Incidence of clinical and sub-clinical bovine mastitis caused by \textit{Staphylococcus aureus} in Proddatur region of Andhra Pradesh

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Abstract

Staphylococcal mastitis is one of the commonest infection associated with dairy animals, mostly prevails in sub-clinical form and once left unidentified, usually flares up with clinical mastitis resulting in huge economic losses to the dairy owners. Therefore, the present study was undertaken to know the incidence of clinical and sub-clinical mastitis caused by \textit{Staphylococcus aureus} in bovines in the Proddatur region of Andhra Pradesh by means of conventional and molecular methods. A total of 61 clinical mastitis milk samples were collected and 105 milk samples were screened for the presence of sub-clinical mastitis by California mastitis test (CMT). The clinical samples and the samples positive for sub-clinical infection were then subjected for specific culture and morphological identification by gram’s staining. The positive isolates were further confirmed for \textit{Staphylococcus} genus and \textit{S. aureus} by PCR targeting the 16S rRNA and nuc gene respectively. The overall incidence of \textit{Staphylococcus} Sp and \textit{S. aureus} in bovine mastitis was found to be 89% and 54% respectively in clinical mastitis and 71% and 50% in sub-clinical mastitis as confirmed by PCR. These findings clearly indicate the prevalence of \textit{S. aureus} infection in bovine mastitis in this specific region and suggest the need for the routine screening of dairy herds for \textit{S. aureus} associated mastitis infection, use of better management and animal husbandry practices for the prevention and early cure of bovine mastitis.

Keywords: Bovine mastitis, \textit{Staphylococcus aureus}, Incidence, Polymerase chain reaction

Introduction

Livestock sector is a major income source of the poor and especially of women in developing countries. India is one of the largest milk producer in the world and bovine mastitis is one of the important production diseases of dairy animals which directly or indirectly affect the economy of the farmers and ultimately affect the economy of the country. In dairy cattle, mastitis results in severe economic losses from reduced milk production, treatment cost, increased labor, milk withheld following treatment and premature culling \cite{1}. Because of the anatomical position, udder is subjected to outside influence and is prone to both inflammation and non-inflammatory conditions \cite{2}. Infectious mastitis results from the introduction and multiplication of pathogenic microorganisms in the mammary gland and leads to a reduced synthetic activity, changes in the milk composition and elevated milk Somatic Cell Count (SCC) \cite{3}. Almost any bacteria or fungi can opportunistically invade tissue and can cause mastitis. However, most infections are caused by various species of \textit{Staphylococci}, \textit{Streptococci}, and gram-negative rods, especially lactose-fermenting organisms of enteric origin, commonly termed coliforms. \textit{Staphylococcus aureus} infection of cattle is of high economic relevance as \textit{S. aureus} is an important agent of bovine mastitis \cite{4,5,6,7}. Though some cows may flare up with clinical mastitis (especially after calving) the infection is usually sub-clinical, causing elevated somatic cell counts (SCC) but no detectable changes in milk or the udder. The bacteria persist in mammary glands, teat canals and teat lesions of infected cows and are contagious. The infection is spread at milking when \textit{S. aureus} contaminated milk from an infected gland comes in contact with an uninfected gland and the bacteria penetrate the teat canal. A research has shown that 3 percent of all animals are infected with \textit{S. aureus} \cite{8}. However, \textit{S. aureus} represents 10 to 12 percent of all clinical mastitis infections \cite{9}. Interestingly, cows infected with \textit{S. aureus} do not necessarily have elevated SCC.
S. aureus intramammary infections (IMIs) are difficult to eliminate. Once established, S. aureus infections do not respond well to antibiotic therapy and infected cows must be segregated or culled from the herd. The most notable feature of S. aureus is its ability to evade and influence the host immune system. Much research work involving strain typing has shown that important strain differences exist in contagious, persistent and virulent strains of S. aureus [10]. Persistent infection of S. aureus mastitis is attributed to the ability of specific strains of S. aureus to transform into a variant sub-population known as small colony variants (SCVs) that can survive within host cells and subsequently modulate the immune response [11]. Staphylococcal infections are typically associated with death of tissue and evidence suggests intracellular bacteria are capable of inducing apoptosis. S. aureus-mediated apoptosis has been reported in epithelial cells [12, 13, 14], keratinocytes [15] and endothelial cells [16, 17].

Antimicrobial resistance of S. aureus is an increasingly important problem. The most important issue is resistance towards methicillin and all beta-lactam antibiotics (methicillin-resistant Staphylococcus aureus, or MRSA) [18, 19]. Milkers hand introduction has the largest impact on the incidence of Staphylococcal and Streptococcal mastitis particularly the methicillin resistant Staphylococcus aureus (MRSA) mastitis. Prior sanitized human hands and premilking udder preparation considerably reduces the incidence of the disease [20]. Early identification of the prevalence and distribution of causative pathogens is one of the important prerequisites to effectively prevent diseases and to guide treatment. Therefore, the objective of this study was to know the incidence of clinical and sub-clinical mastitis caused by S. aureus in bovines in the Proddatur region of Andhra Pradesh by means of conventional (California mastitis test - CMT, bacterial culture and gram’s staining) and molecular (Polymerase chain reaction) methods.

Materials and Methods

Sample Collection

A total of 61 clinical mastitis milk samples (52 cows and 9 buffaloes) were collected from Veterinary Clinical Complex and Livestock Farm complex, College of Veterinary Sciences, Proddatur, various private farms maintained in Proddatur town and villages nearby. Also, a total of 105 bovine milk samples (42 cows and 63 buffaloes) were screened for the presence of sub-clinical mastitis using California Mastitis Test [21] out of which, 16 samples (7 cows and 9 buffaloes) were positive for infection. Milk samples were collected in 10 ml sterile vials after discarding the initial 1-2 ml of milk during milking. The collected samples were transported in cold condition and stored at 4°C until further processing.

Isolation and Identification of Staphylococcus aureus

Samples were mixed well and two or three loopful of milk was streaked on to Mueller Hinton agar with 7% sodium chloride which is specific for organisms of the genus Staphylococcus and incubated at 37°C for 24-48 hrs. The cultured organisms were then subjected for bacteriological analysis. Gram staining was then performed and only the gram positive cocci which were arranged in clusters were considered and the same individual colonies from the culture plates were streaked on Mueller Hinton agar and incubated overnight at 37°C to obtain good growth of the bacterium. A loop full of obtained culture was inoculated in 2ml Luria broth, incubated overnight at 37°C and this final culture was used for the identification of S. aureus by polymyxinase chain reaction.

Extraction of bacterial DNA

The DNA from S. aureus was extracted following the method described by Christensen et al. 1993 [22] with minor modifications. Overnight culture of Luria broth (2ml) was transferred to an eppendorf tube and pelleted at 12,000 rpm for 10 min. The supernatant was removed and cells were resuspended in 900µl of TE buffer. Then 80µl of SDS and 25µl of proteinase K (20mg/ml) was added to the tube, mixed well by inverting the tube several times and incubated for 30min at 55°C. Added 900µl of phenol/chloroform (1:1) and mixed gently by inverting the tubes several times until it becomes a homogenous milky solution. Centrifuged at 14,000 g for 10 min. Carefully transferred 500µl of the upper aqueous phase into a fresh eppendorf tube. Added 75µl of (3M) sodium acetate and mixed gently. Added 500µl of isopropanol and mixed gently by inverting the tube and centrifuged at 15,000 x g for 10min to precipitate the DNA. The collected DNA was washed with 1ml of 70% ethanol, centrifuged at 10,000 x g for 5 min. The supernatant was removed and the DNA pellet was allowed to dry. The DNA was finally dissolved in 100µl of TE buffer at 37°C for 15min and stored at -80°C.

Staphylococcus genus specific PCR amplification

PCR assay targeting the 16S rRNA gene of Staphylococcus species [23] was performed using the primer set; forward 5′ – AACTCTGTATTAGCGGAAAGACA – 3′ and reverse 5′ – CCACCTTTCCTCCGGTTTGTCACC – 3′ targeting a DNA amplicon of 756 bp. A 25 µl of PCR reaction mix was prepared using 12.5 µl of Dream taq green PCR master mix (Thermo Scientific), 1 µl of forward primer, 1 µl of reverse primer, 5 µl of template and 5.5 µl of nuclelease free water. Amplification was carried out as follows: an initial denaturation of 95°C for 4 minutes; 30 cycles of 95°C for 45 sec, 55°C for 45sec and 72°C for 45 sec and a final extension step at 72°C for 6 min.

PCR for S. aureus

The isolates which were positive for Staphylococcus genus were further screened for S. aureus by targeting the nuc gene according to Brakstad et al. 1992 [24] with slight modification. A 25 µl of PCR reaction mix was prepared using 12.5 µl of master mix, 1 µl of forward primer, 1 µl of reverse primer, 5 µl of template and 5.5 µl of nuclease free water as carried out for Staphylococcus genus. Amplification was carried out as follows: an initial denaturation of 94°C for 5 minutes; 30 cycles of 94°C for 1 min, 55°C for 0.5 min and 72°C for 1.5 min and a final extension step at 72°C for 3.5 min.

Agarose Gel Electrophoresis

The DNA fragments were stained with ethidium bromide and were visualized using Chemi documentation system from Syngene, Biocon, U.S.A. The size of the amplified product was compared by the use of 100 bp DNA ladder, Thermo Scientific.

Results and Discussion

By means of culture and staining (Fig 1), 44% of the samples (27) were positive for Staphylococcus genus out of 61 clinical mastitis milk samples collected from cows and buffaloes. Of all the bovine milk samples examined for sub-clinical mastitis, 14% (16/105) of the samples were positive for infection by CMT (Details are provided in Table 1 and Table 2). Out of 16 samples positive for sub-clinical mastitis by CMT, 14 samples (88%) were positive for Staphylococcus genus by culture and staining. Grams’ staining was done and only the gram positive cocci arranged in clusters were identified as Staphylococcus genus. The isolates positive for Staphylococcus genus by

~ 789 ~
culture and staining were then confirmed for *Staphylococcus* genus and *Staphylococcus aureus* by PCR. 24 clinical isolates (89%) out of this presumptive 27 isolates of clinical mastitis were positive for *Staphylococcus* Sp by PCR using genus specific primers (16S r RNA) which has amplified the expected product size of 756bp (Fig 2). Out of 24 clinical isolates of *Staphylococcus* Sp, 13 (54%) were positive for *S. aureus* by PCR targeting the nuc gene (Fig 3). Out of the 14 sub-clinical mastitis milk samples positive for *Staphylococcus* Sp. by culture and staining, 10 isolates were positive for *Staphylococcus* Sp by PCR (Fig 2). Out of these 10 sub-clinical isolates positive for *Staphylococcus* genus by PCR, 5 isolates (50%) were positive for *S. aureus* by PCR (Fig 3).

Therefore, in the present study, the overall prevalence of clinical mastitis caused by *S. aureus* in bovines was found to be 54% in the specific region of Proddatur as confirmed by PCR. In cows, the prevalence was found to be 47% and in buffaloes it was found to be 80%. The present findings show a higher incidence rate of *S. aureus* bovine mastitis when compared to the findings of Patel et al. 2012 [25] and Sharma et al. 2012 [26], where the prevalence of *S. aureus* mastitis was found to be 30-40 % in India. In an another study done by Bhat et al. 2017 [27], the prevalence of *S. aureus* bovine mastitis in Jammu region was found to be 60.87%. In China, ninety (46.2%, 90/195) samples collected from dairy cows suffering from mastitis were positive for *S. aureus* [28]. The over all percent prevalence of sub clinical mastitis was 54/172 (27%) and clinical mastitis was 8/172 (4%) in buffaloes. Out of 564 quarters examined for mastitis, 320 (56.73%) quarters were found culturally positive showing isolation of *Staphylococcus aureus* [29].

The prevalence of bovine mastitis ranged from 29.34 to 78.54% [30, 31, 32] in cows and 27.36 to 70.32% [33, 34] in buffaloes. Detailed analysis of previous studies conducted in Pakistan [35] revealed that highest prevalence of clinical and sub-clinical mastitis in cattle and buffaloes was due to *S. aureus* with a mean of 46.72%. The incidence of mastitis in crossbred Murrah is considerably high as compared to indigenous breeds of buffaloes and cattle in India [36]. Mastitis rate was lower in Jersey than in Holstein cattle [37]. The involvement of genetic factors causing susceptibility to mastitis has also been established as incidence rates showing higher among relatives of affected individuals than the general populations. The risk of mastitis increases with the increase in parity, attributable to mammary immunocompromisation [38].

The difference in prevalence rates of *S. aureus* associated bovine mastitis with respect to the present findings and the above mentioned data may be due to variation in the sanitary condition of the udder, size of sampling and geographic location. PCR technique has an advantage of giving faster results than the conventional culture techniques [39]. As reported by Hamed and Zaitoun 2014 [40], the culturally negative samples when examined by PCR technique, indicated that 80% of the tested animals were positive to *S.aureus*. Therefore, there is a need for the evaluation of false negative results with reference to the identification of infection by CMT and further identification of *Staphylococcus* Sp by culture and staining.

### Table 1: Incidence of *S. aureus* in bovine clinical mastitis milk

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of samples examined</th>
<th>No. of samples positive for CMT</th>
<th>Presumptive culture and staining method</th>
<th>PCR for <em>Staphylococcus</em> genus</th>
<th>PCR for <em>Staphylococcus aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. of positive</td>
<td>%</td>
<td>No. of positive</td>
</tr>
<tr>
<td>Cows</td>
<td>42</td>
<td>7</td>
<td>6</td>
<td>86</td>
<td>3</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>63</td>
<td>9</td>
<td>8</td>
<td>89</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>16</td>
<td>14</td>
<td>88</td>
<td>10</td>
</tr>
</tbody>
</table>

### Table 2: Incidence of *S.aureus* in bovine sub- clinical mastitis

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of samples examined</th>
<th>Presumptive culture and staining method</th>
<th>PCR for <em>Staphylococcus</em> genus</th>
<th>PCR for <em>Staphylococcus aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of positive</td>
<td>%</td>
<td>No. of positive</td>
</tr>
<tr>
<td>Cows</td>
<td>52</td>
<td>21</td>
<td>40</td>
<td>19</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>9</td>
<td>6</td>
<td>66</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>27</td>
<td>44</td>
<td>24</td>
</tr>
</tbody>
</table>

Fig 1: *Staphylococcus aureus* isolated on Mueller hinton agar agar
with CMT, identification of the source of mastitis infection, pathogenic organism as well as the drug of choice for the treatment by the use of ABST. Also, with reference to some of the limitations with the identification of S. aureus by routine CMT, culture, staining and their sensitivity, there is a need for the use of PCR technique for the confirmatory and faster diagnosis of bovine mastitis caused by S. aureus.

References

Conclusion
In this study, the overall incidence rate of clinical and sub-clinical bovine mastitis caused by S. aureus in Proddatur region and confirmed by PCR was found to be 54% and 50% respectively. Therefore, it can be concluded that the major bacterial pathogen causing clinical and sub-clinical mastitis in cows and buffaloes in the specific region is S. aureus. The infected sub-clinical mastitis milk if consumed without pasteurization may cause serious threat to the humans and also can act as a silent and chronic cause of reduced milk yield, may end with clinical mastitis if undetected and can lead to severe economic loss to the livestock owners. Therefore, there is a need for the routine screening of the dairy herds for the etiology of reduced milk production, screening for sub-clinical mastitis

Fig 2: Staphylococcus genus specific PCR targeting 16S rRNA gene.

Fig 3: Staphylococcus aureus species specific PCR targeting nuc gene.

Lane 1 - 3: PCR amplicons (270 bp) from cow’s mastitis milk samples; Lane 4: Negative control; Lane 5: 100 bp DNA ladder; Lane 6 – 8: PCR amplicons from buffalo mastitis milk samples
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