



A study on Methicillin resistant *Staphylococcus aureus* mastitis in dairy cows

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious problem in dairy animals suffering from mastitis. The study was carried out to evaluate the incidence of Methicillin resistant *S. aureus* from clinical mastitis milk samples and their antibiotic resistance profile and characterised with respect to the molecular features that contributed to the resistance in these pathogens. Isolation and identification of Methicillin resistant *S. aureus* were performed from acute clinical mastitis samples. The isolates were tested using agar disc diffusion method for their antimicrobial susceptibility and modified resazurin assay micro dilution technique for MIC to 8 different antimicrobial drugs. A total of 235 clinical mastitis milk samples from dairy cows were cultured for incidence of *S. aureus*. Methicillin resistant *S. aureus* was isolated from a total of 12 (44.25%) of the 116 *S. aureus* samples. Based on the antimicrobial sensitivity and MIC results, MRSA isolates were found sensitive to gentamicin, enrofloxacin, amoxicillin+sulbactam, ceftriaxone and resistant to amoxicillin, oxytetracycline, penicillin G and oxacillin. Most of MRSA isolates were found to be multi-drug resistant. MRSA alert kit test and *mecA* and *blaZ* target gene PCR were found to be useful in the confirmation of MRSA.

Keywords: Antibiotic susceptibility, Antimicrobial drug resistance, Bovine mastitis, MIC, *mecA*, MRSA, PCR *blaZ*, PVL gene

INTRODUCTION

Bovine mastitis, a multi-factorial disease, is characterized by physical, chemical and microbiological changes in the milk and pathological changes in the glandular tissue of udder (Radostits *et al.*, 2008). Among the various pathogens that affect the bovine mammary gland *Staphylococcus aureus* is the most frequently (45-60 per cent) isolated pathogens and often results clinical or subclinical or chronic bovine mastitis. *S. aureus* is capable of producing several exo and endotoxins e.g coagulase and is also endowed with a capsule that makes it difficult to treat.

In the control of mastitis, the improper use of antimicrobial agents on dairy farm animals is a major concern as it leads to the emergence of resistant zoonotic bacterial pathogens (Piddock, 1996). The antibiotic-resistance of prevalent *S. aureus* strains is another serious concern besides the pathogenicity. The emergence of antibiotic-resistance in *S. aureus* from mastitic dairy animals has been shown in recent years. Beta-lactam antibiotics are frequently used in mastitis therapy and the resistance is due to the production of

beta-lactamases and low affinity penicillin-binding protein, PBP2A (Olsen *et al.*, 2006)

Several strains of *S. aureus* isolated from mastitis case have been reported to show resistance against multiple antimicrobials such as penicillin-G, gentamicin, streptomycin, ampicillin, ciprofloxacin, oxytetracycline (Kumar *et al.*, 2011). β -lactamase resistant penicillins such as methicillin and oxacillin are not used in dairy cows except for cloxacillin that is used in products for intramammary administration (Turutoglu *et al.*, 2006). Among the resistance to commonly used antibiotics, resistance to this bacterium, methicillin (hence termed methicillin resistance *S. aureus*- MRSA) is a serious cause of concern in both human and animals (Witte *et al.*, 2007). Bovine and human MRSA strains are epidemiologically related, which indicates transmission from either cow to human or human to cow possible (Juhász- Kaszanyitzky *et al.*, 2007). This strain is negative for the Pantone Valentine Leucocidin (PVL) genes, which differentiates it from community associated MRSA ST 1, which is positive for PVL genes (Vandenesch *et al.*, 2003). PVL genes have been associated with community-acquired MRSA (CA-

MRSA) rather than hospital-linked human MRSA (HA-MRSA) strains (Blaine *et al.*, 2010). The genes encoding for PVL toxin are shown to be responsible for severe necrotic lesions of the skin and soft tissues and are predominantly present in CA-MRSA (Rankin *et al.*, 2005).

Data on the prevalence of these resistant strains is lacking and this study was undertaken to look at the incidence of Methicillin resistant *S. aureus* from clinical mastitis milk samples and their antibiotic resistance profile. These organisms were also characterised with respect to the molecular features that contributed to the resistance in these pathogens.

MATERIALS AND METHODS

Collection, isolation and identification of *S. aureus*:

Two hundred and thirty five milk samples from acute mastitis cases from Large Animal Clinic Medicine Unit of Madras Veterinary College Teaching Hospital and six dairy farms in Coimbatore district. The guidelines of National Mastitis Council (NMC) were followed for sample collection, transportation, culture and isolation of Staphylococci. A total of 116 *S. aureus* isolates were characterized by their growth on blood agar and mannitol salt agar and the results for catalase and coagulase (CLSI, 2008).

Profiling the antibiotic susceptibility and resistance:

Antimicrobial susceptibility testing was carried out at equivalent to 0.5 McFarland turbidity standard by agar disc diffusion method on Mueller-Hinton agar plates following the guidelines of CLSI (CLSI, 2008). Commercially available antibiotic discs (Himedia, Mumbai) were used in the study viz., Enrofloxacin (EX 10 mcg), Amoxicillin+sulbactam (AMS 15 mcg), Amoxicillin (AMX 10 mcg), Gentamicin (GEN 10 mcg), Ceftriaxone (CTR 30 mcg), Oxytetracycline (TE 30 mcg) and Penicillin G (P 10 units) and Oxacillin (OX 5 mcg). The sizes of the zone of inhibition were recorded and interpreted as either bacteria were susceptible or resistant to the exposed agent (Table 1).

Methicillin resistant *S. aureus* : MSRA Alert™ Kit (Hi-media, Mumbai) was used for confirmation of Methicillin Resistant *Staphylococcus aureus*. The inoculum was prepared from material picked up with sterile loop from atleast 4-5 well isolated colonies on mannitol salt agar of similar type, inoculated into 3 ml sterile nutrient broth and incubated at 37°C. This broth sample was inoculated into the dissolved in the MSRA Alert™ and was incubated at 35°C without shaking. The MRSA Alert test tubes were examined up to 24 hours for the presence of a clot and colour change in the medium from red to yellow indicated positive result (Selepak and Witebsky, 1985).

Minimum inhibitory concentration (MIC): The minimum inhibitory concentrations of different antibiotics for MRSA isolates were determined by modified resazurin assay microdilution technique (Sarker *et al.*, 2007). The MIC panels consisted of

dehydrated antimicrobial agents: amoxicillin, ceftriaxone, enrofloxacin, gentamicin, penicillin, oxytetracycline, amoxicillin + sulbactam and oxacillin in 96-well U bottom microtiter plates and performed in accordance with the guidelines established by the CLSI (CLSI, 2008). The lowest concentration of antibiotic that resulted in complete inhibition of visible growth and did not produce any turbidity was taken as the MIC end point (Table 2).

Molecular characterization of the *S. aureus* and their resistance genes:

A single colony was from the selective agar was suspended in nuclease free water for the different isolates and lysed by boiling for 10 minutes and the lysate was stored at -20°C until use. The lysate were used in a PCR reaction with primers targeting the *gap* gene (Yugueros *et al.*, 2001) to confirm these isolates to be *S. aureus*. The PCR amplicons from some of the samples were sequenced and analysed by BLAST search to confirm their identity. On confirmation of these isolates to be *S. aureus* PCR was performed in these lysates to detect the *mecA* and *blaZ* genes that contribute to methicillin resistance in these bacteria and also with primers to determine the PVL genes that contribute to the transmission of these isolates across species. The details of primers used in the study are presented in Table 3.

PCR amplification was performed in a total reaction volume of 25 µl. The reaction mixture contained 12.5 µl of the master mix, 20 pmol of the forward and reverse primer and 15 µl of the test lysate. The amplification profile for the PCR for detection of the different genes were 94°C 5 min; 35 cycles of 95°C for 45 sec, 55°C for 1 min, 72°C for 1 min; final extension of 72°C for 7 min. The PCR products were separated by gel electrophoresis in 1.5 per cent agarose gel and visualized staining with ethidium bromide.

RESULTS

A total of 235 clinical mastitis milk samples revealed 116 samples positive for *S. aureus*, of which 12 samples (10.34%) were MRSA.

Bacterial cultures (Staphylococci) from mastitis samples were obtained on blood agar and mannitol salt agar plates. The colonies of haemolytic staphylococci were smooth, circular, moist, 1-2 mm in diameter, pinhead, raised, convex having entire margins and golden yellow in colour. Standard tests used for biochemical characterization of all the isolates obtained from mastitis samples confirmed the isolates as *S. aureus*.

Profiling the antibiotic susceptibility and resistance:

MRSA showed maximum sensitivity to enrofloxacin and amoxicillin and sulbactam (75 per cent) each, gentamicin (33.3 per cent) and ceftriaxone (58.3 per cent) (Table 1). The isolates showed highest resistance to methicillin (100 per cent), amoxicillin (91.7 per cent), followed by penicillin G (83.3 per

Table 1. Antibiotic susceptibility profile determined by agar disc diffusion method for MRSA isolates.

Isolates	Gen	OTC	Am+Su	Ceft	Enro	Amox	Pen	Oxa
1	R	R	S	R	S	R	R	R
2	R	S	S	R	R	S	S	R
3	R	S	S	R	S	R	R	R
4	S	R	S	S	S	R	R	R
5	R	S	R	R	S	R	R	R
6	R	S	S	S	R	R	R	R
7	S	R	R	R	S	R	R	R
8	S	R	S	S	S	R	S	R
9	R	R	R	R	S	R	R	R
10	R	R	S	R	S	R	R	R
11	R	S	S	S	R	R	R	R
12	S	R	S	S	S	R	R	R

R = Resistant, S=Susceptible; Gen =Gentamicin, OTC = Oxytetracycline, Am+Su = Amoxicillin+Sulbactam, Ceft = Ceftriaxone, Enro = Enrofloxacin, Pen = Penicillin G and Oxa = Oxacillin

Table 2. Minimum inhibitory concentrations (MIC) of drugs against MRSA (n=12) isolated from udder of cows.

	MIC ($\mu\text{g/ml}$) (n=12)										MIC ₅₀	MIC ₉₀
	125	62.5	31.25	15.62	7.8	3.9	1.95	0.97	0.48	MIC range		
Gentamicin	33.3	25	16.7	-	8.3	8.3	8.3	-	-	1.95-125	31.25	125
Oxytetracycline	25	41.7	16.7	-	-	8.3	8.3	-	-	1.95-125	62.25	125
Ceftriaxone	16.7	33.3	33.3	-	8.3	8.3	-	-	-	3.9-125	62.25	62.5
Enrofloxacin	-	16.7	16.7	16.7	8.3	25	8.3	-	8.3	0.48-62.5	15.62	62.5
Amoxicillin	33.3	50	16.7	-	-	-	-	-	-	31.25-125	62.5	125
Pencillin G	33.3	41.7	25	-	-	-	-	-	-	31.25-125	62.5	125
Amoxicillin + Sulbactam	8.3	25	16.7	-	-	33.3	16.7	-	-	1.95-125	31.25	62.5
Oxacillin	66.7	33.3	-	-	-	-	-	-	-	62.5-125	125	125

Numbers indicate percentage of isolates. Numbers in bold indicate percentage of isolates with MIC values greater than the highest concentration in the dilution range and vertical line indicate clinical breakpoints, with the value to the right of the line being susceptible and those to the left being resistant. MIC₅₀ and MIC₉₀ values are concentrations at which $\geq 50\%$ and $\geq 90\%$ of isolates are inhibited respectively.

Table 3. Primer sequences, amplicon size and target genes.

Bacteria	Primer sequence (5'-3')	Amplicon size (bp) and reference	Target gene
Methicillin resistant <i>S. aureus</i>	AAA ATC GAT GGT AAA GGT TGG C	533	<i>mecA</i>
	ACT TCT GCA GTA CCG GAT TTG C	(Lee, 2003)	
	ACT TCA ACA CCT GCT GCT TTC	173	<i>blaZ</i>
	TGA CCA CTT TTA TCA GCA ACC	(Martineau <i>et al.</i> , 2000)	
ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	433	PVL	
GCA TCA AST GTA TTG GAT AGC AAA AGC	(Lina <i>et al.</i> , 1999)		

cent) and oxytetracycline (41.7 per cent) (Table 1).

Methicillin resistant *S. aureus*: The incidence of MRSA mastitis by MRSA Alert kit test was 10.34 per cent (12/116). Twelve *S. aureus* samples were positive for clot and color change from yellowish to red confirm the MRSA.

Minimum inhibitory concentration (MIC): Minimum inhibitory concentration of different antibiotics for

MRSA isolates are presented in Table 2.

The MIC breakpoints for gentamicin, oxytetracycline, ceftriaxone, enrofloxacin, amoxicillin, penicillin G, amoxicillin + sulbactam and oxacillin were 8, 8, 4, ≥ 0.5 , 0.25, 0.12, 4, and 2 $\mu\text{g/ml}$ for MRSA respectively (Febler *et al.*, 2010).

Based on the breakpoints the result indicate that MRSA is sensitive to gentamicin (24.9 percent),

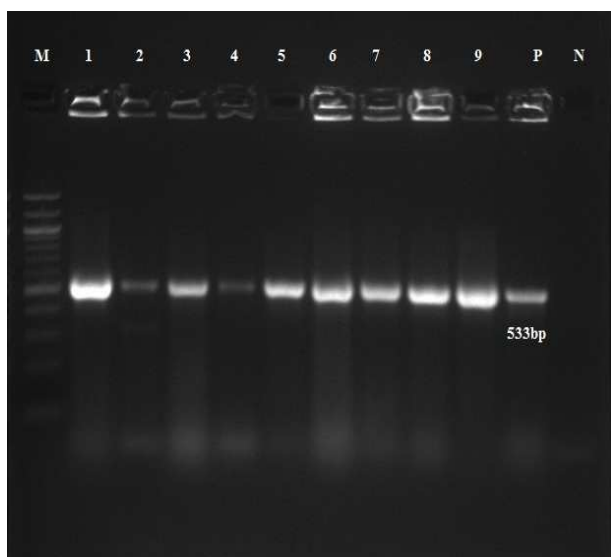


Fig. 1. PCR results of *mecA* gene of MRSA. M – 100bp ladder, Lane 1-9 – Test samples, P- Positive control, N- Negative control.

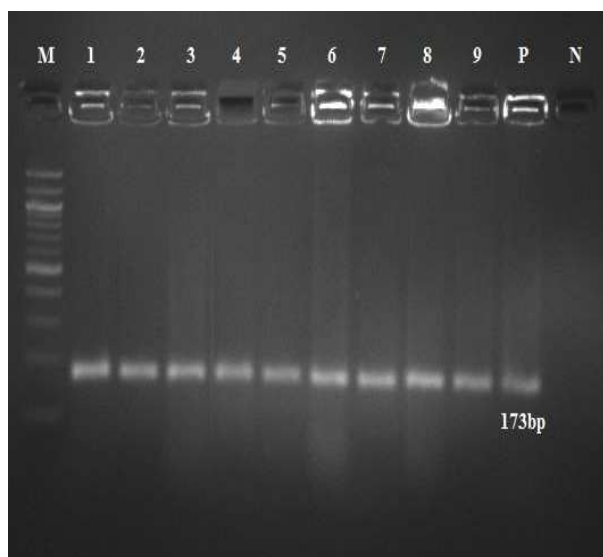


Fig. 2. PCR results of *blaZ* gene of MRSA. M – 100bp ladder, Lane 1-9 – Test samples, P- Positive control, N- Negative control.

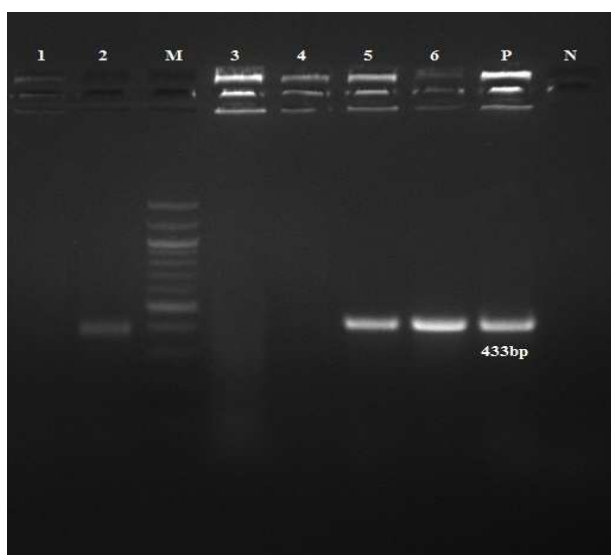


Fig.3. PCR results of *PVL* gene of MRSA. M – 100bp ladder, Lane 1-6 – Test Samples, P- Positive control, N- Negative control.

enrofloxacin (8.3 percent), amoxicillin + sulbactam (50 percent), ceftriaxone (8.3 percent) and resistant to amoxicillin (100 per cent), oxytetracycline (73.4 percent), penicillin G (100 per cent) and oxacillin (100 percent) and this concur with findings of (Febler *et al.*, 2010).

Molecular characterization of the *S. aureus* and their resistance genes: The incidence of MRSA mastitis by targeting specific gene *mecA* (Fig.1) and *blaZ* (Fig.2) in the study was 10.34 per cent (12/116). 7 out of 12 MRSA strains showed amplification of PVL genes (Fig.3).

DISCUSSION

Antibiotic resistant bacteria pose a growing problem of concern, worldwide. Mastitis is the most common

cause for antibiotic use in dairy herds. Effectiveness of current treatments and ability to control infectious diseases in both animals and humans may become hazardous. However, improper use of antibiotics creates problems such as the emergence of bacterial resistance to antibiotics (Chambers, 1997). One of these problems, the occurrence of methicillin resistance, has been observed frequently in recent years (Febler *et al.*, 2010). Therefore, infections by MRSA require rapid and accurate diagnosis for elimination at an early stage, because these strains can cause severe damage to infected sites and may be widespread in the environment (Lee *et al.*, 2004). In most routine microbiological settings, the detection of methicillin resistance among staphylococcal isolates is based on phenotypic assays such as the disk diffusion test and MIC determination. Genetic confirmation of positive findings based on detection of the *mecA* gene has also been reported (Udo *et al.*, 1996).

MRSA strains were multi-drug resistant which might be due to production of betalactamase and PBP2a (penicillin binding protein) (Kumar *et al.*, 2010). Indiscriminate use of antibiotics and intramammary preparations used by the owner without the prescription of the veterinarian is attributed to be one of the reasons for increasing incidence of these strains.

MRSA alert kit test was found to be useful in the detection of methicillin resistance in β lactamase resistance *S. aureus* (Selepak and Witebsky, 1985).

The MIC break point for penicillin G, oxytetracycline, amoxicillin and oxacillin in MRSA mastitis in the present study was 0.12, 8, 0.25 and 2 $\mu\text{g}/\text{mL}$. Hence, they were considered as resistance. The high resistance of penicillin G, oxytetracycline, amoxicillin and oxacillin in MRSA mastitis in the present study could be attributed to the indiscriminate use of these drugs and intramammary preparations (Cloxacillin) used by

the owner without the prescription of the veterinarian. Enrofloxacin was found most effective against MRSA (Kenar *et al.*, 2012) and results obtained are similar to those obtained by the above said authors. All MRSA were resistant to members of the penicillin family, such as ampicillin, oxacillin and penicillin.

Detection of *mecA* and *blaZ* gene by PCR was gold standard test for confirmation of methicillin resistance (Loeffler and Lloyd, 2010). This high incidence in the present study might be due to indiscriminate use of antibiotics and intramammary preparations used by the owner without the prescription of the veterinarian.

MRSA from bovine hosts could also contain PVL genes (Fig.3). Since cattle has a close contact with the cattle rearing community. MRSA from an animal reservoir has entered the human population and it is responsible for causing >20% of all MRSA infections (Van Loo *et al.* 2007). Avoiding routine antimicrobial use in food animals, to decrease selection pressures, might decrease the prevalence of MRSA among cows.

Conclusion

The incidence of MRSA mastitis was 10.34 per cent. MRSA isolates were found sensitive to gentamicin, enrofloxacin, amoxicillin+sulbactam, ceftriaxone and resistant to penicillin G, amoxicillin, oxytetracycline and oxacillin and were Multidrug resistance. MRSA alert kit test and targeting *mecA* and *blaZ* genes were useful in the confirmation of MRSA. The study suggests a high risk for transmission of AMR bacteria from milk or milk products to human populations.

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