis.

Materials and Methods
Bacterial strains
A total of 68 *Staphylococcus aureus* isolates from bovine clinical (n=28) and sub clinical (n=40) mastitis milk were revived in Brain Heart Infusion broth. The isolates were further confirmed using molecular test.

Susceptibility tests

The cefoxitin disk diffusion test was performed by the disc diffusion method (Wayne 2002) [12]. The test colony was inoculated overnight in nutrient broth at 37 °C. About 100 µl of the growth culture was spread on Mueller-Hilton agar plates with a sterile L-shaped spreader and the antibiotic discs of 5 mm were stuck to the plates with forceps, belonging to cefoxitin (30 µg). All antibiotic disc-containing plates were incubated for 18-24 h at 37 °C. The zones of inhibition were measured using a calibrated zone scale (Hi-media, India) and recorded for further interpretation according to the guidelines of CLSI. The isolates that showed resistance to cefoxitin were considered as phenotypic MRSA and were further examined by molecular means.

Template DNA preparation for PCR

The template DNA was created using the phenotypic confirmed MRSA isolates by the boiling and snap-chilling method. To pellet the bacterial cells, 1.5 ml of an overnight-grown culture in nutrient broth were transferred to a 2 ml microfuge tube and centrifuged at 5,000 rpm for 10 min. After discard of the supernatant 1 milliliter of normal saline was added to the bacterial pellet and vortexed. The centrifugation at 5,000 rpm for 4 minutes was performed and supernatant was discarded. 200 µl of distilled water was added to the pellet, which was then placed under boiling water for 8 minutes. Immediately after boiling, the tube was moved to a temperature of –20 °C and defrosted at room temperature before use with centrifugation at 4,000 rpm for three minutes.

Standardization of PCR targeting *mec*A and *mec*C gene

A PCR assay was optimized for amplification of the *mec*A and *mec*C genes fragment of MRSA isolates as per the method described by Farbo (2021) [13]. The PCR reaction mixture was prepared in 25 µl reaction volume each containing 2.5 µl 10X PCR buffer, 0.5 µl of dNTP mixture (10 mM each), 2 µl (10 pmol/µl) of forward and reverse primers, 1µl (1 unit) Taq DNA polymerase, 5 µl of bacterial lysate and 12 µl nuclease-free water.

The PCR amplification reaction for amplification of *mec*A gene was used with an initial denaturation of 94 °C for 5 min, followed by 30 amplification cycles of denaturation at 94 °C for 30 secs, annealing at 53 °C for 1 min, and elongation at 72 °C for 30 secs with a final elongation phase at 72 °C for 2 min. Similarly, the PCR amplification reaction for amplification of *mec*C gene was optimized with an initial denaturation phase of 94 °C for 5 min, followed by 32 amplification cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min with a final elongation phase at 72 °C for 10 min.

The detection of *mec*A and *mec*C was performed by loading of 2.5 µl of 100 bp DNA ladder into the first well and 8 µl of amplified PCR products mixed with 2 µl of 6x loading dye in the remaining wells of 1.5% agarose gel containing 0.5 mg/mL of ethidium bromide in Tris-acetate-EDTA buffer. The DNA bands were observed and recorded under Gel documentation system (Vilber, France).

Results and Discussion

Currently, the cefoxitin disk diffusion test has been recommended for the phenotypic characterization of MRSA which can foretell the presence of *mec*A gene in *S. aureus* with a high degree of sensitivity and specificity (Swenson *et al.* 2005) [14]. The present work revealed that out of 68 tested isolates, 52 isolates (76.47%) were resistant to cefoxitin and categorized phenotypically as MRSA. Further, 77.50% (31/40) *S. aureus* isolates from bovine subclinical mastitic milk samples and 75.00% (21/28) from bovine clinical mastitic milk were identified as MRSA by phenotypic method. The sampling area wise distribution of phenotypic MRSA showed that all isolates from Danapur, Maner, Khagaul, and Phulwari Sharif were MRSA while 80.00% isolates of Raja Bazar, 73.33% isolates of advance diagnostic laboratory, Bihar Veterinary College, 66.67% from Digha & Ramna Road, 33.33% from Kautilya Nagar, and 7.14% from ILFC, BASU, Patna were also MRSA. Such findings come in harmony with the previous literature showing the prevalence of MRSA that was 47.83% to 52.20% (Falagas *et al.* 2013 [15]; Elhaig and Selim 2014 [16]; Hassan *et al.* 2016) [17].

Methicillin resistance occurred mainly due to the presence of the *mec*A or *mec*C gene on the *S. aureus* chromosome that is responsible to produce Penicillin-binding protein PBP2a (Ito *et al*. 2003) [18]. Hence in the present study for the identification of MRSA encoded by the *mec*A and/or *mec*C gene, PCR assays were standardized to detect 138 and 162 bp amplicon size at annealing temperature of 53.0 and 56° C for 1 min, respectively. Further, a total of 52 *S. aureus* isolates that showed phenotypic resistance with cefoxitin were tested for the presence of the *mec*A and *mec*C genes and a total of 15.38% (8/52) isolates were found positive to give *mec*A gene amplicon of 138 bp in agarose gel electrophoresis (Fig. 1), while, 5.77% (3/52) isolates were found positive to give *mec*C gene amplicon of 162 bp (Fig. 2).

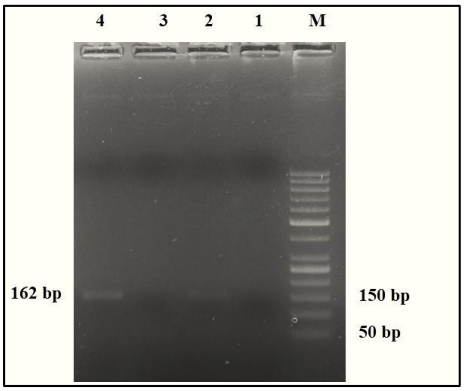

Fig 1: PCR amplification of *mec*A gene of MRSA.
M: 50 bp DNA ladder
L2 & L4: Positive amplicons of 162 bp
L1 & L3: No amplicon

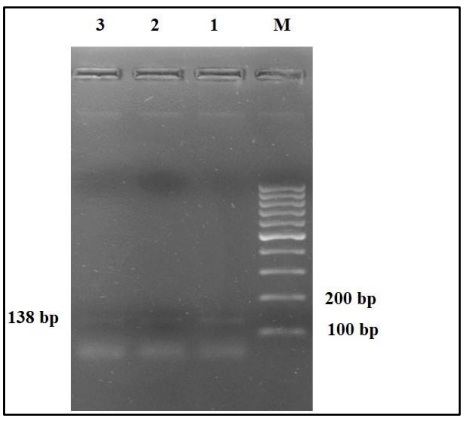

Fig 2: PCR amplification of *mec*A gene of MRSA.
M: 100 bp DNA ladder
L1 - L3: Positive amplification of 138 bp

In the present study, a total of 11 (21.15%) cefoxitin resistant *S. aureus* isolates harbour either *mec*A or *mec*C gene. Like the present study, different workers also performed studies for *mec*A gene detection among MRSA isolates. Kutar *et al*. (2015) [19] reported the presence of the *mec*A gene in 9.61% of *S. aureus* isolates from cattle and buffaloes with clinical or subclinical mastitis in Uttar Pradesh, India, Whereas, Mausam *et al*. (2016) [20] reported the detection of *mec*A gene among 29.33% of *S. aureus* in bovine milk samples of Bihar. The presence of the *mec*A gene among *S. aureus* isolates were also reported from different part of the world. Qolbaini *et al*. (2021) [21] reported the prevalence of 10.50% MRSA (*mec*A^+) among milk specimens with subclinical mastitis. Similarly, Hoque *et al*. (2018) [22] reported the detection of the *mecA* gene among 20.0% of *S. aureus* isolates from cow mastitis in Bangladesh, whereas, Shrestha *et al*. (2021) [23] reported the presence of the *mec*A gene among 6.9% of *S. aureus* isolates from milk samples in Chitwan, Nepal. Contrary to this study, a higher distribution of the *mec*A gene among 35.7% isolates was reported by Selim *et al*. (2022) [24]. In this study, 5.77% *phenotypic* MRSA isolates were found to harbour *mec*C gene that agreed to the previous studies of Paterson *et al*. (2014) [25] who reported *mec*C MRSA among 10 (2.15%) dairy farms samples in England and Wales. However, in contrast to the findings of present study, lack of *mec*C gene was also reported among MRSA isolates (Paterson *et al*. 2014 [25]; Klibi *et al*. 2019 [26]).

Conclusions

Based on the findings of present study, it could be concluded that *S. aureus* including methicillin resistance could be a major pathogen involved in bovine sub-clinical and clinical mastitis in and around Patna, Bihar. The *mec*A gene was more frequent than the *mec*C gene in MRSA isolates from clinical and subclinical mastitis. The changing molecular basis of MRSA isolates not only impacts new treatment strategies against MRSA, but also hampers the control of MRSA infections. Molecular testing of MRSA for *mec*A and *mec*C genes further add in molecular epidemiology and to understand the rise of multidrug resistance in MRSA.

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