Analysis of Virulence Potentials of Community Acquired *Staphylococcus aureus*, Isolated from a Slam Population of West Bengal, India

Prithwiraj Mukherjee, Amit Karmakar, Smritikana Biswas, Srayose De, Saikat De, Chandradipa Ghosh *

Department of Human Physiology with Community Health, Vidyasagar University, Medinipur-West, Pin 721102, West Bengal, India

**Abstract**—The worldwide spread of community acquired *Staphylococcus aureus* (CASA) skin infection is becoming an emerging problem. This study was conducted to delineate the virulence properties and characteristics of *Staphylococcus aureus* isolated from children belonging to low socio-economic classes in Midnapore town, West Bengal, India. Samples were collected from affected children by aseptic means from areas of infection with proper medical intervention. These samples were confirmed first as *Staphylococcus aureus* by PCR amplification of 16S rRNA. Further morphology, antibiotic susceptibility, capability of secreting several enzymes, biofilm forming abilities was studied. PCR was carried out for detection of the presence of enterotoxins (seA and seB), exfoliatin toxins (etA and etB) and toxic shock syndrome toxin (tsST) genes. Results reveal that 33% community acquired *Staphylococcus aureus* strains were methicillin resistant and 22% were vancomycin resistant and those strains were capable of biofilm formation. Besides, all of the pathogenic and non-pathogenic strains were harboring exfoliatin toxin gene (etA). So it can be concluded that inducible multidrug resistant community acquired *Staphylococcus aureus* with their diverse pathogenic toxic potentials giving emerging alarm in that geographical location in India.

**Keywords**—*Staphylococcus aureus*, *Staphylococcal Scalded Skin Syndrome*, *Staphylococcal toxins*, *vancomycin*, *biofilm*.

### I. INTRODUCTION

*Staphylococcal* scalded skin syndrome (SSSS) is a syndrome of acute exfoliation of the skin (Acland et al., 1999). One of two *Staphylococcal* exfoliatin toxin (A and B) binds to desmoglein-1 in desmosome causing it to break down. That leads to a red rash and separation of the epidermis beneath the granular cell layer (Ladhani et al., 1999; Adhisivam et al., 2006; Amagai et al., 2002). Children are more at risk because of lack of immunity and immature renal clearance capability (Baartmans et al., 2006). It is rare in adults (Cribier et al., 1994). Mortality rate from SSSS in children is very low (1-5%), whereas in adults it is higher (20-30%) (Chi et al., 2006). No gender predilection was documented in children. In adults, the male-to-female ratio was approximately 2:1 (El Helali et al., 2005).

*Staphylococcus aureus* is the leading cause for a broad spectrum of diseases that ranges from superficial skin lesions and invasive inflammations to life-threatening diseases. Toxic shock syndrome belongs to a class of toxin-mediated disease that evokes multisystem disorders in the human host due to the *Staphylococcal* toxic shock syndrome toxin (TSST-1) (Abdulmula et al., 2006). According to serological classification, six *Staphylococcal* enterotoxin (SE) group have been recognized: SEA, SEB, SEC, SED, SEE and SHE (Su et al. 1995). These enterotoxins are small peptides (26 to 29 kDa) and have a great deal of similarity at their amino acid level (Manisha et al., 2000). They are the main source of food poisoning and cause intensive intestinal peristalsis.

Reports are increasing implicating community-acquired multidrug resistant *Staphylococcus aureus* as a cause of SSSS (Ito et al., 2002). Multidrug resistant *Staphylococcus aureus* is thought to emerge in a community when a locally prevalent antibiotic susceptible strain acquires genetic element and utilizes mobile genetic elements and nucleotide polymorphisms to establish local and geographic niches (Kennedy et al., 2008). The formation of biofilms is also another example of a phenotypic change. Biofilm, consists of multiple layers of bacteria encased within secreted exopolysaccharide matrix material that protects the enclosed bacteria from host defenses and impedes delivery of at least some antibiotics (Stewart et al., 2002).
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CASA cases are being reported from all over the world, yet there have not been many studies regarding the actual incidence of CASA infections in people of low socioeconomic classes specifically in children community in different geographical regions in India. In this study, attempts were undertaken primarily to screen out the community-acquired *Staphylococcus aureus* strains from children belonging to low socioeconomic condition from Midnapore town, India. We tried to search out the presence of essential virulence genes for scalded skin syndrome in those strains and their ability of elaborating different enzymes, biofilm formation and response towards different antibiotics. Genes coding for enterotoxins and toxic shock syndrome toxin were also screened out for proper characterization of their overall virulence potential.

II. MATERIALS AND METHODS

2.1 Collection of Samples and culture media:

This study was carried out in children those had superficial skin infection of two to nine years age group from slam areas surrounding Midnapore town, West Bengal, India. Superficially scar tissues were collected using standard microbiological procedure with proper medical intervention. Samples were cultured in bacto brain heart infusion medium (HiMedia, India) overnight at 37°C in a shaker incubator (Scigenics Biotech, India). All bacterial samples were grown further on nutrient agar (HiMedia, India) media and purified by a single colony isolation technique on nutrient agar containing 10% sodium chloride.

2.2 Identification of *Staphylococcus aureus* bacteria from purified isolates:

Identification of *Staphylococcus aureus* were performed by PCR amplification of 16S rRNA. The oligonucleotide primer pairs for 16S rRNA are listed in Table1. The reaction condition used was reported previously (Astrid et al., 2004).

2.3 Biochemical characterization of *Staphylococcus aureus* bacteria from identified isolates:

The identified isolates were characterized by traditional biochemical tests, including Gram staining, catalase, coagulase, urea and gelatin hydrolysis test (Murray et al., 1978; Ronald et al., 1995; Bannerman et al., 2003). The bacterial samples were inoculated on MacConkey's agar for 24-48 hours and examined for bacterial growth.

2.4 Test for Hemolytic ability:

To identify the hemolytic ability, the bacterial isolates were screened on blood agar media by the method mentioned by Ronald et al., (1995).

2.5 Antibiotic susceptibility testing:

The antibiotic-resistance profile was determined by the Disc agar diffusion (DAD) technique (Bauer et al., 1966; Acar, 1980), using ten crucial antibiotic discs. Among those antibiotics all were obtained commercially from HiMedia, India. *E. coli*, an all-sensitive reference strain, was used as a quality control strain for the DAD test.

2.6 Biofilm Assay:

Cells from colonies grown overnight on LB agar plates at room temperature, were resuspended in LB broth to an optical density of 0.3 at OD600. 3μl of the cell suspension was added to 300μl of LB in 10/75-mm borosilicate glass tubes. Cultures were incubated at room temperature without shaking for various times. At the desired end-point, non-adherent cells were removed by rinsing with distilled water. Biofilms were stained by the addition of 350μl of 1% crystal violet (Sigma, India) for 25 min followed by rinsing with distilled water, as described elsewhere (Watnick et al., 2001; Lauriano et al., 2004). The cell-associated dye was solubilized in 400μl of dimethyl sulfoxide (DMSO) and quantified by measuring the OD570 value of the resulting solution. All assays were performed in triplicate.

2.7 Detection of enterotoxins, exfoliatin toxins and toxic shock syndrome toxin using PCR:

Genomic DNA was prepared by modification of a method described elsewhere (Chakraborty et al., 2001). Oligonucleotide primers are listed in Table1. Those primers sequences were chosen from published primer sequences and were obtained commercially from GeNei, India. The amplification was performed in an automated thermo cycler with a hot bonnet (Eppendorf). The optimized thermal cycling conditions for multiplex PCRs were 30 cycles of denaturation at 95°C for 1 min (2 min for the first cycle), annealing at 55°C for 47 sec, and polymerization at 72°C for 1 min 37 sec (5 min for the last cycle). For amplification the following components were used: 1.5 mM MgCl2, 200 mM each of dATP, dTTP, dGTP and dCTP, 2 mM of each primer, 0.1 mg template DNA, and 1.25 U Taq polymerase (GeNai). For prevention of primer-dimmer formation and denaturation of template DNA, after addition of template DNA (5 to 10 ng), the mixture was incubated at 95°C for 5 min and stored in ice before addition of 1.25 U of Taq DNA polymerase. Amplified products (10 μl) were visualized by 1.2% agarose gel electrophoresis at 100V for 1 hour. The gel was previously stained with ethidium bromide (HiMedia), and the bands were visualized under UV illumination (Eppendorf) at 254nm.
III. RESULT AND DISCUSSION

Bullous impetigo is a common disease among children caused by \textit{Staphylococcus aureus}. CASA incidence varies among country by country. Low frequency (around 1%) found among European countries and a high frequency (>60%) found in Asian countries (Deurenberg et al., 2007). From those community strains it was found that 19-51% strains were from the group of MRSA. Report from China in 2008 showed 10.4% of isolates from skin and soft tissue infections to be MAS (Ho et al., 2008). The rate was increased to 54.1% in the report of Yao et al. 2010. In India, Nagaraju et al. (2004) reported that an isolation rate of MRSA from community associated pyoderma to be 10.9% (Nagaraju et al., 2004).

CASA is defined as CASA isolated from outpatient with no history of hospitalization within the past one year and who presented no other established risk factors. On that background we collected a number of bacterial isolates from skin infections of children and from them through PCR analysis of 16S rRNA using specific primers we identified eighteen isolates to be \textit{Staphylococcus aureus}. Those isolates produced round opaque colonies of golden yellow color and all of them were Gram positive bacteria. We did not proceed with other strains which did not give 16S rRNA amplification product. Staphylococci form an irregular cluster of cells and staphyloxanthin is responsible for its characteristic golden yellow color (Clauditz et al., 2006). For definitive characterization by using biochemical or enzyme based way, we found those strains have the ability to grow at a temperature range of 15 to 45 degrees and at NaCl concentrations as high as 15 percent (Duguid et al., 1996). All of them were catalase positive and fifteen of them were coagulase positive. Previous reports reveal that coagulase positive \textit{Staphylococci aureus} are more virulent (Aarestrup et al., 1994; Blair, 1962; Sperber et al., 1975). The pathogenic ability of \textit{Staphylococcus aureus} also depends to a small extent on their capability to degrade the RBC. For determining the pathogenic ability of the coagulase positive \textit{Staphylococcus aureus} hemolysis test using blood agar media was performed and found that coagulase positive bacteria produced clearing zone surrounding their growth. In blood agar media blood was incorporated into medium to provide growth factor required by the fastidious pathway of bacteria those have hemolyzing power of blood (RBC) by secreting enzyme hemolysin.

We have found that those among eighteen, sixteen \textit{Staphylococcus aureus} were capable of biofilm formation and two strains were defective in that. This ability to form biofilms is of significant interest, as biofilm formation influences the efficacy of antimicrobial therapy and the subsequent outcome of an infection (Gilbert et al., 2002) This ability is also important for virulence as that helps the organism to survive within a habitat (Gotz, 2002). \textit{Staphylococcus aureus} has been shown by confocal laser-scanning microscopy to form a biofilm-like glyocalyx and to congregate in microcolonies (Akiyama et al., 2003). The \textit{ica} operon, which encodes a polysaccharide intercellular adhesin, is the best understood mediator of biofilm development (Cramton et al., 1999), however, ica-independent biofilm development, biofilm-associated protein (Bap) and the \textit{Staphylococcus aureus} surface protein (SasG) have all been implicated in biofilm development and regulation (Cucarella et al., 2007; O’Neill et al., 2007; Corrigan et al., 2007). The \textit{Staphylococcus aureus} quorum-sensing system is encoded by the accessory gene regulator (agr) locus is also involved in biofilm formation (O’Neill et al., 2007).

We have found that those isolate showing sensitive or resistance towards different antibiotic environment. Since the beginning of the antibiotic era \textit{Staphylococcus aureus} has responded to the introduction of new drugs by rapidly acquiring resistance by a variety of genetic mechanisms including acquisition of extrachromosomal plasmids or other types of DNA insertion and by mutations in chromosomal genes. Aminoglycosides such as kanamycin, gentamycin, streptomycin, etc. were once effective against \textit{Staphylococcal} infections until the organism evolved mechanisms to destroy the aminoglycosides action (Carter et al., 2000). The \(\beta\)-lactamase-resistant penicillins (methicillin, oxacillin, cloxacinil and flucloxacillin) were developed to treat penicillin-resistant \textit{Staphylococcus aureus} and are still used as first-line treatment. Methicillin was the first antibiotic in this class to be used (it was introduced in 1959), but the first case of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) was reported in England (Jevons, 1961).

We have found 22% strains to be vancomycin resistant among these eighteen. The first case of vancomycin-intermediate \textit{Staphylococcus aureus} (VISA) was reported in Japan in 1996 (Hiramatsu et al., 1997), but the first case of \textit{Staphylococcus aureus} truly resistant to glycopeptides antibiotics was only reported in 2002 (Chang et al., 2003). Three cases of VRSA infection have been reported in the United States in the year 2005 (Menichetti et al., 2005). A number of studies have investigated the role of the bacterial cell wall in vancomycin susceptibility in \textit{Staphylococcus aureus} (Sieradzki et al., 1999; Cui et al., 2000). Increased resistance to vancomycin in Mu50 \textit{Staphylococcus aureus} was associated with increased peptidoglycan synthesis, reduced peptidoglycan cross-linking and thickening of cell wall (Cui et al., 2000). Thickening of cell walls correlated with the trapping of vancomycin in the outer layers and was considered to be the mechanism of resistance (Cui et al., 2000). Other studies have suggested that structural changes in cell wall teichoic acids may also play a role in the resistance mechanism by reducing the rate of cell wall degradation thus maintaining a correlation between wall thickness, and decreasing susceptibility to vancomycin (Sieradzki et al., 1999).
Recently, there has been evidence to support the exchange of genetic material among VRSA bacteria (Srinivasan et al., 2002; Lowy et al., 2003; Weigal et al., 2003). Genetic analysis suggests that the in-vivo transfer of vancomycin resistance from E. faecalis to an MRSA strain occurred to produce the Michigan VRSA isolate (Weigal et al., 2003). Acquisition of the vanA gene in the Michigan isolate occurred via the interspecies transfer of Tn1546 (the vanA transposon, harboured within a multiresistant conjugative plasmid) from co-isolated vancomycin-resistant E. faecalis (Weigal et al., 2003). This isolate achieved vancomycin resistance by altering the terminal peptide of D-alanyl-D-alanine to D-alanyl-D-lactate, which only occurs with exposure to low concentrations of vancomycin, and the new dipeptide seems to have a reduced affinity for vancomycin (Lowy et al., 2003).

Our data clearly show that etA gene was amplified in multiplex PCR from all of these eighteen isolates and we did not find any other toxin gene. The two ETs i.e., ETA and ETB, in conjunction or independently, are implicated in causing SSSS (Iandolo et al., 1989; Sakurai et al., 1995; Johnson et al., 1991). Reports obtained in case of strains of Staphylococcus aureus producing one or both of two immunologically distinct exfoliative toxins, ETA or ETB have also been observed to be associated with a series of impetiginous Staphylococcal diseases referred to as SSSS (Iandolo et al., 1989; Marrack et al., 1990; Lee et al., 1987). Although ETA and ETB have identical biological activity and a degree of genetic similarity (Marrack et al., 1990). The gene coding for ETA is chromosomal whereas the gene coding for ETB is plasmid linked (Lee et al., 1987; Yamaguchi et al., 2002). In Nigeria, several authors argued that exfoliatin A would be the most frequent (Adesiyun et al., 1991; Ladhani et al., 2001). The association of multi drug resistance and exfoliatin genes was common about 1.4% in Tunisia (Ben et al., 2006).

The emergence of multi-drug resistance strains in the community is a growing concern. Although hospital associated strains can be transmitted in the community, some authors argue that the increasing dominancy of multi-drug resistant strains in the community cannot be explained by the spread of hospital strains (Loughman et al., 2009). Strains in the community are often different from clinical strains and it appears to have risen outside the health care environment because of the selective pressure of antibiotics (Buckingham et al., 2004; Fridkin et al., 2005). However, lower rates of CASA observed in Europe and this is due to a better standard of hygiene and good policies in using antibiotics reduces the movement of bacteria in those communities (Trystram et al., 2002).

IV. CONCLUSION

We have collected a number of samples from skin and soft tissue infection from children and identified eighteen were Staphylococcus aureus by polymerase chain reaction. Those isolates have hemolytic power and can use biofilm mode of growth. We have found 33% were MRSA and 22% were VRSA. They were all exfoliatin toxin positive strains. This genotypic technique, however, allows the detection of gene-harborng strains independent of their expression. Hence, a positive result in the PCR is indicative only of the presence of the organism and does not indicate the viability or pathogenic toxic potential. The emergence of inducible VRSA in Midnapore, India and its intrageneric transfer is alarming. This may soon become a global problem, unless antimicrobial agents are used more prudently.

V. ACKNOWLEDGEMENTS

Authors are thankful to all the children for their assistance to conduct the study. We also extend thanks to all the parents, for their kind cooperation.

REFERENCES


8
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FIG. 1

A comparison of 18 h biofilms made by *Staphylococcus aureus*. Biofilms stained with crystal violet and quantification of biofilm-associated crystal violet is shown here. Error bars are standard errors of means.

FIG. 2

Sensitivity of strain PM4 towards antibiotic vancomycin, polymyxin B, chloramphenical, streptomycin and kanamycin. Bacterial smear was prepared on nutrient agar plate and inhibitory zone was measured using standard procedure.

FIG. 3

Strain PM12 on nutrient agar plate.

FIG. 4

Agarose gel electrophoresis of multiplex PCR products showing 119bp and 228bp band represent *etA* and 16S rRNA respectively of strain PM4, PM7 and PM12.

**Table-1: Staphylococcus aureus exotoxin-specific oligonucleotide primer sequences.**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Oligonucleotide Sequence (5'-3')</th>
<th>Source</th>
</tr>
</thead>
<tbody>
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</table>
Analysis of virulence potentials of community acquired staphylococcus aureus, isolated from a slam...

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>seA-F</td>
<td>GCAGGGACACAGTTTAGGC</td>
<td>Astrid et al. 2004</td>
</tr>
<tr>
<td>seA-R</td>
<td>GTTCTGTGAAGTATGAACACG</td>
<td>Astrid et al. 2004</td>
</tr>
<tr>
<td>seB-F</td>
<td>ACATGTAATTGGATTTGCACTG</td>
<td>Astrid et al. 2004</td>
</tr>
<tr>
<td>seB-R</td>
<td>TGCAAGGACATGTGATACCA</td>
<td>Astrid et al. 2004</td>
</tr>
<tr>
<td>etA-F</td>
<td>CTAATGCAATTGGTTACAACG</td>
<td>Becker et al. 1998</td>
</tr>
<tr>
<td>etA-R</td>
<td>TGCAATGCAACCAGTTACCC</td>
<td>Becker et al. 1998</td>
</tr>
<tr>
<td>etB-F</td>
<td>ACGCCCATATACATTCAATTCG</td>
<td>Becker et al. 1998</td>
</tr>
<tr>
<td>etB-R</td>
<td>AAAGTTATTCATTTAATGCACTG</td>
<td>Becker et al. 1998</td>
</tr>
<tr>
<td>tssT-F</td>
<td>GCTTGCGACAACTGCTACAG</td>
<td>Astrid et al. 2004</td>
</tr>
<tr>
<td>tssT-R</td>
<td>TGGATCCGTCATTCATTGTTAT</td>
<td>Astrid et al. 2004</td>
</tr>
<tr>
<td>16s rRNA-F</td>
<td>GTAGGTGGCAAGCGTTATCC</td>
<td>Astrid et al. 2004</td>
</tr>
<tr>
<td>16s rRNA-R</td>
<td>CGCACATCAGCTGTTACCG</td>
<td>Astrid et al. 2004</td>
</tr>
</tbody>
</table>

F indicate forward primer, R indicate reverse primer (Astrid et al. 2004; Becker et al. 1998)

Table 2: Biochemical Characterization of Staphylococcus aureus.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Gram Stain</th>
<th>Catalase Ability</th>
<th>Coagulase Ability</th>
<th>Urease Ability</th>
<th>Gelatinase Ability</th>
<th>Growth on MacConkey Agar</th>
<th>Hemolysin Ability</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tbody>
</table>

+ indicate positive result, - indicate negative result (judged by eye).

Table 3: Susceptibility of Staphylococcus aureus to 10 antimicrobial agents.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>(%) Sensitive</th>
<th>(%) Resistant</th>
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<tbody>
<tr>
<td>Ampicillin(30mcg)</td>
<td>38.88</td>
<td>61.12</td>
</tr>
<tr>
<td>Streptomycin(10mcg)</td>
<td>66.66</td>
<td>33.34</td>
</tr>
<tr>
<td>Kanamycin(30mcg)</td>
<td>83.33</td>
<td>16.67</td>
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<tr>
<td>Chloramphenicol(25mcg)</td>
<td>83.33</td>
<td>16.67</td>
</tr>
<tr>
<td>Tetracycline(50mcg)</td>
<td>44.44</td>
<td>55.56</td>
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<tr>
<td>Polymixin B(50mcg)</td>
<td>88.88</td>
<td>11.12</td>
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<tr>
<td>Oxacillin(1mcg)</td>
<td>55.55</td>
<td>44.45</td>
</tr>
<tr>
<td>Methicillin(10mcg)</td>
<td>66.66</td>
<td>33.34</td>
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<tr>
<td>Vancomycin(30mcg)</td>
<td>77.77</td>
<td>22.23</td>
</tr>
<tr>
<td>Novobiocin(30mcg)</td>
<td>88.88</td>
<td>11.12</td>
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