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**Research Article**

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**ABSTRACT**

A total of 90 market milk samples collected from Tirupati and its surrounding areas were used for isolation of *Staphylococcus aureus*. Out of 90 milk samples, 48 isolates were found as purple coloured cocci in clusters on gram staining. All the 48 isolates were confirmed as pathogenic *Staphylococcus aureus* by a positive coagulase test. The biochemical tests like IMViC tests, urease test, oxidase test, nitrate reduction test and catalase tests, confirmed the presence of *Staphylococcus aureus*. DNase test revealed the presence of blue to purple coloured colonies with clear zones around the colonies which are the characteristic colonies on DNase agar. On blood agar plates, the positive isolates produced β haemolysis. A panel of 14 antibiotic discs were tested by using the standard disc diffusion method. Among the 48 isolates all of them were not resistant to Vancomycin (0%). Maximum resistance was observed for cefotaxim (77.50%), followed by, ampicillin (48.24%), penicillin (43.96%), gentamycin (43.00%), streptomycin (38.80%), ciprofloxacin (31.50%), tetracycline (31.20%), cefoperazone (25.60%), erythromycin (23.86%), cephotaxin (23.11%), oxacillin (17.83%) Azithromycin (15.70%) and cephalothin (14.82%).

**INTRODUCTION**

*Staphylococcus aureus* is a common human pathogen which can be capable of producing a wide variety of diseases starting from skin and soft tissue infection to life threatening endocarditis, bacteraemia and necrotizing pneumonia (Gordon and Lowy, 2008). Infections caused by *Staphylococcus aureus* have assumed new public health significance due to development of multi drug resistant strains particularly MRSA and its epidemic clones that are increasingly being found in hospitals and communities like Hospital associated - MRSA (HA-MRSA) and community associated –MRSA (CA-MRSA). Detection of MRSA in animals has been reported by various scientists in the previous studies. The prevalence of MRSA in farm or domestic animals like goat, sheep, cattle, horses and further in different companion animals such as dogs and cats (Walther et al., 2008; Saleha et al., 2010) were reported and revealing the fact that MRSA has emerged as a potential zoonotic pathogen. These studies of MRSA among the domestic and companion animals have raised the curtains for extensive further studies to address the issue of MRSA colonization and transmission to human beings particularly those who are in close contact with the animals (Khanna et al., 2010).

Methicillin is grouped under narrow spectrum beta lactamase resistant penicillin. The mechanism of action is by interfering primarily with the synthesis of bacterial cell wall and will be responsible for binding of methicillin to penicillin binding proteins (PBPs) (Walther et al., 2008). *Staphylococcus aureus* has the ability to develop resistance to any antibiotic that comes under clinical use (Pantosti et al., 2007). Methicillin resistance to *Staphylococcus aureus* is due to the acquisition of the mec A gene that encodes a new protein designated as PBP 2a which belongs to a family of enzymes in building the bacterial cell wall.PBP 2a has very low affinity for beta lactamasases (Pantosti et al., 2007). The mec A gene is placed on a mobile genetic element, which is called as Staphylococcal cassette chromosome mec (ssc mec) inserted in the Staphylococcal chromosome upstream of x (Katayama et al., 2000). Different type of ssc mec can be distinguished on the basis of different key elements present, that are the mec gene complex, comprising mec A and its regulatory gene mec I and mec R1.

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There have been a number of reports stating that animals may serve as reservoirs for MRSA infection of human’s. In the last two decades, new generations of MRSA have emerged with the ability to transfer to human beings and food producing animals. There is a transferring MRSA from food animals to human beings. The potential of MRSA to become a dangerous zoonotic pathogen could affect the epidemiology of MRSA in humans. As the prevalence of MRSA in animals is continuous to rise, there is an inherent risk for new MRSA colonies to evolve secondary to horizontal gene transfer and host selection pressure and then spread to human hosts. Thus the presence of MRSA in animals is a concern not only to a veterinarians and animal health care workers but also to public health.

**MATERIALS AND METHODS**

The specimens selected for this study were milk samples. These samples were collected by using sterile test tubes. Test tubes were sterilized in hot air oven at a temperature of 160°C for 1 hour. Milk samples were collected from the local vendors by using a sterile dipper. A total of 90 milk samples were collected aseptically in sterile test tubes. The collected specimens were processed within 2 to 24 hours of collection.

For the isolation and identification of *Staphylococcus aureus* Tryptic soy broth was used for enrichment of inoculum. Baird Parker agar supplemented with 5% sterile egg yolk tellurite suspension was used for isolation of *Staphylococcus aureus*. Loop full of inoculum from the tubes was transferred to tryptic soy broth tubes and the tubes were incubated at 37°C for 24 hours. After incubation a loop full of inoculum from tryptic soy broth tubes was streaked over Baird Parker agar with egg yolk tellurite plates and the plates were incubated at 37°C for 48 hours.

A smear was prepared from the growth on Baird Parker agar and it was stained with Grams method of staining. Gram positive bacteria were identified up to genus level as staphylococcus based on morphology. All the isolates were identified up to species level based on biochemical and sugar fermentation tests as per the methods described by Barrow and Felthan (1993) and Bergeys manual of systemic bacteriology (1984).

For confirmation of *Staphylococcus aureus*, the biochemical tests conducted were catalase test, DNase test, nitrate reduction test, oxidase test, urease test and IMViC tests, the sugars used for sugar fermentation tests were raffinose, sucrose, maltose, d-mannitol and d-mannose.

**Tube coagulase test**

A heavy suspension of the bacterial culture under test was inoculated into 0.5 ml of undiluted human plasma in a test tube and incubated in a water bath maintained at 37°C. The tube was examined for coagulation at 30 min, 2 h, 4 h, 6 h and overnight. A positive reaction was indicated by the conversion of plasma into stiff gel, best recognized by its remaining in place when the tube was tilted or inverted.

**Test for haemolysis**

Isolates of *S. aureus* grown over night on Baird parker agar plates were spot inoculated on tryptose soya agar plates containing 5% of disseminated sheep blood cells. The plates were incubated at 37°C for 24 hrs and appearance of haemolytic zone around the colony was recorded.

**DNase test**

About 42 grams of DNase test agar base was suspended in 1000 ml distilled water and the same was heated with frequent agitation to dissolve the medium completely and 0.1 gm Toluidine Blue (FD051) was added to the medium. The medium was sterilized by autoclaving at 12 to 15 lbs pressure (118°C to 121°C) for 15 minutes. After sterilization the medium was cooled to 45°C and approximately 15 to 20 ml of the medium was added to the sterile petriplates. The plates were incubated at 35-37°C for 18-24 hrs. The plates were observed for blue to purple coloured colonies with clear zones around the colonies.

The biochemically confirmed strains of Staphylococcus aureus were preserved by taking a loop full of the isolated organism and the same was added to the sterile tryptone soya glycerol broth vials and mixed well in vortex mixer. The vials were then labelled and stored at -20°C.

**Antimicrobial susceptibility testing**

Antimicrobial sensitivity against 14 antibiotics was done as per the standard disc diffusion method of Bauer et al (1966).

Bacterial suspension was made by transferring 4-5 colonies from primary isolated medium i.e. Baird Parker agar with 5% egg yolk tellurite to 5ml of tryptic soya broth by touching the top of the colonies with a flame sterilized and cooled platinum loop. The resulting culture after incubation at 37°C for 8 hours was compared with the turbidity standard prepared separately for adjustment of bacterial suspension.

The turbidity standard was prepared by adding 0.5 ml of (1.17% w/v) Barium chloride dehydrate (BaCl₂ 2H₂O) solution to 1% Sulphuric acid. The turbidity standard was placed in a tube identical to the one used for the broth sample and was stored in the dark at room temperature. The turbidity was equivalent to 10⁸ cfu/ml which is half the density of a Mac Farland 0.5 standard. The standard was agitated on a vortex mixer immediately before use. If the culture was found less turbid than the turbidity standard it was further incubated for 2-8 hours at 37°C until turbidity was equivalent to the standard. If the turbidity exceeds that of the standard the culture solution was diluted with tryptic soya broth to equitate with the standard.

Commercially available standard antimicrobial discs (Hi-Media) were procured and stored at 2-8°C in the refrigerator. Unopened disc containers were removed from the refrigerator 1-2 hours before use, to bring them to room temperature. The antimicrobial discs with known concentrations as noted in micrograms (µg) or International Units (IU) per disc were used to study the antimicrobial susceptibility of the isolates. The antimicrobial discs used in this study are given in Table 1.

and the ccr genes complex comprising to different ccr recombinases that are responsible for the mobility of the element (Ito et al., 2001; Ma et al., 2002).

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Muller–Hinton agar, the recommended medium for disc diffusion test was employed in this study. The prepared medium was autoclaved, when the temperature of medium reached between 45 – 50°C, it was mixed well and approximately 15-20ml was added to the sterile petridishes and incubated overnight at 37°C for sterility testing and the uncontaminated plates were wrapped with aluminium foil and they were stored at 4°C till use.

The sterile cotton swab was dipped in the standardized inoculum (turbidity so adjusted) and rotated several times. Then the cotton swab was gently pressed on the upper inside wall of the test tube to remove excess inoculum. The swab was then streaked over the entire surface of the muller-hinton agar plate for three times. The plate was turned at 60° angle between each streak to ensure even distribution of the inoculum. A final sweep of the swab was made around the agar rim.

Allowed the inoculums to dry for 5 to 15 minutes. Selected antimicrobial discs were placed at least 24mm apart by using a disc dispenser and gently pressed down on to the agar surface to provide uniform contact. The inoculated plates were inverted and incubated at 37°C for 24-48 hours. Each plate was examined after incubation for the diameter of zones of complete inhibition including the diameter of the disc was measured up to the nearest whole millimetre with ruler in non-reflecting background. The zone margin was the area where no obvious growth was visible and the readings were compared with that specified readings in the interpretive chart supplied by the manufacturer of the antibiotic discs and the results were documented as sensitive (S), intermediate (I) and resistant (R).

**RESULTS**

All the milk samples collected from the local vendors were inoculated into tryptic soy broth and incubated at 37°C for 24 hours. After the incubation period a loop full of inoculum from trypticase soy broth tubes was streaked on Baird Parker agar plates by following all the aseptic precautions. The plates were incubated at 37°C for 48 hours. The plates were observed for black and shiny colonies on Baird Parker agar plates (Fig. 1). All the 48 isolates were subjected to Gram’s staining and found purple coloured cocci in clusters (Fig. 2). All the isolates were confirmed as pathogenic *Staphylococcus aureus* by a positive coagulase test as shown in Fig. 7.

![Fig. 1 Plate showing the growth of black and shiny colonies surrounded by clear zone of Staphylococcus aureus on Baird Parker Agar](image1)

![Fig. 2 Gram’s staining of Staphylococcus aureus exhibited characteristic cocc clusters](image2)

![Fig. 3 Tests showing results of Voges-Proskauer](image3)

The isolates were subjected to the biochemical tests like IMViC tests, urease test, oxidase test, nitrate reduction test, DNase test, blood agar plate test and catalase tests.

All the isolates were negative for Indole and Citrate utilization tests. Whereas all the isolates produced bright red colour in methyl red test and red colour in Voges – proskauer test.
(Fig.3). All the biochemical reactions confirmed the presence of *Staphylococcus aureus*. Further all the isolates were subjected to urease test (Fig. 4), oxidase test, nitrate reduction test (Fig. 5) and catalase test (Fig. 6). The results revealed that all the isolates were positive for urease test, catalase test and nitrate reduction tests, on the other hand all of them were negative for oxidase test.

**DNase test**

The sterilized plates of DNase test agar base were streaked with the inoculums took from positive Baird Parker agar plates. The plates were incubated at 35-37°C for 18-24 hrs and observed for blue to purple coloured colonies with clear zones around the colonies (Fig.9).

**Blood agar plate test**

For further confirmation of *Staphylococcus aureus*, all the isolates were streaked on blood agar plates and incubated at 37°C / 24 hours. The results revealed that all the isolates produced β haemolysis on blood agar plates (Fig.10).

**Antimicrobial sensitivity testing**

To detect the resistant/sensitivity pattern of *S. aureus* from different sources, *in-vitro* antibiotic sensitivity was carried out by disc diffusion as per the method of Bauer et al., (1966).
using 14 commercially available antibiotic discs. Muller Hinton agar plates showing the sensitivity, intermediate sensitivity and the resistance patterns of various antibiotic discs were shown in Table.1 and Figure 11 &12.

**Antimicrobial sensitivity of isolates from samples**

A total of 48 isolates were resistant to different antibiotic discs used in this study. The sensitivity patterns of the isolates for various antibiotic discs are given in Table.2. Among the 48 isolates all of them were not resistant to vancomycin (0%). Maximum resistance was observed for cefotaxim (77.50%), followed by, ampicillin (48.24%), pencillin (43.96%), gentamycin (43.00%), streptomycin (38.80%), ciprofloxacin (31.50%), tetracycline (31.20%), cefoperazone (25.60%), erythromycin (23.86%), cephoxitin (23.11%), oxacillin (17.83%) Azithromycin (15.70%) and cephalothin (14.82%).

**DISCUSSION**

*Staphylococcus aureus* causes severe animal diseases such as suppurative diseases, mastitis, arthritis and urinary infections that are associated with various virulent factors such as the production of extracellular toxins and enzymes. For humans this organism is an important cause of food poisoning, pneumonia, post operative wound infections and nosocomial bacteraemia. Human isolates of *S. aureus*, unlike animal isolates, are frequently resistant to the penicillinase resistant penicillins (Kloos WE and Bannerman TL, 1995). An organism exhibiting this type of resistance is referred to as Methicillin (oxacillin) Resistant *Staphylococcus aureus* (MRSA). Such organisms are also frequently resistant to most of the commonly used antimicrobial agents including the aminoglycosides, macrolides, chloromphenicol, tetracyclines and fluoroquinolones (Mandell et al, 1995). In addition MRSA strains should be considered to be resistant to all cephalosporins, cephems and other β-lactams such as ampicillin, sublactam, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactum and the carbapenems, regardless of the in-vitro test results obtained with those agents (National Committee for Clinical Laboratory Standards, 2001). The resistance to cefotaxim was 77.50% in this study. Similar type of results were obtained by Vidhani S et al (2001) where they could found 78.50% resistance to cefotaxim. The resistance to ampicillin was 48.24% in this study and the percentage of resistance can be considered as less when compared to the results of Kandle et al (2003) who reported 90% resistance to ampicillin, Saravanan et al (2013) reported 100% resistance, Kitara et al (2011) found 75% resistance, Tekalign Kejela and Ketema Bacha (2013) observed 76.3% resistance to ampicillin on the contrary Dachen Tsering et al (2011) reported only 4.81% resistance to ampicillin.

The resistance to cephoxitin was 23.11% in this study. Similar type of results were obtained by Vidhani S et al (2001) where they could found 78.50% resistance to cefoxitin. The resistance to penicillin was 23.86% in this study and the percentage of resistance can be considered as less when compared to the results of Kandle et al (2003) who reported 90% resistance to ampicillin, Saravanan et al (2013) reported 100% resistance, Kitara et al (2011) found 75% resistance, Tekalign Kejela and Ketema Bacha (2013) observed 76.3% resistance to ampicillin on the contrary Dachen Tsering et al (2011) reported only 4.81% resistance to ampicillin.

**Table.2. Antimicrobial sensitivity/intermediate/resistant-pattern of S. aureus from different sources**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Antimicrobial agent</th>
<th>Pattern of antibiogram</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pattern of antibiogram</td>
<td>Sensitive (%)</td>
</tr>
<tr>
<td>1</td>
<td>Cefotaxim</td>
<td>8(16.66)</td>
</tr>
<tr>
<td>2</td>
<td>Ampicillin</td>
<td>20(41.66)</td>
</tr>
<tr>
<td>3</td>
<td>Penicillin</td>
<td>21(43.75)</td>
</tr>
<tr>
<td>4</td>
<td>Gentamycin</td>
<td>19(39.58)</td>
</tr>
<tr>
<td>5</td>
<td>Streptomycin</td>
<td>19(39.58)</td>
</tr>
<tr>
<td>6</td>
<td>Ciprofloxacin</td>
<td>30(62.5)</td>
</tr>
<tr>
<td>7</td>
<td>Tetracycline</td>
<td>17(35.41)</td>
</tr>
<tr>
<td>8</td>
<td>Cefoperazone</td>
<td>15(31.25)</td>
</tr>
<tr>
<td>9</td>
<td>Erythromycin</td>
<td>22(45.83)</td>
</tr>
<tr>
<td>10</td>
<td>Cephoxitin</td>
<td>23(47.91)</td>
</tr>
<tr>
<td>11</td>
<td>Oxacillin</td>
<td>29(60.41)</td>
</tr>
<tr>
<td>12</td>
<td>Azithromycin</td>
<td>15(31.25)</td>
</tr>
<tr>
<td>13</td>
<td>Cephalothin</td>
<td>32(66.66)</td>
</tr>
<tr>
<td>14</td>
<td>Vancomycin</td>
<td>48(100.00)</td>
</tr>
</tbody>
</table>

*Fig. 10 Plate showing beta haemolysis of Staphylococcus aureus on blood agar*

*Fig. 11 & 12 Plates showing antibiotic resistance of Staphylococcus aureus on Muller-Hinton agar*
Prabhakar (2011), Saravanan (2013), Pandey et al (2012) and Hoerle Jairo and Adriano Brandelli (2009) have reported 100% resistance to penicillin whereas Kandle et al (2003) observed only 90% resistance.

The Staphylococcus aureus isolates in this study exhibited 43% resistance to gentamicin. Similar type of results were obtained by Jayatilleke and Bandara (2012) where they could found 44% resistance to gentamyacin, whereas a high resistance to gentamycin than in the present study was observed by Saravanan et al (2013) who found 100% resistance, Bilal Ahmed Mir and Srikanth (2013) observed 91.8% resistance, Nwankwo Emmanuel Onwubiko and Nasiru Magaji Sadiq (2011) reported 92.4% resistance and 97.6% resistance was found by Syed Zahid Bukhari et al (2011), whereas Kitare et al (2011) observed no resistance to gentamycin.

In the present study 38.80% of resistance was observed for streptomycin by the isolates. A little higher than the present results obtained in this study of Nwankwo Emmanuel Onwubiko and Nasiru Magaji Sadiq (2011) who reported 44.2% resistance to streptomycin. And further Adekunle Odunayo Adejuwon et al (2010) reported 23%resistance to streptomycin by the S.aureus isolates.

S.aureus isolated in this study was highly resistant to ciprofloxacin (31.50%), whereas results obtained from the work carried out by Syed Zahid Bukhari et al (2011) who found 75.80% resistance to ciprofloxacin was observed, Saravanan et al (2013) observed a lower resistance of 12.50%, Nwankwo Emmanuel Onwubiko and Nasiru Magaji Sadiq (2011) found 78.0% resistance, Shanthi and Uma Sekar (2009) observed 60% resistance, Jayatilleke and Bandara (2012) showed 54% resistance to ciprofloxacin.

The resistance to tetracycline was 31.20% in this study, similar results were obtained by Nwankwo Emmanuel Onwubiko and Nasiru Magaji Sadiq (2011) where he found 31.2% resistance to tetracyclines where as Narasinga Rao and Prabhakar, (2011) found 68.5% resistance and Kitare et al (2011) observed 45.3% resistance to tetracyclines. In the present study 25.60% resistance was observed for cefoperazone by the isolates. Similar type of results were obtained by Edward Malinowski (2002) were they observed 27.7% resistance.

In the present study 23.86% of resistance was observed for erythromycin by the isolates. Abdul Rahman Baidani (2011) reported 31% resistance to erythromycin which was almost similar to the results obtained in this study, whereas high percentage of resistance was observed by Narasinga Rao and Prabhakar (2011), where they observed 95.6% resistance, 98.3% resistance was found by Syed Zahid Bukhari et al (2011), 90% resistance by Kandle et al (2003), 52.4% resistance by Nwankwo Emmanuel Onwubiko and Nasiru Magaji Sadiq (2011), 62.5% resistance by Shanthi and Uma Sekar (2009), on the other hand least resistance of 4.12% and 7.8% was observed by Dachen Tsering et al (2011) and Kitare et al (2010) respectively.

The resistance of cephoxitin in the present study is 23.11% which is almost similar to the resistance observed by Saravanan et al (2013) where he observed 25% resistance to cephoxitin.

The resistance to oxacillin in this study is 17.83%. High resistance to oxacillin than in the present study was observed by Abdul Rahman Baidani et al (2011) where they identified 86.2% resistance to oxacillin and similar to the results of the present study Bilal Ahmed Mir and Srikanth (2013) observed 10.6% resistance to oxacillin.

The staphylococcus aureus isolates in this study exhibited 15.7% resistance to Azithromycin. Similar type of results were obtained by Antonia pointa and Cristina Tuchilus (2011).

Cephalothin has exhibited 14.82% of resistance in this study but a high percentage of resistance than in the present study was observed by Syed Zahid Bukhari et al (2011). In the present study all the isolates were sensitive to vancomycin. Similar to the results of the present study, Indian Network for Surveillance of Antimicrobial Resistance (INSAR) group, India (2013), Hoerle Jairo and Adriano Brandelli (2009), Pandey et al (2012), Abdul Rahman Baidani et al (2011) reported that they have not observed any resistance to vancomycin by the isolates where as Dacher Tsering et al (2011) observed 20.27% resistance and 3% resistance was observed by Telign Kajela and Ketema Bacha (2013).

Although it is extremely difficult to explain these conflicting data with regards to both time and place of study, the variation is probably due to differential clonal expression and drug pressure in community.

Acknowledgment
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