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**Original Research Article**

# Isolation and Identification of Multidrug Resistant and Methicillin Resistant *Staphylococcus aureus* from Bovine

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**A B S T R A C T**

The present study was undertaken with the aim to isolate and characterize Methicillin resistant *Staphylococcus aureus* (MRSA) from milk of clinical cases of bovine mastitis and nasal swabs of healthy dairy cattle. A total of 75 milk samples and 35 nasal swabs were collected and processed in the laboratory. Isolation rate of *Staphylococcus aureus* from mastitic milk and nasal swab was found to be 100%. The screening of MRSA was done by disk diffusion method using Oxacillin, in which 18 MRSA were recovered from mastitic milk, while 10 MRSA were recovered from nasal swabs. All 28 MRSA isolates showed amplification of *mec A* gene in PCR, while *femA* gene was detected in 23 isolates. The antibiogram study of *S. aureus* isolates was done against 9 antibiotics viz. Amoxicillin, Ampicillin, Gentamycin, Ciprofloxacin, Cephalexin, Lincomycin, Methicillin, Penicillin and Streptomycin. Among milk isolates (75), multi drug resistance was exhibited by 7 isolates for 2 antibiotics, 3 isolates for 3 antibiotics, 1 isolate each for 4 and 5 antibiotics. Highest number of isolates (7, 16.00%) showed resistance to Streptomycin followed by Lincomycin (4, 9.33%), Ampicillin (4, 5.33%) and Penicillin (1, 1.33%). Out of 35 nasal swab isolates, 4 isolates showed resistance for 2 antibiotics, 2 for 4 antibiotics, 3 for 5 antibiotics and one isolate each for 6, 7 and 9 antibiotics. Highest number of isolates (10, 28.57%) was found resistant to Amoxicillin and Ampicillin each, followed by 8 isolates (22.85%) to Penicillin G, 7 isolates (20.00%) to Lincomycin and 5 isolates (14.24%) to Cephalexin.

**Keywords**

Methicillin resistant *Staphylococcus aureus* (MRSA), Multidrug resistance (MDR), Bovine mastitis

## Introduction

*Staphylococcus aureus* is one of the major causes of mastitis in dairy animals and its resistance against multiple antimicrobials always remains crucial concern. It is also a common resident of skin and nasal mucosa of human and animals (Lee, 2003). In recent past, a group of *Staphylococcus aureus* resistant to beta lactam has emerged as great threat to public causing nosocomial and community acquired infections (Pesavento *et al.*, 2007). These are known as Methicillin resistant *Staphylococcus aureus* (MRSA). MRSA infected farm animals can easily disseminate the pathogen to their milk, meat and farm workers. There are evidences of existence of MRSA in animals and their transmission between man and animals (Farreira *et al.*, 2011; Christaine *et al.*, 2015; Smith, 2015). The present study was designed to isolate and characterize

Methicillin resistant *Staphylococcus aureus* (MRSA) associated with clinical cases of bovine mastitis and to find out their presence in nasal swabs of healthy dairy cattle.

## Materials and Methods

### Collection of sample

Milk samples were collected from the mastitic cows reared at Instructional Livestock Farming Complex (ILFC) and from cases brought at Teaching Veterinary Clinical Complex (TVCC) of the College of Veterinary Science according to the method recommended by NMC (National Mastitis Council, 1990).

Approximately 10 ml of milk sample of 75 cows was collected aseptically from mastitic quarter into sterile vials and transported on ice to the laboratory. Similarly, a total of 35 nasal swabs samples were collected from healthy milking cows maintained at ILFC. The swabs were immediately placed into the sterile test tube and brought to the laboratory on ice. The samples were either immediately cultured or stored at 4°C for a maximum of 24 hr.

### Isolation and identification of *S. aureus*

The milk samples were primarily screened for mastitis using California mastitis test (CMT) and the samples showing strong positive reaction were selected for isolation of *S. aureus*. The isolates showing typical colony on mannital salt agar (MSA) were picked up on nutrient agar and subjected to gram staining. Further identification was done by biochemical tests viz. Catalase, Coagulase, Nitrate, fermentation of glucose, mannitol and maltose sugars and haemolysis on blood agar as per the procedure described by Cappuccino and Sherman (1992).

### MRSA screening and Antibiogram study

*S. aureus* isolates were tested for Methicillin resistance by using Oxacillin disc (1 µg) (Hi-Media) and recorded as resistant in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (Wayne, 2013). Antibiogram study of *S. aureus* included various antibiotics viz. Amoxycillin, Ampicillin, Cephalexin, Ciprofloxacin, Gentamicin, Lincomycin, Methicillin, Penicillin G and Streptomycin. The isolates were grown on Mueller Hinton agar using modified disk diffusion method (Bauer *et al.*, 1966).

### Molecular characterization of *S. aureus*

*S. aureus* isolates were subjected to PCR for finding out the presence of *mecA* and *femA* gene specific for methicillin resistance. DNA from the isolates was extracted using snap chilling method as described by Franco *et al.*, (2008). The PCR amplification of *mec A* gene and *fem A* gene was done using protocols standardized by Murakani *et al.*, (1991) and Manikandan *et al.*, (2011), respectively. The published oligonucleotide sequence of *mecA* (*F AAAATCGATGGTAAAGGTTGGC*, R *AGTTCTGCAGTACCGGATTTTGC*) and *femA* (F *AAAAAAGCACATAACAAGCG*, R *GATAAAGAAGAAACGAGCAG*) genes was synthesized by Merck India. The PCR was performed in final volume of 25 µL containing 12.5 µL of 2x master mix, 2.5 µL (50 pmol) of forward and reverse primer, 2.5 µL of DNA template and nuclease free water 5.0 µL. The PCR cycling condition for *mecA* gene included initial denaturation at $94^0$C for 30 sec followed by 40 cycles at $94^0$C for 30 sec, annealing at $55^0$C for 30 sec, elongation at $72^0$C for 1 min and final extension at $72^0$C for 5 min. While *femA* gene amplification was done at $94^0$C, 30 sec for initial denaturation followed by 30

cycles at $94^0$C for 30 sec, annealing at $50^0$C for 30 sec, elongation at $72^0$C for 30 sec and final extension at $72^0$C for 10 min. The amplified products were imaged by running them in 1.5% agarose gel containing 0.5ug/ml ethidium bromide as per the method of Sambrook and Russell (1989).

## Results and Discussion

The present study was conducted to isolate and characterize methicillin resistance *S. aureus* (MRSA) from the cases of clinical bovine mastitis and nasal swabs of healthy dairy cattle and to study the antibiogram in order to find out the antibiotic resistance pattern prevailing in the study area. The milk samples were first screened by CMT and those found strongly positive were chosen for further study. *S. aureus* was successfully isolated from all 75 milk samples following inoculation onto MSA. Similarly, all 35 nasal swabs samples yielded *S. aureus* when inoculated onto MSA. The appearance of characteristic pink-yellow coloured colonies on the MSA and gram positive cocci in clusters on Gram' staining confirmed the isolation of *S. aureus*.

The isolates were further subjected to haemolysis test, catalase test (slide and tube) coagulase test, nitrate reduction test and fermentation of glucose and lactose sugars. Out of 75 *S. aureus* isolates from mastitis milk, 68 (90.66%) isolates tested positive for catalase, while 59 (78.66%) isolates were positive in slide coagulase test and 62 (82.66%) isolates were positive in tube coagulase test. β haemolytic property on sheep blood agar was exhibited by 52 (69.33%) isolates and nitrate was reduced by 65 (86.66%) *S. aureus* isolates. Among nasal swab isolates, 32 out of 35 (91.42%) showed catalase positive test while 28 (80%) and 29 (82.85%) isolates showed positive reaction with slide and tube coagulase test, respectively. Nitrate reduction ability was exhibited by 30 (85.71%) isolates and β haemolysis on sheep blood agar was exhibited by 25 (71.42%) isolates. Glucose and lactose sugars were fermented by 64 (85.33%) and 57 (76.00%) *S. aureus* isolates from milk samples and 28 (80.00%) and 26 (74.28 %) isolates from nasal swabs, respectively (table 1). The screening of MRSA was done by disc diffusion method using oxacillin disk in which, 18 out of 75 (24.00%) milk isolates were found MRSA, while10 out of 35 (28.57%) nasal swab isolates exhibited resistance. These methicillin resistant isolates were further confirmed by PCR by targeting *mecA* and *femA* genes. All 18 isolates from the mastitic milk and 10 isolates from nasal swabs showed amplification of *mecA* gene with a product size of 533 kb. The presence of *femA* gene was revealed by 510 kb amplicon, which was exhibited by 15 MRSA from mastitic milk and 8 MRSA from nasal swabs (table 1).

The antibiogram study of all *S. aureus* isolates was done against 9 antibiotics using Amoxicillin, Ampicillin, Gentamycin, Ciprofloxacin, Cephalexin, Lincomycin, Methicillin, Penicillin and Streptomycin disks which showed sensitivity pattern as given in table 2.0. All the *S. aureus* isolates from mastitic milk were sensitive to Amoxicillin, Gentamicin, and Ciprofloxacin. Highest resistance was seen against Streptomycin (12, 16.00%) followed by Lincomycin (7, 9.33%), Ampicillin (4, 5.33%), Penicillin (1, 1.33%). Among nasal swabs isolates of *S. aureus*, 10(28.57%) isolates showed resistance to Amoxicillin and Ampicillin each followed by 8(22.85%) isolates showing resistance to Penicillin G, 7(20.00%) isolates to Lincomycin, 5(14.24%) isolates to Cephalexin and 2(5.71%) isolates to Gentamicin and Ciprofloxacin each.

**Table.1** Identification of Methicillin Resistance *Staphylococcus aureus* (MRSA) isolates from milk and nasal swab samples

| Characterization criteria | Tests performed | Positive isolates of milk sample (N= 75) | Positive isolates of nasal swabs (N=35) |
|---|---|---|---|
| | | Number (%) | Number (%) |
| Mannital salt agar | Growth | 75 (100.0) | 35 (100.0) |
| Biochemical test | Catalase | 68 (90.66) | 32 (91.42) |
| | Slide coagulase | 59 (78.66) | 28 (80.00) |
| | Tube coagulase | 62 (82.66) | 29 (82.85) |
| | Nitrate reduction | 65 (86.66) | 30 (85.71) |
| | β- haemolysis | 52 (69.33) | 25 (71.42) |
| Sugar fermentation | Glucose | 64 (85.33) | 28 (80.00) |
| | Lactose | 57 (76.00) | 26 (74.28) |
| Methicillin Resistance | Disk method | 18 (24.00) | 10 (28.57) |
| | *mecA* gene | 18 (24.00) | 10 (28.57) |
| | *femA* gene | 15 (20.00) | 8 (22.85) |

**Table.2** Antibiotic sensitivity range of *S. aureus* isolates from mastitic milk and nasal swabs

| **Antibiotics (Hi-Media)** | **Conc. (µg/disc)** | **Mastitic Milk** | | **Nasal Swabs** | |
|---|---|---|---|---|---|
| | | **No. of resistant isolates** | **No. of susceptible isolates** | **No. of resistant isolates** | **No. of susceptible isolates** |
| **Amoxicillin** | 10 | - | 75(100.00%) | 10(28.57%) | 25(71.42%) |
| **Ampicillin** | 25 | 4 (5.33%) | 71(94.66%) | 10(28.57%) | 25(71.42%) |
| **Gentamicin** | 50 | - | 75(100.00%) | 2(5.71%) | 33(94.28%) |
| **Ciprofloxacin** | 10 | - | 75(100.00%) | 2(5.71%) | 33(94.28%) |
| **Cephalexin** | 30 | 3 (4.00%) | 72(96.00%) | 5(14.28%) | 30(85.71%) |
| **Lincomycin** | 2 | 7 (9.33%) | 68(90.66%) | 7(20.00%) | 28(80.00%) |
| **Penicillin G** | 2 | 1 (1.33%) | 74(98.66%) | 8(22.85%) | 27(77.14%) |
| **Streptomycin** | 25 | 12 (16.00%) | 63(84.00%) | 3(8.57%) | 32(91.42%) |
| **Methicillin** | 5 | 18 (24.00%) | 57(76.00%) | 10(28.57%) | 25(71.42%) |

There were some isolates of *S. aureus* that exhibited multiple drug resistance. Out of 75 milk isolates, 7 isolates showed resistance to 2 antibiotics, 3 isolates to 3 antibiotics and one each showing resistance to 4 and 5 antibiotics, respectively. Similarly, among nasal swab isolates, 4 isolates exhibited resistance for 2 antibiotic, one isolate for 3 antibiotic, 2 isolates for 4 antibiotics, 3 isolates for 5 antibiotics, one isolate each for 6 and 7 antibiotics and one isolate exhibited resistance for all 9 antibiotics.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is emerging as an important zoonotic and veterinary pathogen of public health importance. It causes nosocomial and community onset infections. Since, the first instance of MRSA in animals was reported in milk from mastitic cows (Devries *et al.*, 1972), it has now become an important study material in Veterinary Microbiology.

Present study was designed to isolate and identify the MRSA associated with clinical

bovine mastitis. The association of *S. aureus* with clinical bovine mastitis was very evident from the present finding as all 75 milk samples tested positive for CMT, yielded *S. aureus* onto mannitol salt agar. Large numbers of authors have reported it as most common cause of mastitis in bovine milk (Adwan, 2006; Gentilini, *et al.,* 2002; Kumar *et al.,* 2010; Sharma and Prasad, 2002). In the present study, attempt was also made to find out their presence in the nasal passage of the healthy cows and all 35 nasal swabs samples yielded *S. aureus* onto the mannitol salt agar. *S. aureus* is well known for its colonization in the mucosa of upper respiratory tract and digestive tract of all mammals and birds (Hajek *et al.,* 1988). The biochemical characterization of the isolates using catalase, coagulase, haemolysis, nitrate reduction and sugar fermentation test revealed typical characteristics of *S. aureus*. The high specificity and sensitivity of coagulase test have made it a standard method for identification of *S. aureus* in the milk. In the present study, 82% isolates were coagulase positive, which is considered as an important phenotypic determinant of virulence in *S. aureus* (Erasmus, 1985; Higgins and Chartier, 1984; Hajek *et al.,* 1988; Tyagi *et al.,* 2013). β haemoysis was also exhibited by 69- 71% isolates indicating their virulent trait (Ali-Vehmas *et al.,* 2001). The carriage of *S. aureus* in anterior nares plays a key role in epidemiology and pathogenesis of staphylococcal infection (Baptiste *et al.,* 2005). Sarkar *et al.,* (2014) have reported high prevalence of pathogenic *S. aureus* from the nasal swabs of farm workers of a closed dairy herd.

Methicillin resistance among *S. aureus* strains have emerged as a global problem in last few years (Akande, A., 2010; Mathai *et al.,* 2013). Initially isolated from hospitalized patient in U.K. in 1961, the strains have now been recovered from general human and animal population (Lee, 2003; Christaine *et al.,* 2015). In present study, 24% isolates from mastitic milk and 28.57% isolates from nasal swabs appeared MRSA in screening test with overall isolation rate of 25.45%. In the previous studies, the incidence of MRSA has been reported to be 32.8% (Mathur *et al.,* 1994), 24% (Pulimodd *et al.,* 1996), 32% (Witte *et al.,* 1995) and 51.6% (Maniknandan *et al.,* 2011) from different places. This implies that the incidence of MRSA infection keeps changing every year and it is on the rise when compared to last few years. All phenotypically positive MRSA isolates also showed amplification of *mecA* gene which was in concordance with conventional methods. Similar findings have been reported by Shanehbandi *et al.,* (2014). On the contrary, *femA* gene amplification was not exhibited by all phenotypically positive MRSA. In a similar study, Jonas *et al.,* (2002) found combination of *mecA* and *femB* gene in 36 isolates out of 439 swabs tested (throat, nose, groin, perineum, wound, and drainage) using duplex PCR, whereas some of the MRSA showed only *mecA gene*. On the contrary, Manikandan *et al.,* (2011) observed *femA* gene in all MRSA isolates from clinical pus sample. So, this implies that all the genes determining resistance may not necessarily be present in all isolates. Secondly, conventional method of screening samples takes 2 or 3 days before definitive MRSA identification can be achieved while PCR for *mecA* and *femA* provides reliable and unequivocal results for MRSA identification within 18 h.

Antibiotic resistance has become a major public health problem throughout the world. Bovine mastitis is a single most common cause of use of antibiotic in dairy cattle. Consequently, many *S. aureus* strains have acquired resistance to commonly used antibiotics leading to treatment failure

(Aires-de-sousa *et al.,* 2007; Kumar *et al.,* 2010; Oliver *et al.,* 2005; Sumathi *et al.,* 2008; Sundhakar *et al.,* 2009). *S. aureus* isolates recovered from mastitic milk and nasal swabs were tested for antibiotic resistance against 9 antibiotics. The isolates from mastitic milk were found sensitive to Amoxycillin, Gentamicin, and Ciprofloxacin and these findings are in the agreement with Sudhakar *et al.,* (2009) who have recorded highest sensitivity to Ciprofloxacin. The milk isolates exhibited resistance for Streptomycin (16.00%), Ampicillin (5.33%) and Penicillin (1.33%). Similarly, Kumar *et al.,* (2011) have also reported higher resistance to Streptomycin (36.4%), Ampicillin (29.9%) and Penicillin-G (28.9%). The present findings indicate that the isolates showed resistance to antibiotics (Streptomycin, Ampicillin, and Cephalexin) that are frequently used for the treatment of mastitis in field. The most interesting finding was that only one milk isolate was found resistant to Penicillin-G which might be due to discontinuation of its use for a long time in this area. Among the nasal swabs isolates, highest (28.57%) resistance was seen against Ampicillin and Amoxicillin followed by Penicillin (22.85%), Lincomycin (20.00%). There are many reports of raising trend of antibiotic resistance due to increase antibiotic use (Adwad, 2006; Gentilini *et al.,* 2002; Ombui *et al.,* 2000; Pesavento *et al.,* 2007 and Sumathi *et al.,* 2008).

Multiple drug resistance has emerged as a great problem in treatment of bacterial infection in last few years. There are large numbers of reports pouring in from different parts of the world that describe increased trend of developing multiple drug resistance strains (Adwan, 2006; Neyra *et al.,* 2014; Ombui *et al.,* 2000; Pulimodd *et al.,*1996; Rinsky *et al.,* 2013). In present study also, 3 milk isolates exhibited multi drug resistance for 3 antibiotics, while 1 isolate each showed resistance to 4 and 5 antibiotics. Similar finding has been reported for milk isolates from same area by Tyagi *et al.,* (2013). In comparison to milk samples, multi drug resistance phenomenon was more prevalent in nasal swabs isolates as evidenced by 1 isolate for 3 antibiotics, 2 isolates for 4 antibiotics, 3 isolates for 5 antibiotics and one each for 6 and 7 antibiotics. The most interesting observation was that 1 isolate showed resistance to all the antibiotics. The multiple drug resistance in staphylococcal isolates has been reported previously by several other investigators (Adwan, 2006; Gentilini *et al.,* 2002; Kumar *et al.,* 2010; Neyra *et al.,* 2014; Ombui *et al.,* 2000; Wang *et al.,* 2008). These healthy animal carriers of MDR strains can serve as potential threat for transmission to human (Lee, 2003, Oliver *et al.,* 2005)

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