Molecular Detection of *bla*Z and *mec*A Genes and Study of Antibiotic Resistance Pattern in Clinical Isolates of *Staphylococcus aureus* from Bovine Mastitis in Coastal Andhra Pradesh

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Abstract

**Introduction**: *Staphylococcus aureus* is known to cause sub clinical and clinical intra mammary infections like mastitis in milch animals. *S. aureus* demonstrates a distinctive ability to quickly develop a resistance mechanism, starting with penicillin, until against even the most recent, linezolid and daptomycin.

**Materials and Methods**: In the present study, 100 milk samples from mastitis of buffaloes in coastal Andhra Pradesh were collected. These isolates were characterized initially by biochemical tests like Mannitol fermentation, Catalase test, Coagulase test, Spot Oxidase test, Voges – Proskauer test and the Haemolytic activity on 5 % Sheep Blood agar. Provisionally confirmed isolates of *S. aureus* were further processed for molecular detection of *S. aureus* with species specific oligonucleotide primers Staur 4 and Staur 6. The sensitivity of confirmed isolates of *S. aureus* to different antimicrobials was tested against 10 antimicrobials by Kirby-Bauer disc diffusion method. The presence of antibiotic resistance genes like b-lactamase (*bla*Z) and methicillin resistance (*mec*A) were screened by PCR using specific primers.

**Result**: Out of 100 isolates, 69 samples were provisionally positive for *S. aureus* by biochemical methods and 34 isolates (34.0%) by species specific PCR. High resistance was recorded to Ceftriaxone+Tazobactum, Oxacillin and Amoxycillin (100%), followed by Ceftriaxone+ Sulbactum (93.55%) and Methicillin (83.87%), Penicillin (80.65%), Ampicillin (70.97%) and Ceftriaxone (38.71%). Low resistance was observed for Cefoxitin (6.47%) and Amoxycillin+ Clavulanic acid(0). A high frequency of *mec*A (61.29%) and *bla*Z (45.16%) antibiotic resistance genes was found in *S. aureus* isolates. Both *mec*A and *bla*Z genes were present in 22.58% of *S. aureus* isolates.

**Conclusion**: The results show that the organisms are acquiring resistance against commonly employed antimicrobials to treat mastitis by acquiring antibiotic resistance genes at an alarmingly fast rate.

**Key Words**: *Staphylococcus aureus*, antibiotic resistance, Mastitis, *bla*Z, *mec*A.

Introduction

In India, losses due to mastitis in dairy animals are estimated to be about Rs. 6053.21 crore per annum (1). Approximately 8% to the GDP of Indian economy is contributed by dairy industry. But the onslaught of diseases likes mastitis, not only in India but globally, leads to enormous losses to the dairy sector. As a consequence, this big economic sector is suffering several setbacks including bacterial...
pathogens, requiring scientific interventions urgently needed to curb the menace of such emerging diseases of livestock in the country. One of such disease is mastitis, causing serious wastage and undesirable milk quality in dairy development of tropics. Subclinical mastitis is common in India, varying from 10–50% in cows and 5–20% in buffaloes than the clinical mastitis (1–10%) (2). *Staphylococcus aureus* is one of the most important causative agents in sub clinical and clinical intra mammary infections like mastitis in milch animals. The Indian dairy industry is facing economic losses by mastitis due to its adverse impact on milk production of cows and buffaloes (2 & 3).

The ability of *S. aureus* to develop or acquire strategies which provide resistance to different antimicrobials is an additional approach in the impressive arsenal of this pathogen. Antibiotic therapy is a significant tool used in the scheme of mastitis control. The development of resistance among different bacterial strains is because of misuse or intensive use of antibiotics for treatments (4). One of the diagnostic tools accessible to practitioners to assist with the selection of a suitable treatment is the *in vitro* testing of isolates against a representative panel of antimicrobial drugs. The susceptibility pattern of the implicated strain enables crucial action to be taken by the veterinarian in terms of treatment (5). This avoids the needless application of ineffective antimicrobials and prevents unnecessary costs from being incurred.

Extensive and inadvertent use of antimicrobials both in human and in veterinary medicine is the key reason for emergence of resistant strains of *S. aureus* (6). Emergence of MRSA (Methicillin Resistant *S. aureus*) and VRSA (Vancomycin Resistant *S. aureus*) have been reported in livestock in past (7-14). There is an enormous increase and emergence of *S. aureus* strains resistance to the antibiotic methicillin (MRSA strains) and also to the beta lactam antibiotics like penicillin and its derivatives over the past few decades. The antimicrobials generally used for the treatments of infections caused by *S. aureus* were penicillin and its derivatives, including methicillin (15). The presence of meCA and blaZ genes in a particular strain is responsible to the intrinsic resistance to these antimicrobials. Due to the development of antibiotic resistance in *S. aureus*, conventional antibiotic treatment which was used frequently at field level is not satisfactory for preventing the establishment of chronic infection or in eliminating existing disease. There are three different types of MRSA namely Hospital Acquired MRSA (HA-MRSA), Community Acquired MRSA (CA-MRSA) and Livestock Associated MRSA (LA-MRSA) (16). As the treatment options for this highly zoonotic MRSA are limited, these infections have gained importance. There is a drastic rise in number of reports of MRSA in domestic dairy animals (7-11). Cross-infection of certain strains of MRSA between humans and animals, were also reported (17). It was reported in several parts of the world that MRSA causes life threatening sepsis, endocarditis and osteomyelitis in human beings. Animals can thus act as potential source of MRSA infection to in-contact human beings (18). Thus the development of multidrug resistance in MRSA possesses a serious public health concern too.

In this context, the present investigation has been carried out with an aim to know the antibiotic sensitivity pattern of *Staphylococcus aureus* isolates with special reference to MRSA. A total of 100 mastitis milk samples were collected from three different districts from Coastal Andhra Pradesh, namely Krishna, Guntur and East Godavari and were evaluated for the presence of *S. aureus*. The *S. aureus* isolates were tested for their susceptibility to a panel of antimicrobial agents which are frequently used for the treatment of *S. aureus* in human beings. The present study enables to assess the potential zoonotic threat of *S. aureus* from animal origin which are resistant to multiple drugs commonly employed in human antimicrobial therapy.

**Materials and Methods**

**Sample Collection:** A total of 100 milk samples were collected from buffaloes affected with...
Molecular Detection of Genes and Antibiotic Resistance

Table 1. Oligonucleotide primers and PCR test conditions for S. aureus and detection of antibiotic resistant genes for blaZ and mecA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Product size</th>
<th>Sequence (5'- 3')</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus 23s rRNA</td>
<td>Staur 4, Staur 6</td>
<td>1250bp</td>
<td>ACGGAGTTACAAGGACGAC AGCTCAGCCTAAGGACGAC</td>
<td>94°C /45sec</td>
<td>64°C/60sec</td>
<td>72°C/2min</td>
</tr>
<tr>
<td>BlaZ</td>
<td>blaZ F, blaZ R</td>
<td>517</td>
<td>AAGAGATTTGCGCATGCTTC GCTTGACCACCTTATCAGC</td>
<td>94°C/4min</td>
<td>94°C /60sec</td>
<td>55°C/60sec</td>
</tr>
<tr>
<td>(Vesterholm-Nielsen et al., 1999)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MecA</td>
<td>mecA F, mecA R</td>
<td>162</td>
<td>TCCAGATTCAAATTCACCCAGG CCACTTCATCTTGAAGG</td>
<td>94°C/45sec</td>
<td>50°C/30sec</td>
<td>72°C/30sec</td>
</tr>
<tr>
<td>(Stegger et al., 2012)</td>
<td></td>
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F= Forward primer R=Reverse Primer
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of 5ìl Go Taq Green Master Mix (Promega, USA), 0.25ìl of forward and reverse primer each, 0.5ìl of DNA template to which 4.0ìl of distilled water was added. The master mix contained 2X Green Go Taq Reaction Buffer (pH 8.5), 3 mM MgCl₂ and 400ìM of each dNTP. The PCR conditions starts with initial denaturation and subsequent denaturation carried out at 94°C for 2 min and 45 sec respectively, followed by annealing at 64°C for 60 sec, continued by extension and final extension at 72°C for 2 min and 10 min respectively. A total of 35 PCR cycles were run with the same conditions.

The amplified PCR products were analyzed by electrophoresis on a 1.7% agarose gel stained with 0.5ìg of ethidium bromide / ml in Tris Borate EDTA (TBE) buffer. Electrophoresis was performed at 90 V for 120 min in submarine gel electrophoresis unit (M/s Atto Corporation, Japan) and finally the PCR products were visualized in InGenius Gel Documentation System, (M/s Syngene, U.K) along with a ProxiO 100bp DNA ladder (M/s BioLit, SRL, India).

**Antimicrobial Susceptibility Testing by Disc Diffusion Assay:** All the positive *S. aureus* isolates were tested for susceptibility for the following 10 antimicrobial agents: amoxicillin (30 mcg), amoxicillin + clavulanic acid (30/15 mcg), ceftriaxone (30mcg), ceftriaxone + tazobactam (30/10 mcg), ceftriaxone + sulbactam (30/15 mcg), oxacillin (5 mcg), penicillin (10 mcg), ampicillin (10 mcg), methicillin (5 mcg), and cefoxitin (10 mcg). The disc diffusion assay and zone interpretation of each antimicrobial agent was done according to Clinical and Laboratory Standards Institute, 2012 (CLSI, 2012).

**Molecular Detection of blaZ and meCA Genes by PCR:** The PCR assays were performed for the detection of antibiotic resistance genes *meCA* responsible for methicillin resistance and *blaZ* gene responsible for penicillin resistance. The primer sequences and PCR conditions were given in the table 1. PCR was run for 35 cycles with initial denaturation at 94°C for 4 min and final elongation at 72°C for 10min for *blaZ* oligonucleotide primer sets. And 35 cycles with initial denaturation at 94°C for 5 min and final elongation at 72°C for 10min for *blaZ* oligonucleotide primer sets.

**Results**

The study has been conducted by using 100 mastitis milk samples were collected and tested for the presence of *S. aureus*. Out of 100 milk samples 40 cases were clinical mastitis whereas 60 were subclinical mastitis after testing by California Mastitis Test.

**Isolation of S. aureus:** Out of 100 samples collected, 69 (69.0%) isolates were mannitol fermenting when cultured on Mannitol Salt Agar (MSA) medium and all the isolates were gram positive cocci.

**Biochemical Characterization:** All the 69 isolates were catalase, Vogus Proskauer and coagulase positive whereas oxidase negative.

**Haemolysis:** Out of 69 isolates 20 isolates were α haemolytic, 10 isolates were â haemolytic and 5 isolates shown both α and â haemolysis on 5% sheep blood agar medium. Remaining 34 isolates were non haemolytic.

**Detection of S. aureus by PCR:** 34 isolates out of 69 were confirmed as *S. aureus* by PCR test (Fig 1). These 34 isolates were further tested to study the antimicrobial resistance pattern to different antimicrobial agents.

**Antimicrobial Susceptibility Testing by Disc Diffusion Assay:** Antimicrobial Susceptibility Testing to the molecular confirmed *S. aureus* isolates was performed by Disc Diffusion Assay and the results are shown in Fig 2. A total of 10 different antimicrobials were tested against 34 *S. aureus* isolates. Maximum resistance was recorded to oxacillin, ceftriaxone and tazobactam (100%), followed by ceftriaxone+ sulbactam (93.55%) and methicillin (83.87%). These were followed by Penicillin (80.65%), Ampicillin (70.97%) and Ceftriaxone (38.70%). Minimum resistance was found to Cefoxitin (6.47%) and Amoxycillin + Clavulanic Acid (0.0%). The results show that the organisms are acquiring resistance...
Molecular Detection of Genes and Antibiotic Resistance against commonly employed antimicrobials to treat mastitis.

**Detection of mecA and blaZ in S.aureus Isolates:** Total of 34 isolates were screened for the presence of antibiotic resistance genes namely, blaZ and mecA. Out of which in 14 (45.16%) isolates blaZ gene was present and in 19 (61.29 %) isolates mecA gene was present. Besides, in 7 (22.58%) isolates both blaZ and mecA genes were present (Fig 4).

**Discussion**

Bovine mastitis is a significant intramammary infection in animals and *S. aureus* is one of the major pathogen in bovine mastitis. It causes serious loss in agriculture and animal husbandry. *S. aureus* is known for its dynamic virulent characteristics (25). The distribution of \( \alpha, \beta \)-haemolysin and coagulase was in agreement with earlier findings (25,26 & 23). In another study done by us, it has been found that 68% of the samples were positive for *S.aureus* (27) of bovine mastitis in coastal Andhra Pradesh whereas in the present investigation only 31% were positive for *S. aureus*. Similar to our findings, other workers from India like Chavan et al. (28) in Hissar found prevalence of *S. aureus* in Mastitis cases 38.66% coagulase positive and
29.33% of coagulase negative *S. aureus*. Sharma *et al*, (29) and Roychoudhury and Dutta (30) have also reported many positive cases of *S. aureus*. Many workers have found *S. aureus* to be more prevalent than other species of the same genus (31, 32, 33 & 34) in mastitis. However, very few systematic reports were available for bovine mastitis problem in coastal Andhra Pradesh where dairy industry is one the major source of income for farmers.

Therefore in the present study we have tried to address this problem. A total of 100 *S. aureus* isolates from milk samples were tested for antibiotic resistance to obtain crucial information regarding the potential threat of antibacterial resistance in animal diseases and its possible zoonotic potential. A huge number of isolates were observed to demonstrate resistance to multiple antimicrobials. Frequent and long-term use of a particular antibiotic in a specific region creates a selection pressure in the organisms, resulting in development of resistance in bacteria. (23,35 &36) In the present investigation, a very high level of resistance was recorded to oxacillin, ceftriaxone and tazobactam (100%), followed by ceftriaxone+ sulbactam (93.55%) and methicillin (83.87%). These were followed by Penicillin (80.65%), Ampicillin (70.97%) and Ceftriaxone (38.70%). Minimum resistance was found to Cefoxitin (6.47%) and Amoxycillin + Clavulanic Acid (0.0%). Followed by Cefoxitin(3.3%), Ceftriaxone and Linezolid (9.68%), Streptomycin (12.90%), Neomycin (25.81%) and Enroflaxacin (29.03%). This result is alarming as the organisms are acquiring resistance against commonly employed antimicrobials for Gram positive bacteria used in human beings. The percentage of penicillin-G resistant isolates (80.65%) in this study was higher than those reported in American herds and European herds (37, 38 & 39). There was a higher prevalence of MRSA (48.39%) as compared with those in similar reports given by Moon *et al*, Kumar and Van den Eede *et al* (36, 23 & 40). Moreover, the multidrug resistance proportion was higher in MRSA than in MSSA isolates for various antimicrobials (41 & 23). Unlike the findings of Pankaj *et al*, (42), in the present study *S. aureus* isolates (100%) were resistant to ceftriaxone. Studies conducted by several workers (43,28,30 & 44) have showed increased resistance towards different traditional and newly introduced antimicrobials. In support to these studies, the antibiogram obtained in the current study indicated higher resistance towards newer and older antimicrobials. This proves that *S. aureus* demonstrates a distinctive ability to quickly respond to newer antimicrobials with the development of an appropriate resistance mechanism. The exact mechanism of development of resistance requires a thorough investigation since it creates an alarming situation of non-responsiveness of antibiotic and transmission of resistance across other genera.

Further the positive *S. aureus* isolates were screened for the presence of antibiotic resistant genes like *blaZ* which is responsible for penicillin resistance and *mecA* gene responsible for methicillin resistance. In the present investigation the percentage positive for *mecA* was 61.29%, whereas in the investigation carried by Memon *et al*. (45) there was no presence of *mecA* gene in *S. aureus* isolates from bovine mastitis. Lee (46) isolated 525 *S. aureus* isolates, out of which 19 (3.61) were positive for *mecA* gene from bovine mastitis cases. In Finland out of 135 isolates only one isolate of *S. aureus* from bovine mastitis was *mecA* positive (47) and in another study carried in West Bengal only 18.42% was positive for the presence of *mecA* gene in *S. aureus* from bovine mastitis cases. In Tamilnadu (48), the percentage positivity for *mecA* was 10.34. The present study is clear evidence that is a high risk of emergence of MRSA in India with special reference to Coastal Andhra Pradesh. This may probably due to improper use of antimicrobials by unqualified people, which is prevailing in this area, though we don’t have conclusive treatment history of individual animals that are tested. Unlike to the results of Memon *et al*. (45) where they got 82% of *blaZ* positive in the present work the percentage positive of *blaZ*
gene was 45.16%, whereas in Tamilnadu it was 10.34%.

Depending upon the type of organisms and use of antimicrobials in a particular region antibiotic resistance patterns vary among different farms, regions, states and countries. Prudent use of antimicrobials in the dairy animal is important, necessary and worthwhile. Therefore, antimicrobial sensitivity test is recommended before institution of treatment, so that injudicious antibiotic usage and thus the development of resistance is prevented. Moreover, prophylactic management measures against mastitis, rather than therapeutic management using antimicrobials has to be encouraged in dairy industry. The information obtained from the present study will be of helpful not only in prioritizing mastitis control efforts but in zoonotic transmission control as well.

**Conclusion**

Milk samples from 100 mastitic buffaloes in four districts of Andhra Pradesh were collected and antibiotic sensitivity test was done to assess the resistance pattern. It has been found that the organisms possess multiple antibacterial resistance, characterized by b-lactamase (45.16%) and methicillin resistance genes (61.29%). Since the farmers live in close proximity to the animals, it can be of a significant public health concern.

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