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Prevalence and identification of toxic shock syndrome toxin producing wound isolates of *staphylococcus aureus* from Namakkal District of Tamil Nadu

V.Zambare[1]*, M.Ponraj[2], V.Rubeni[2]

[1]South Dakota School of Mines and Technology, Rapid City, SD 57701, (USA)

[2]Department of Microbiology, Vivekanandha College of Arts and Science for Women, Elayampalayam, Namakkal Dist, TN, (INDIA)

E-mail : vasuzambare@gmail.com; goldking1977@gmail.com

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ABSTRACT

Nine- skin infection, eight- burn and nine- accident wound samples were analyzed for the isolation of *Staphylococcus aureus* on selective Mannitol salt agar (MSA) media and identified by biochemical tests. Out off 31 samples, 10 samples showed presence of *S. aureus* and out of which burn samples had highest (50%) prevalence of *S. aureus.* All *S. aureus* showed multiple antibiotic resistance especially against penicillin (100%), vancomycin (90%), methicillin (80%) and oxacillin (60%). The lowest resistance (30%) was against chloramphenical, erythromycin and trimethoprim. Highest antibiotic resistance of 74, 56.6 and 33.3% were obtained from accident wound samples, burn samples and skin infection isolates, respectively. Among the 10 isolates, 5 isolates produced the toxic shock syndrome toxin (TSST) gene and was confirmed by PCR amplification. TSST gene was present only in skin infection samples and few burn samples but not in accidental wound samples. All 5 isolates with TSST gene were also producing toxin TSST and was confirmed by SDS-PAGE. To the best of our knowledge this is the first report on PCR detection of the TSST-1 gene in *S. aureus* from Namakkal District of Tamil Nadu, India.

KEYWORDS

Toxic shock syndrome toxin (TSST);
Wound isolates;
Staphylococcus aureus;
Antibiotic resistant;
Protein profile.

INTRODUCTION

Staphylococcus aureus is a Gram positive, coagulase positive cocci of the family Staphylococcaceae. They are ubiquitous and form the most common cause of localized supparative lesions and are the most predominant members of the normal flora of the skin of human beings. The genus *Staphylococcus*, is currently composed of 32 species[1] and 15 subspecies[2,3] depending on the cell wall composition, they may be pathogenic commensals or parasites. The pathogenic *staphylococci* includes *S. aureus, S. epidermis, S. saprophyticus, S. lugdunesis* and *S. haemolyticus*. The commensals include *Staphylococcus capitis. S.*

aureus is the only coagulase positive human species. All other human species collectively are referred to as the coagulase-negative *Staphylococci*. *S. hominis, S. hemolyticus* and *S. simulans* and other species, to an even lesser extent, account for a small percentage of infections. *Staphylococci* are wide spread in nature, although they are mainly found living on the skin, skin glands and mucous membranes of mammals and birds. They are sometimes found in the mouth, blood, mammary glands and intestinal, genitourinary and the upper respiratory tract. *Staphylococcal* disease is multifactorial and is usually due to the production of several pathogenic factors like peptidoglycan, teichoic acid, capsular polysaccharide, protein-A, extracellular enzymes like lipases, hyalronidase, nucleases, protein receptors and coagulase. Also, some cytolytic toxins like hemolysin and leukocidin, enterotoxins, toxic shock syndrome toxin (TSST) and exfoliative toxin were produced by *S. aureus*[4].

S. aureus is the causative agent of different opportunistic infections in humans and animals[3]. *S. aureus* is a primary pathogen of nosocomial and community acquired infections, endocarditis, bacterimia, superficial infections and deep skin and soft tissue infections. The toxin mediated diseases includes gastro enteritis, staphylococcal scalded skin syndrome and toxic shock syndrome[5,6]. *S. aureus* produces a variety of extracellular toxins and virulence factors that contributes to its pathogenicity. A number of staphylococcal enterotoxins (SEs), classified as A, B, C_1, C_2, C_3, D or E, can be produced by some strains[7]. Most *S. aureus* strains isolated from patients with toxic shock syndrome (TSS), causes a severe acute illness that rapidly leads to multi organ system failure, by producing a toxin known as toxic shock syndrome toxin-1 (TSST-1).

Antibiotics are widely used to treat infectious diseases in both humans and animals. But the emergence of antibiotic resistance strain among bacterial population is a very serious threat during the recent days. Resistance to various antibiotics can be acquired by means of changes in the genetic code of housekeeping genes or by uptake of foreign DNA containing antibiotic resistance genes. The emergence of antibiotic resistant microorganisms and their spread is threatening to medical community. This is particularly true in case of *S. aureus* which is the most common agent of nosocomial infections. It is of increasing concern that the organism is gaining drug resistance which often complicates treatment. Many isolates of *S. aureus* have been found to be resistant to new semi synthetic beta lactam antibiotics like methicillin, oxacillin and flucloxacillin. The resistance shown by *S. aureus* to antibiotics like methicillin is due to the presence of plasmid DNA and this plasmid encodes resistance to gentamycin and chloramphenicol[8]. Methicillin resistant *Staphylococcus aureus* (MRSA) is *also* a serious threat to hospitalized patients globally and it represents a challenge for public health; as community associated infections appear to be on the increase in both adults and children in various regions and countries[9]. The overall incidence of MRSA isolates has gradually increased around 30% or more in some countries[10]. It was estimated that MRSA strains accounted for 84% of hospital – acquired *S. aureus* isolates and 45% of non hospital acquired *S. aureus* isolates in Taiwan, 1998[11]. In Indian hospitals, MRSA is one of the common hospital acquired infection in different hospitals which accounts for 30 to 80% methicillin resistance based on antibiotic sensitivity tests[12]. Nosocomial MRSA isolates are mostly multidrug resistant. MRSA strains are resistant not only to methicillin but also to all other beta lactam antibiotics. There are two basic mechanisms responsible for the resistance of *S. aureus* to beta lactam anti microbial agents. The mechanism is the production of beta lactamases and the presence of low affinity penicillin binding proteins that make them resistant to anti staphylococcal penicillins including methicillin. *S. aureus* can develop resistance to antibiotics with amazing efficiency[13].

There is considerable genetic heterogenecity through natural populations of *S. aureus*. Many different techniques are available for tracing the spread of single *S. aureus* strain of human and animal origin, such as antibiotyping, the biochemo typing, the phage typing, and protein electrophoresis. SDS-PAGE of proteins has been using increasingly concerning bacterial systematics both at genus and the species level, and more recently type determination. SDS-PAGE of whole-cell proteins provides additional criteria for the study of the epidemiology and evolution of *S. aureus* strains. In this study our aim was to characterize whole cell proteins of TSST producing S. *aureus* isolates from wound infection, which had common antibiotic susceptibility test

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results. The purpose of this study was to investigate isolation and identification of multidrug resistance strains of *S. aureus* containing toxic shock syndrome toxin (TSST) gene for production of toxin protein TSST.

EXPERIMENTAL

Study population

A total of 31 swab wound samples were collected from patients of various private hospitals in and around Namakkal area and categorized into three groups. Group I, II and III which included 8, 9 and 9 samples from skin infection, burn and accidental patients, respectively. Swab samples were immediately inoculated into peptone water. The samples were properly labeled indicating the source, and the samples were transported to the Microbiology Laboratory for bacteriological investigations within 4–6 h of collection and preserved at 4-8°C until observed.

Isolation and identification of *staphylococcus aureus*

Loopful of culture from peptone water was streaked on the Mannitol salt agar (MSA) containing (g/L) 1-peptone, 10-beef extract, 75.0-sodium chloride, 10-mannitol, 15-agar, 0.025-phenol red (pH 7.4). The plates were incubated at 37°C for 24-48 hrs. The isolated colony from selective media of MSA was used for further analysis. The test tubes were incubated at 37°C for 16-24 hrs and then stored under refrigerator for further study. The clinical isolates were identified on the basis of colony characteristics, Gram stain morphology, and biochemical tests like sugar fermentation (glucose, sucrose, lactose maltose, mannitol), triple sugar iron, indole production, methyl red, Voges-Proskauer, citrate utilization, catalase and oxidase tests[3]. All the strains were preserved in MSA slants at 4^0C, till further use.

In vitro antibiotic susceptibility

All the finally identified *S. aureus* strains were subjected to in vitro antibiotic susceptibility test by antibiotic disc diffusion method[14]. The nutrient broth was prepared and sterilized at 121°C and inoculated with the isolates, then incubated at 37°C for 24 hrs. After incubation period the broth cultures were inoculated onto the surface of Mueller-Hinton agar plates and nine commonly used antibiotics discs (chloramphenical, erythromycin, gentamycin, methicillin, oxacillin, penicillin, tetracyclin, trimethoprim, vancomycin) of 30 µg concentration were placed, then the plates were incubated at 37°C for 18 to 20 hrs. The zone of inhibition and resistance was measured, recorded and interpreted according to the recommendation of the disc manufacture.

PCR amplification of TSST gene of *S. aureus*

Genomic DNA of all *S. aureus* isolates were isolated according to the method described by Capaval et al.[15]. All isolates of *S. aureus* were subjected in PCR assay according to El-Ghodban et al.[16] procedure with some modification for TSST gene amplification. The primers for TSST gene amplification of 5'-ATGGCAGCATCAGCTTGATA-'3 and 5'-TTTCCAATAACCACCCGTTT-'3 were purchased from Sigma Chemicals (USA) and used for PCR studies. Each PCR reaction mixture (20µl) contained 1 µl of template DNA (Genomic DNA), 2µl of 10 X PCR buffer, 0.5 µl of 2.0 mM of each primers, 1µl of 25 mM of each deoxynucleotide triphosphate and 0.5µl of Taq DNA polymerase (5U/µl) and 15.5µl of molecular grade water. A brief spin was given to settle down the materials then the tubes were kept in theromocycler (Genei). After initial denaturation at 94°C for 5 min, the samples were subjected to 30 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min and extension at 72°C for 1 min. A final extension was performed at 72°C for 7 min. Following PCR, the reaction mixtures were analyzed by electrophoresis on a 2% agarose gel, containing ethidium bromide (0.2mg/ml), in the presence of an appropriate DNA molecular weight marker. Then the amplified bands were observed under UV transilluminator and the genetic diversity from *S. aureus* was determined.

Preparation of whole cell lysate

Whole cell proteins of all strains were extracted and analyzed according to Kumar et al.[17]. All clinical isolates of *S. aureus* were plated on MSA plates and incubated at 37°C for 24 hrs. A sweep of 4-6 colonies from these plates were inoculated into flasks containing 10ml of MS broth and incubated at 37°C overnight in an orbital shaker. The resulting broth cultures were centrifuged at 10,000g for 15 min. at 4°C. The superna-

tant was discarded and the pellets were washed twice in sterile PBS (pH 7.2). The pellets were finally resuspended in equal volume of sterile PBS and disrupted by shaking with glass beads for 2-3 min under constant cooling by liquid CO_2 till 90% of the cells were broken. The unbroken cells were deposited by centrifugation at 12,000g for 30 min. at 4°C and the supernatant was preserved at –70°C till further use. The protein content of the samples was estimated by method of Bradford[18] and adjusted to give a final concentration of 2 mg/ml.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Whole-cell protein profile of all *S. aureus* isolates was analyzed by SDS-PAGE following the discontinuous buffer system of Laemmli[19]. Samples were prepared for PAGE by mixing in proportions of one part sample to three parts of sample buffer and boiling for 5 min. Solubilized samples (20µl) were applied to wells in a 4% acrylamide stacking gel over a 10% acrylamide separating gel. Electrophoresis was performed using an electrophoresis apparatus (Genei, Bangalore). The gels were run at constant voltage of 60 V until the bromophenol blue dye had reached the bottom over a period of 45 min-1hr. Medium range molecular weight markers (Genei, Bangalore) were also run for molecular weight estimation of bands of interest. After the completion of electrophoresis the gels were removed and stained with Coomassie Brilliant Blue R-250. The gels were photographed and protein profiles of the isolates were compared.

RESULTS AND DISCUSSION

S. aureus are Gram positive cocci in clusters. They cause a variety of superficial and deep seated infections, in most cases with pus-formation in man. They are frequently found as contaminants in clinical specimens taken from the body surfaces, for example, swab from skin, nose, throat, wounds, burns and bed-sores. A total number of 31 patients with different types of wounds were collected during the study period. Out off 31 wound samples only 10 samples (32.2%) showed presence of *S. aureus* (Figure 1).

Identification of *S. aureus* was confirmed by golden growth on MSA (Figure 2) plates and was found to be positive for sugar fermentation (glucose, sucrose, lactose maltose, mannitol), methyl red, catalase, negative indole production, Voges-Proskauer, citrate utilization and oxidase tests with acid production on triple sugar iron. There was a distribution of isolates from the different types of wound infections, Burn wound infections were 7 samples and yielded 3 isolates (42.8%); Skin infection wound samples were 6 and yielded 2 isolates (33.3%); Accident wounds were 18 sample and yielded 5 isolates (27.7%) of *S. aureus* (TABLE 1). This is in agreement with the findings of Pruitt et al.[20] and observed more than 50% of injuries by traffic accidents and flame burns. Also, Okesola et al.[21] reported 35% of *S. aureus* isolated from infected wounds in Ibadan.

Antibiotic susceptibility

All the confirmed *S. aureus* strains were subsequently tested for antibacterial drug resistance based on Kirby-Bauer disk diffusion method. The drug resistance patterns of *S. aureus* isolated from wound samples were found to be highly variable. Almost all the 10 strains were resistant to one or more antibiotics (TABLE 1). Strains of *staphylococci* with multiple drug resistance are widespread. They appear and spread rapidly after the introduction of new antimicrobial agents. However, a single mutational event results only in a slight increase in resistance to a single drug, as is shown by in vitro studies[22]. The development of antimicrobial resistance nearly always has followed the therapeutic use of antimicrobial agents. Since antibiotic use became widespread 50 years ago, bacteria have steadily and routinely developed resistance. Therefore monitoring of normal microbial towards antibiotic resistance serves important issues in relation of emergence pathogens[23]. Among the 10 isolates of *Staphylococcus*, isolates Sa 8 and Sa 9 showed highest resistance of 88.8%. Also, isolates Sa 4 and Sa 7 showed 66.6% resistance followed by Sa 2 and Sa 4 (44.45%). The lowest antibiotic resistance was observed in Sa 1 and Sa 5 (22.2%). The multiple antibiotic resistance (MAR) index was depicted in Figure 3A. Among the 9 types of antibiotics used, the highest resistance was seen against penicillin (100%) and the second most was vancomycin (90%) followed by methicillin (80%) and oxacillin (60%). The lowest resistance pattern was against chloramphenical,

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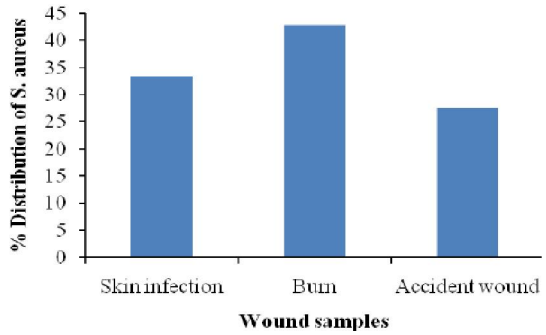
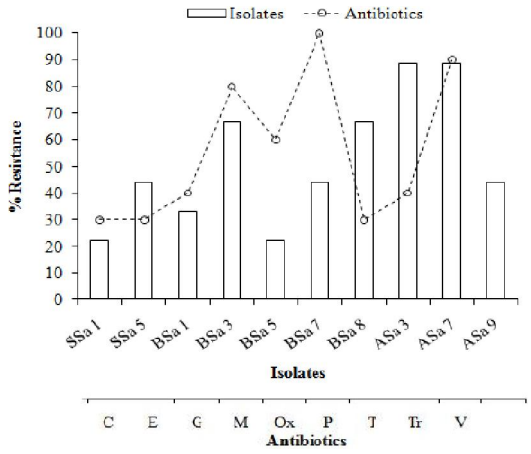


Figure 1 : % distribution and occurrence of *Staphylococcus aureus* from different wound samples

Figure 2 : *S. aureus* growth on Mannitol salt agar plate (MSA)



(A)

(B)

Figure 3 : Antibiotic resistance patterns for *Staphylococcus aureus* isolates. (A)-% multiple antibiotic resistance of *S. aureus* strains, (B)-*S. aureus* strain with antibiotic sensitivity and resistance pattern on agar plate

TABLE 1 : Prevalence and isolation of *Staphylococcus aureus* from wound samples

No.	Types of wound samples	Isolates code	% occurrence of *S. aureus*
1	Skin infection	[a]SSa 1	22.2
2		SSa 5	
3	Burn	[c]BSa 1	62.5
4		BSa 3	
5		BSa 5	
6		BSa 7	
7		BSa 8	
8		[c]ASa 3	
9	Accident wound	ASa 7	33.3
10		ASa 9	

[a]SSa *S. aureus* from skin infection wound sample; [b]BSa- *S. aureus* from burn wound sample; [c]ASa- *S. aureus* from accident wound sample

TABLE 2 : Multiple antibiotic resistance patterns of *Staphylococcus aureus*

Isolate code	[d]C	[e]E	[f]G	[g]M	[h]Ox	[i]P	[j]T	[k]Tr	[l]Va
[a]SSa 1	[m]S	S	S	S	S	[n]R	S	S	R
SSa 5	S	S	S	R	R	R	S	I	R
BSa 1	S	S	S	R	S	R	S	S	R
[b]BSa 3	S	I	R	R	R	R	R	R	R
BSa 5	S	S	S	S	S	R	S	R	S
BSa 7	S	I	R	R	R	R	S	S	R
BSa 8	S	R	S	R	R	R	S	R	R
[c]ASa 3	R	R	R	R	I	R	R	R	R
ASa 7	R	R	R	R	R	R	R	S	R
ASa 9	R	I	S	R	R	R	I	I	R

[a]*S. aureus* from skin infection wound sample; [b]*S. aureus* from burn wound sample; [c]*S. aureus* from accident wound sample, [d]Chloramphenical, [e]Erythromycin, [f]Gentamycin, [g]Methicillin, [h]Oxacillin, [i]Penicillin, [j]Tetracyclin, [k]Trimethoprim, [l]Vancomycin, [m]Sensitive, [n]Resistance

erythromycin and tobramycin (30%), (Figure 3A). The pattern of antibiotic sensitivity and resistance is shown in figure 3B. Sensitive strains showed clear zone and resistance strains showed growth around the antibiotic disc. *S. aureus* had developed multidrug resistance in many regions of the world[24], although reported prevalence rates indicate that wide variations exist regionally and even from herd to herd. Penicillin often predicts susceptibility to other β-lactamase-sensitive antimicrobial agents, for example ampicillin, and this has been used in several studies of *S. aureus* resistance.

In an Argentinian study, penicillin resistance was re-

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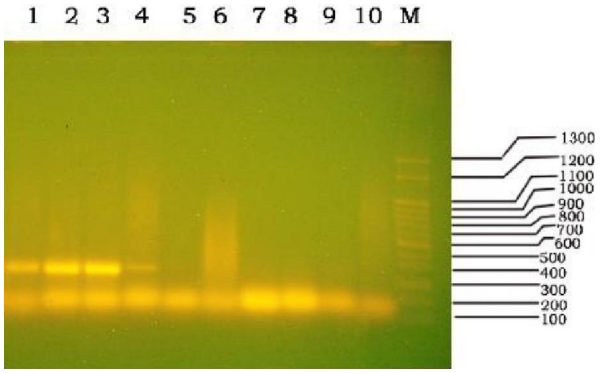

Figure 4 : PCR amplification of TSST gene from *S. aureus* (Lane 1-SSa, Lane 2-SSa5; Lane 3-BSA1; Lane4-BSa3; Lane5-BSa5; Lane 6-BSa7; Lane7-BSa8; Lane8-ASa3; Lane9-ASa-7; Lane10-ASa9; LaneM-100bp DNA ladder)

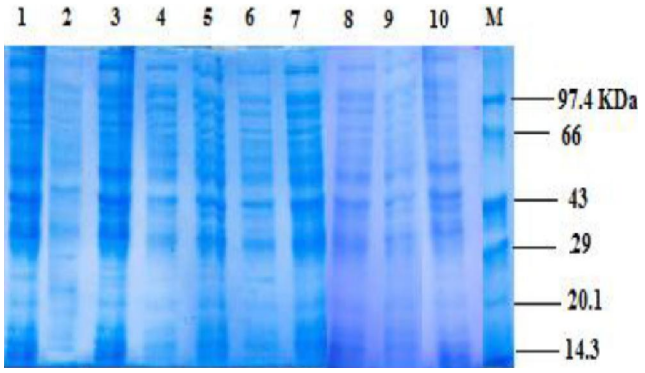

Figure 5 : Protein profile of *S. aureus* on SDS-PAGE (Lane 1-SSa, Lane 2-SSa5; Lane 3-BSA1; Lane 4-BSa3; Lane 5-BSa5; Lane 6-BSa7; Lane 7-BSa8; Lane 8-ASa3; Lane 9-ASa-7; Lane 10-ASa9; Lane M- Medium range protein marker)

ported to be 30%[25]. In Finland, more than 50% of the *S. aureus* isolates are penicillin-resistant[26], which is significantly higher than data reported from other Nordic countries, for example, 30% in Denmark[27] and 7% (*S. aureus* isolated from acute clinical mastitis) in Sweden[28]. The high penicillin resistance amongst *S. aureus* in Finland is likely due to the wide use of intramammary preparations containing combinations and broad-spectrum antibiotics. Considering the some of the major districts of Tamilnadu state (India) there were only one report available on prevalence and antibiotic susceptibility pattern of MRSA as nosocomial pathogen[29]. In that case out of 906 strains of *S. aureus* isolated from clinical and carrier samples, 250 (31.1%) and 39 (37.9%) were found to be methicillin resistant, respectively. Almost all clinical MRSA strains (99.6%) were resistant to penicillin, 93.6% to ampicillin, and 63.2% towards gentamicin, co-trimoxazole, cephalexin, erythromycin, and cephotaxime. All MRSA strains (100%) of carrier screening samples had resistance to penicillin and about 71.8% and 35.9% were resistant to ampicillin and co-trimoxazole, respectively. Overall multidrug resistance was observed among 63.6% of clinical and 23% of carrier MRSA isolates[29]. In Assam, 34.78% MRSA isolates were reported by Saikia et al.[30]. All MRSA isolates, showed 50% constitutive resistance, 9.38% inducible resistance.

Amplification of TSST gene from *Staphylococcus aureus*

TSS is an acute multisystem disease characterized by high fever, hypotension, vomiting, diarrhea, myalgias, nonfocal neurologic abnormalities, conjunctival hyperemia, strawberry tongue, and an erythematous rash with subsequent desquamation on the hands and feet. TSST-1 has been associated with wound infection, nasal packing, sinusitis, tracheitis, pneumonia, empyema, abscesses, burns, osteomyelitis and primary bacteremia[31]. In this PCR assay, all isolates of *S. aureus* were subjected according to the previous study. Among the 10 isolates of *S. aureus,* 5 isolates (50%) produced the TSST gene and was confirmed by PCR. The distribution of toxic gene from skin infection isolates has 100% (2 isolates) and 60% (3 isolates) from burn sample (Figure 4).

Similar correlation between methicillin resistance and TSST-1 production in clinical isolates has been reported by Kimura et al.[32]. The background of this relationship was unknown, but we speculate that *tst* and the methicillin-resistant gene *(rnecA)* may be coregulated, as TSST-1 production[16]. Several studies have been reported on TSST-1 gene for prevalent in methicillin-resistant *S. aureus* than in methicillin-susceptible *S. aureus*[32,33].

SDS PAGE analysis of *S. aureus*

Recently, *S. aureus* strains have been shown to have an association to the type of infection. In order to compare the whole cell protein profile of *S. aureus* strains associated with skin, burn and accidental wound infection, whole cell lysates of isolates of all 10 isolates were subjected to SDS-PAGE. Both deep and superficial isolates showed almost identical protein profile which consisted of 30-35 bands with major bands having molecular weights of approximately 120, 92, 80, 66,

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55, 42, 36, 29, 24, 20, 16 and 14 kDa (Figure 5). Likewise protein profile of whole cells of *S. aureus* in patients with deep-seated and superficial staphylococcal infections were observed by Kumar et al.[17]. Most of the isolates had similar bands patterns but lane 2 and lane 3 had polymorphic bands compared to others lanes. The protein profiles of isolates within the same group were grossly similar. More than 90% of the bands were qualitatively and quantitatively identical with minor quantitative difference in the intensity of the others. No significant inter strain variation was found among the skin, burn and accidental wound isolates regarding their whole cell protein profile. Two isolates of each burn and accidental wound samples were not showing presence of TSST protein band in range of 22-24 kDa.

CONCLUSION

Total ten *S. aureus* were isolated and identified from wound samples collected from hospitals of Namakkal District. Among them 50% of *S. aureus* were producing TSST gene and its toxin protein which was confirmed by PCR amplification and SDS-PAGE, respectively. All isolates were resistance to multiple antibiotics. TSST gene producing *S. aureus* were represented from skin infected and burn wound samples. These findings will help in inhibiting or minimizing the spread up of such infectious strains and also is a potential to find many alternative remedies.

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