Prevalence and Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Mastitis in Dairy Cattle in Jabalpur, Madhya Pradesh

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**ABSTRACT**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a dreaded pathogen in human and veterinary medicine. MRSA as a cause of mastitis in dairy cattle has got profound economic and serious public health significance. A total of 111 dairy cattle were screened for mastitis by CMT from six dairy farms of Jabalpur. The 85 mastitis milk samples were screened for MRSA by bacterial culture method using cefoxitin for enrichment. The MRSA strains were identified and characterized by phenotypic and genotypic methods for virulence determinants and methicillin resistance. The MRSA strains showed β-haemolysis as a predominant haemolysis pattern. The strains were positive for *mecA* gene which is considered as a gold standard for the confirmation of methicillin resistance. The prevalence of MRSA mastitis in dairy cattle was 16.47%. The MRSA strains were positive for the virulence factors associated with pathogenicity. The economic and public health implication of the finding is discussed.

**Keywords:** MRSA, Prevalence, mastitis, dairy cattle

Mastitis is a global problem as it adversely affects animal welfare, quality of milk and the economies of milk production, affecting every country involved in dairying. (FAO, 2014). The economic losses due to mastitis in India have increased about 115 folds in the last five decades and presently the loss due to mastitis is to the tune of ₹ 7165.51 crores per annum (Bansal and Gupta, 2009). In India the prevalence of bovine mastitis due to *Staphylococcus aureus* (*S. aureus*) is around 30-40% (Patel et al., 2012; Sharma et al., 2012).

* S. aureus is a contagious mastitis pathogen and the infected udder is the main source of infection to the herd. Transmission is thought to occur from cow to cow primarily via the milkers’ hands, udder cloths or milking machine (Radostits et al., 2006). Although recently it has been recognized that its epidemiological behaviour is not clear cut, with strains demonstrating contagious and/or environmental transmission patterns (Fernandez et al., 2013).

* S. aureus is important in veterinary medicine as it causes mastitis in bovines which is difficult to treat, control and prevent. The efforts to control and prevent *S. aureus* mastitis are often disappointing due to false negative results from bacteriological culture as the organism is shed intermittently, resulting in undetected cases that may re-infect the rest of the herd, and the poor response of *S. aureus* mastitis to treatment (Barkema et al., 2006). The poor cure rate is attributed to a range of surface-exposed (protein A, fibrinogen- and fibronectin-binding proteins) and secreted (enterotoxins, haemolysins and coagulase) virulence factors that allow it to colonize, invade and multiply in bovine mammary epithelial cells which in turn
make antimicrobial agents poorly effective (Le Marechal et al., 2011; Spoor et al., 2013).

*S. aureus* is known to acquire antimicrobial resistance very quickly. Methicillin resistance is of particular relevance because it imparts resistance to all β-lactam anti-microbial agents- penicillins, cephalosporins, carbapenems (Weese and van Duijkeren, 2010). Historically, Methicillin-resistant *S. aureus* (MRSA) was considered as human pathogen. The earlier reports of MRSA as a cause of mastitis in bovines were shown to be of human origin (Devriese and Hommez, 1975; Juhasz-Kaszanyitzky et al., 2007) but through the past decade MRSA reported as cause of mastitis are bovine adapted and has also been isolated world-wide from other food producing animals like pigs and poultry (Fluit, 2012). Additionally several molecular epidemiological studies have demonstrated host specificity is a lineage specific trait that can rapidly evolve and various host shift between human and bovine has occurred in recent and distant past. (Sakwinska et al., 2011; Resch et al., 2013). This has raised public health concerns. It is therefore important that bacterial populations at the animal-human interface should be monitored routinely.

The WHO Global Strategy for Containment of Anti-Microbial Resistance (AMR) 2001(WHO, 2012) identifies effective surveillance as a corner stone of national and international efforts to control AMR. Tracking antibiotic usage and the emergence and spread of resistant strains of bacteria provides information, insights and tools needed to guide and evaluate policy and measures which has to be taken to promote appropriate anti-microbial use at all levels- local, national and international. The detection of AMR and monitoring its spread requires laboratory based surveillance.

The prevalence of MRSA in human hospitals of Madhya Pradesh is reported to be around 30 – 40% (Joshi et al., 2013; Tripathi, 2015). The prevalence of MRSA in veterinary setting in the state of Madhya Pradesh had been lacking. This study was designed to study the prevalence scenario of MRSA in dairy cattle of the Jabalpur.

**MATERIALS AND METHODS**

A total of 111 cows were screened from six dairy farms at Jabalpur for the presence of mastitis by clinical examination and California mastitis test (Ruegg and Reinemann, 2002; Dhakal, 2006). Milk from infected quarter or udder with CMT score of ≥ 1 was aseptically collected in sterile tubes transported in ice and stored at -20 °C until further use.

Isolation of Methicillin-resistant *Staphylococcus aureus* was carried out according to the protocol published by EFSA, 2012 with some modifications. Briefly the milk samples were subjected to one freeze-thaw cycle and were mixed thoroughly before pre-enrichment in Mueller-Hinton (MH) broth (HiMedia) supplemented with 6.5 % NaCl at 35 °C for 16-20 hours; followed by enrichment in Tryptone Soya Broth (HiMedia) with 3.5 mg/L cefoxitin (Sigma) and 75 mg/L aztreonam (Sigma) at 35 °C for 16-20 hours. The HiChrome MeReSa agar plates with cefoxitin and methicillin supplement (HiMedia) were used as selective agar and were incubated at 35 °C for 16-20 hours. Up to five blue-green colonies indicative of being MRSA were chosen and were streaked in Tryptone soy agar and incubated at 35 °C for 16-20 hours.

The presumptive isolates were phenotypically characterized using Catalase test, Gram’s reaction, Voges-Proskauer test, Clumping factor test, Tube coagulase test, Haemolysis in Sheep blood agar, Novobiocin and Polymyxin B resistance, Mannitol fermentation in Mannitol salt agar and Maltose fermentation in Purple agar (National Mastitis Council, 2004; Tille, 2014). The isolates were further confirmed using Staphytect Plus Latex agglutination test kit (Oxoid, United Kingdom) performed as per the manufacturers instruction.

The Methicillin-resistance was phenotypically characterized using cefoxitin (30 μg) disk by disc...
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diffusion assay (CLSI, 2012) and by Penicillin Binding Protein-2a (PBP2a) Latex Agglutination Test (LAT) for the detection of PBP-2a (Oxoid, United Kingdom) as per the manufacturer’s instruction.

The genotypic characterization of the Methicillin-resistant *S. aureus* (MRSA) was done using the multiplex –PCR for the detection of *spa* and *mecA* genes (EFSA, 2012). *S. aureus* ATCC 43300 was used as positive control for the *spa* and *mecA* gene.

The PCR-amplified samples were analysed by agarose gel electrophoresis by using a horizontal 1.5% (w/v) agarose gel in 1X Tris Borate EDTA (TBE) buffer (pH 8.3; 89.0 mM Tris, 89.0 mM boric acid, 2.0 mM EDTA) at 80 Volts for 2 hours. The PCR products were visualized and photographed on a Gel documentation unit (Alpha Innotech). The 100 base pair (bp) ladder molecular weight marker was run in parallel with the samples. The *spa* fragment resulting from the amplification was variable in size and ranges from 180-600 bp depending on the *spa* type present and this fragment should be amplified from all *S. aureus* strains. It is the method of identification and an important virulence determinant. The amplification of the *mecA* gene with a size of 162 bp confirms methicillin resistance.

**RESULTS AND DISCUSSION**

**Prevalence of clinical and subclinical mastitis in bovines**

A total of 111 dairy cows from six dairy farms were screened for mastitis by California Mastitis test (CMT). A total of 85 quarter or composite samples of milk from 111 dairy cows with CMT score ≥ 1 were collected.

Out of the 85 milk samples collected from dairy cattle, a total of 32 samples were of clinical mastitis and 53 samples were of subclinical mastitis. A prevalence of 28.83% and 47.75% of clinical and subclinical mastitis respectively was found in 111 dairy cattle. Mir *et al.* (2014) found a higher prevalence of subclinical mastitis (57.8%) when following the criteria of both CMT and culturally positive samples. However, these results were obtained from farms with machine milked cows. Sharma *et al.* (2012) also reported the prevalence of 39.8% and Bangar *et al.* (2015) determined the pooled prevalence of subclinical mastitis in cattle in India to be 46.4%. In another study of 263 cows in Karnataka, the prevalence of clinical mastitis was 4.7 – 8% depending on the diagnostic tests (Kurjogi and Kaliwal, 2014). The variation in prevalence of mastitis between the different studies might be partly due to different types of diagnostic tests, sampling procedures and criteria for mastitis and to factors such as stage of lactation, parity number and breed of the animals included in the studies.

A total of 21 (24.70 %) samples were found to be culture negative, which indicates that these quarters did not shed bacteria in a sufficient amount or that the infection had been eliminated. However, mastitis causing bacteria can occur in substantial quantities also in growth-negative milk samples (Taponen *et al.*, 2009a; Kuehn *et al.*, 2013) meaning that an infection cannot be excluded even with a negative sample.

**Prevalence and characterization of Methicillin-resistant *S. aureus* (MRSA) from milk**

In human medicine *S. aureus* is considered as a major and common pathogen of genus *Staphylococcus* and is often considered synonymous with coagulase positive staphylococci (CoPS). The case in veterinary medicine is not very straightforward where although *S. aureus* is considered as a major coagulase positive staphylococci like *Staphylococcus intermedius* (Robertson *et al.*, 1992; Krithiga *et al.*, 2011) and *Staphylococcus pseudintermedius* (Pilla *et al.*, 2011) have also been reported to be cause of mastitis in bovines. Additionally the coagulase-negative staphylococci (CoNS) like *S. epidermidis*, *S. chromogenes* and *S. simulans* which are considered as emerging cause of subclinical mastitis in bovines are present as normal microflora in bovine skin and commonly carry the gene for methicillin resistance (Taponen *et al.*, 2009b; Feßler *et al.*, 2010). In this context the accurate identification of the *Staphylococcus* species and the demonstration of virulence factors in it cannot be over-emphasized.

A total of 85 milk samples were screened for the Methicillin-resistant *S. aureus* (MRSA) following the two step enrichment in Mueller-Hinton broth with 6.5% sodium chloride (NaCl) and growth in Tryptone Soy broth (TSB) with cefoxitin (CX = 3.5 mg/L) and aztreonam (AT = 75mg/L). Out of 85 samples, 25 samples showed typical bluish green coloured colony of MRSA in HiCrome
MeReSa agar with both the MeReSa selective supplement and cefoxitin supplement.

Using phenotypic tests – catalase, Gram’s reaction, clumping factor, tube coagulase and Voges-Proskauer tests, a total of 14 of these strains were presumptively identified as *S. aureus* as per the recommended criteria of NMC, 2004 (Table 1).

Table 1: Screening of milk samples for Methicillin-resistant *S. aureus*

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Particulars</th>
<th>No. of isolates positive for the test</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MH broth + 6.5% NaCl</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Growth in TSB + CX + AT</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Growth in HiCrome MeReSa Agar</td>
<td>25</td>
<td>29.41</td>
</tr>
<tr>
<td>4</td>
<td>Catalase</td>
<td>25</td>
<td>29.41</td>
</tr>
<tr>
<td>5</td>
<td>Gram positive cocci</td>
<td>25</td>
<td>29.41</td>
</tr>
<tr>
<td>6</td>
<td>Clumping Factor</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>Voges-Proskauer test</td>
<td>14</td>
<td>16.47</td>
</tr>
<tr>
<td>8</td>
<td>Tube coagulase</td>
<td>14</td>
<td>16.47</td>
</tr>
</tbody>
</table>

The 14 isolates were phenotypically characterized by mannitol and maltose fermentation, susceptibility to Novobiocin (5 µg disc), resistance to Polymyxin B (300 µg disc) and haemolysis in Sheep blood agar (SBA).

The 14 isolates (100%) were positive for mannitol fermentation in mannitol salt agar and for maltose in Purple agar (P agar). Similarly all the 14 isolates (100%) were susceptible to Novobiocin (zone diameter ≥ 17 mm) and resistant to Polymyxin B (zone diameter ≤ 10 mm) in Kirby Bauer disk diffusion assay.

The alpha (α) and beta (β) haemolysis was studied in 5 per cent Sheep blood agar (HiMedia). While 3 isolates (21.42 %) and 8 isolates (57.14 %) showed alpha (α) and beta (β) haemolysis respectively, a total of 3 isolates (24.56%) showed both alpha (α) and beta (β) haemolysis (Table 02).

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Particulars</th>
<th>No. of isolates positive for the test</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coagulase</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Mannitol fermentation</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Maltose fermentation</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Novobiocin susceptibility</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Polymyxin B resistance</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>α haemolysis</td>
<td>3</td>
<td>21.42</td>
</tr>
<tr>
<td>7</td>
<td>β haemolysis</td>
<td>8</td>
<td>57.14</td>
</tr>
<tr>
<td>8</td>
<td>α and β haemolysis</td>
<td>3</td>
<td>21.42</td>
</tr>
<tr>
<td>9</td>
<td>Staphytect Plus test</td>
<td>14</td>
<td>100</td>
</tr>
</tbody>
</table>

All the 14 isolates (100%) were positive for *S. aureus* in the Staphytect Plus latex agglutination test which detects the clumping factor, Protein A and capsular polysaccharide present in the cell wall of *S. aureus*.

The surface exposed (fibrinogen and fibronectin-binding proteins, the staphylococcal protein A or spa) and secreted (coagulase, haemolysins -alpha-toxins, Luk *MF*’; Luk *ED*; thermo-nuclease, enterotoxins) are some of the virulence factors commonly studied in *S. aureus* mastitis in bovines (Le Thanh et al., 2008; Singh et al., 2011)

In this study the virulence determinants phenotypically studied included the coagulase production, haemolysin in sheep blood agar and the ability to ferment mannitol. The protein A and capsular polysaccharides were detected immunologically by Staphytect Plus Latex agglutination test kit (Oxoid, UK). Additionally the staphylococcal protein A or *spa* gene was also genotypically detected using PCR. The strains which produced all these virulence factors were only included in the study. Haemolysin in blood agar represents an important criterion for rapid presumptive identification of *S. aureus* isolates of bovine origin (Boerlin et al., 2003). The phenotypic alpha- and beta-haemolysis shows geographical variation. (Le Thanh et al., 2008). Among the MRSA strains in this study the predominant pattern was β haemolysis. Virtually...
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all mastitis strains produce toxins that are active on neutrophils. All contain at least one leukotoxin gene: a γ-haemolysin (hlg) variant is present in all strains, LukED is present in most strains and lukMF-PV in 15–86% of strains (Monecke et al., 2007; Yamada et al., 2005). The α-haemolysin (hla) gene is present in most strains, and β-haemolysin (hlb) is more common in strains of bovine origin than those of human origin, and both toxins may play a role in the pathogenesis of mastitis (Bramley et al., 1989).

The methicillin resistance of the isolates was characterized by phenotypic methods – Resistance to cefoxitin (30 µg) disc in disk diffusion assay, production of Penicillin binding protein – 2a (PBP2a). The 14 (100%) MRSA isolates were resistant to cefoxitin. All the isolates (100%) produced PBP-2a as determined by the latex agglutination assay. This is in agreement with the study of Lee et al. (2004) which showed the test had sensitivity and specificity of 100%. The study found this test was a reliable and rapid method of detecting MRSA in the veterinary field.

After phenotypic characterization the 14 isolates were genotypically characterized for spa and mecA gene by multiplex PCR. All the 14 isolates (100%) were positive for spa and for mecA gene (Fig. 2). So the prevalence of 16.47 % MRSA mastitis in dairy cattle was found in this study.

Fig. 2: Agarose gel electrophoresis analysis showing multiplex PCR amplification products for the detection of the spa and mecA gene (168 bp) in the MRSA isolates. The Lanes 1, 2, 4-12 shows the mecA gene (168 bp) in the MRSA isolates. P = positive control S. aureus ATCC 43300, N = negative control

Methicillin-resistant S. aureus (MRSA) includes those strains that have acquired genes (mecA or mecC) conferring resistance to methicillin and essentially all other β-lactam antibiotics. MRSA was initially reported as a nosocomial pathogen in human hospitals (or hospital-associated MRSA, HA-MRSA). Since the 1990s, community-acquired or community-associated MRSA (CA-MRSA) infections have also been reported to affect people having no epidemiological connection with hospitals (Deurenberg and Stobberingh, 2008). In 1972 a cow in Belgium with mastitis was the first reported MRSA infection in an animal (Devriese et al., 1972).

More recently, MRSA has been isolated from most food-producing animals and foods of animal origin, raising public health concerns. MRSA strains have been isolated from cows’ or small ruminants’ milk and various dairy products in many countries. Since early 2000s we are witnessing the epidemic wave 6 of MRSA which is for the first time related to livestock (Chatterjee and Otto, 2013). The MRSA prevalence in milk and dairy products recorded in different countries or even regions of the same country differs significantly. High MRSA prevalence have been recorded in milk produced in most African countries, for instance as high as 60.3% in Ethiopia. The MRSA prevalence in Asian countries varies from high e.g. 28.3% in Iran to less than 1% in Japan (Pexara et al., 2013). The prevalence has been reported to be 13.9 % in South Korea (Park et al., 2016). In most European countries, the MRSA prevalence in milk and dairy products has been generally found to be low. In the US and Canada, zero to low MRSA prevalence estimates have been reported (Pexara et al., 2013). The investigation of MRSA prevalence in milk may serve as a tool for assessing both the sanitary conditions employed in dairy herds and the health risks that humans may encounter when infected with antibiotic-resistant strains.

In India the prevalence of MRSA in bovine milk as detected by mecA gene has been reported to vary widely between 5.11 to 27% (Prashanth et al., 2011; Singh et al., 2011; Chandrasekhran et al., 2014; Kutar et al., 2015).

The methicillin resistance in the mastitis causing pathogen incurs greater economic loss to the farmer as it leads to decreased treatment options as the β-lactam antibiotics are not effective, increased cost of treatment due to increased therapeutic failures, poor prognosis and occupational hazard (Barkema et al., 2006; Bardiau et al., 2013).

Staphylococcus aureus is a well-known commensal and opportunistic pathogen of both bovines and humans. S. aureus is a clonal organism. However while S. aureus are
quite ubiquitous in terms of host species, different animals tend to harbor different genetic lineages. The genetic lineages of S. aureus in general and MRSA in particular has been extensively defined by various typing methods like staphylococcal protein A or spa typing, Multi Locus Sequence Typing (MLST) and Staphylococcal Cassette Chromosome (SCC) mec typing. (Deurenberg and Stobberingh, 2008). The molecular typing of MRSA of both human and bovine origin has demonstrated that some of the clones are host specific while others have wider host specificity (Fournier et. al., 2008; Piccinini et. al., 2010). The prevalence of MRSA as a cause of mastitis is of great concern as it has got wider implications on animal welfare and public health. The predominance of β-haemolysis in the MRSA strains in this study is suggestive of human origin of the isolates. However this needs to be validated using spa, MLST and SCCmec typing which will provide the true picture in terms of epidemiology, zoonotic risk and antimicrobial resistance.

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