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To Characterize & Determine the Virulence Factors of *Staphylococcus aureus* in a Tertiary Care Hospital

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Abstract

Context: *Staphylococcus* is a gram-positive, non-motile, nonsporing bacteria that include different opportunistic pathogenic species, responsible for human and animal infections. *Aims*: To isolate and identify *Staphylococcus aureus* from different human clinical samples and to determine various virulence factors of these isolates. *Settings and Design:* Prospective study conducted in Dept of Microbiology, SKIMS J&K. *Methods and Material:* Various clinical samples eg pus, blood, sputum etc received in the Microbiology laboratory were processed for isolation of Gram positive cocci. Identification of *Staphylococcus aureus* was done by standard microbiological methods and various virulence factors were determined. *Statistical Analysis Used:* Descriptive statistics (frequency and percentage) was used. *Results:* A total of 217 strains of *Staphylococcus aureus* were isolated from 1100 clinical specimens. All the isolates had ability to produce free and bound coagulase enzyme. Out of 217 strains, 42 (19.35%)strains showed only α hemolysin production, 84 (38.70%) strains showed only β hemolysin production and rest 91(41.93%) strains showed both α and β hemolysin production. Among all the strains, 203 (93.54%) were positive for producing biofilm and rest of 13 were negative for producing biofilm. Out of 217 strains of S.aureus only 49 (22.58%) strains were able to produce the staphyloxanthin pigment and rest 168 (77.41%) were found to be negative. All strains of *S.aureus* were sensitive to Linezolid, Teicoplanin and vancomycin. On the other hand strains showed high resistance towards Cefoxitin (65.89%). *Conclusions:* Prevalence of methicillin rsistance was high in IPD setting (65.89%) and production of various virulence factors like coagulase, hemolysin production, biofilm production and staphyloxanthin pigment production was more common in MRSA than in MSSA thus suggesting that infection control policies should be adhered positively.

Keywords: *Staphylococcus Aureus*; Prevelance; Virulence Factors.

Introduction

Staphylococcus is a gram-positive, non-motile, nonsporing ubiquitous bacteria that include different opportunistic pathogenic species, responsible for human and animal infections. They are facultative anaerobes. They appear as grape like clusters when viewed under the microscope and has large, round, golden- yellow colonies, often with hemolysis when

grown on blood agar plates [1]. On the basis of the ability to clot blood plasma, they are divided into two groups: *coagulase negative staphylococci (CoNS),* and *coagulase positive staphylococci (CoPS*) that include *Staphylococcus aureus* specie.

Staphylococcus aureus is one of the major pathogens of humans; it causes various suppurative diseases, food poisoning, pneumonia, and toxic shock syndrome [2,3]. About 20% of the population is always colonized with S. aureus, 60% are intermittent carriers, and 20% never carry the organism. In some, but not all, developed countries, many nosocomial infections are caused by *S. aureus* strains that are multiple resistant to antibiotics—known as Methicillin resistant *Staphylococcus aureus* (MRSA) [4-5]. Methicillin resistance is determined by the presence of a penicillin-binding protein with decreased affinity to penicillin. The mecA gene encodes this protein and is located on the staphylococcal cassette chromosome mec (SCCmec) [6-11].

Virulence of S.aureus

It is well known that *S. aureus* produces many virulence factors, such as hemolysins, leukocidins, proteases, enterotoxins, exfoliative toxins, and immune-modulatory factors [2,12-14]. The expression of these factors is tightly regulated during growth. The relative importance of host factors versus bacterial virulence determinants in disease pathogenesis is unknown, but it is widely held that bacterial factors including toxins, cell wall-associated adhesins, and secreted exoproteins are involved in the process [15]. Thus, the pathogenicity of both *S. aureus* and CoNS, comes from their production of an impressive repertoire of virulence factors [16] that includes: surface proteins, that promote colonization of hosts tissues; invasions, that promote bacterial spread in tissue[17]; surface factors, that inhibit phagocyte engulfment [18].

Thus this study was undertaken to isolate *Staphylococcus aureus* from various clinical isolates and to determine various virulence factors of these isolates.

Subjects and Methods

This study was done in the Department of Microbiology SKIMS J&K in 2016 Sample Collection: Various clinical samples eg pus, wound swabs, urine, sputum, blood, various body fluids, tissue and tracheal tips received in the Microbiology laboratory were processed for isolation of Gram Positive Cocci. Identification of *Staphylococcus aureus* was done by standard microbiological methods and various virulence factors were determined such as coagulase activity, hemolysin production, biofilm production and staphyloxanthin pigment production.

Coagulase Activity Test

Coagulase test is based on the ability of *S. aureus* to produce a protein product called coagulase. There are two types of coagulase; Bound coagulase (clumping factor) which converts fibrinogen directly to fibrin without requiring a coagulase reacting factor. This type can be detected by the rapid slide Coagulase (SC) technique [19]. This test was performed on a clean slide using a sterile dropper; a small drop of saline was placed on the appropriate end of the slide as a control then a small drop of human plasma was placed on the opposite end of the slide with a sterilized loop. Cells were collected from one colony and were emulsified in the saline and then a drop of plasma was added, clumping was checked within 10 seconds of adding the bacterial cells to the plasma. On the other hand, the control drop, saline, showed no clumping of bacterial cells. The clumping will become more visible if the slide is rocked gently. The second type of coagulase is free coagulase which converts fibrinogen to fibrin by activating a coagulase reacting factor present in plasma which can be detected by the clumping of bacterial cells in the tube coagulase (TC) technique. Free coagulase activity was determined by the method described by Quinn et al [20] several colonies of each organism were mixed with 0.5 ml of citrated human plasma in a sterile test tube. The tube was incubated at 37°C for 4 hrs and examined after 4 and then kept at room temperature and examined at 24 h. Clot formation at either reading was recorded as positive.

Hemolysin Production

Alpha-hemolysin was evaluated on TSA supplemented with 5% washed human erythrocytes. The plates were incubated for 24 h at 37°C, when positive samples showed a wide zone of complete hemolysis with blurred edges. Beta-hemolysin was evaluated by plating strains on 5% sheep blood TSA. The plates were incubated at 37°C for 24 h and then overnight at 4°C, positive strains showed a wide zone of incomplete hemolysis with sharp edges [21-22].

Biofilm Formation

Quantitative determination was carried out by the

Micro plate method (MP) proposed by Pfaller et al [23]. Using tissue culture plates of 96 flat bottomed wells. Each well was filled with 0.2 ml of 10 [5] CFU/ ml of a bacterial suspension in TSB. After 24h incubation in aerobic condition at 37°C, the contents were aspirated and plates were washed twice with phosphate buffered saline (PBS, pH: 7.2). The wells were stained with 0.1% crystal violet for 2 min. The plates were read in Micro plate reader (BioRad iMark™) to 492 nm. Sterile TSB was used as negative control. All the experiments were repeated at least twice and the values of optical density (OD) were then averaged. A three grade scale was used to evaluate the strains slime producing ability by comparing with OD of negative control or cut off (ODc): nobiofilm producer or (-):= ODc; (Weak): = 2x ODc; (Moderate): 2x ODc < ~ = 4x ODc; (Strong): > 4x ODc.

Staphyloxanthin Assay

The bright golden coloration of this virulence factor facilitates the virulence screening by the simple observation of color [24]. Also, a quantitative carotenoid assay method was adapted from the previous method [25]. In brief, cells were re-inoculated at 1:100 dilutions in TSB medium and incubated for 16 h at 37°C. Cells (1 mL) were then collected by centrifugation at 16,600xg for 1 min and washed with 1 ml of phosphate when buffered saline (PBS). At this point, cell pellets were photographed to compare the staphyloxanthin production. For the extraction of carotenoid pigments, the cell pellets were resuspended in 0.2 mL of methanol by vortexing and this mixture was heated at 55°C for 3 min. Pigment was separated from cell debris by centrifugation at 16,600xg for 10 min. This pigment extraction step was repeated 3 times and the optical densities of collected extractions were measured at 465 nm using a spectrophotometer. Each data point was averaged cells from at least three independent cultures

Antibiotic Susceptibility

Antibiotic Resistance Assay: The standardized Kirby-Bauer disc-diffusion method was performed on Mueller-Hinton agar media using antibiotics Cefoxitin (30mcg), Teicoplanin (30mcg), Linezolid (30mcg) and Penicillin (10 units) and MIC was done for Vancomycin [26,27].

Statistical Analysis

Data were analyzed using SPSS software. Appropriate statistical charts were used to present the data. Chi square analysis was also done. Data was considered statistically significant at the $p < 0.05$ level.

Results

Isolation and Identification of isolates: From a total of 1100 clinical samples, 217 (19.72%) *Staphylococcus aureus* strains were isolated. Among a total of 217 strains of *S.aureus*, 19 (8.7%) were isolated from blood, 07 (3.2%) from body fluids, 93 (42.8%) from pus, 19 (8.7%) from sputum, 02 (1%) from tissue, 02(1%) from tracheal aspirate, 16 (7.3%) from urine and 59 (27.1%) from wound swabs. Among 217 strains obtained, 116 (53.4%) were isolated from females and 101 (46.5%) from males. Out of 217 strains, 143 (65.89) strains were MRSA and rest of 74 (34.10) strains were MSSA.

Among 217 strains, 31 strains (14.29%) were from 0-20 age group, 57 (26.27%) from 21-40 age group, 68 (31.34%) from 41-60, 52 (23.96%) from 61-80 and 9 (4.15%) from age group above 80.

Out of total of 217 strains, 70 were from OPD and rest 147 strains were from IPD. (The data was found to be significant between OPD and IPD patients in case of MRSA and MSSA ($P<0.05$).

Determination of Some Virulence Factors

Coagulase enzyme production :All the isolates had ability to produce free and bound coagulase enzyme.

Hemolysin Production: Different strains of S.aureus showed different types of hemolysis. Out of 217 strains, 42 (19.35%)strains showed only α hemolysin production, 84 (38.70%) strains showed only â hemolysin production and rest 91(41.93%) strains showed both α and β hemolysin production. There was no statistical difference in hemolysin production between MRSA and MSSA isolates ($p > 0.05$) (Table 1).

Biofilm Formation: Among all the strains, 203 (93.54%) were positive for producing biofilm and rest of 13 were negative for producing biofilm and it was found to be statistically significant (p value <0.05). Further it was evaluated that among positive biofilm producers, 62 (28.57%) were weak biofilm producers, 84 (38.70%) were moderate biofilm producers and 57 (26.26%) were strong biofilm producers (Table 2).

Staphyloxanthin Pigment Production: Out of 217 strains of *S.aureus* only 49 (22.58%) strains were able to produce the staphyloxanthin pigment and rest 168 (77.41%) were found to be negative. (Table 3).

Antibiotic Susceptibility assay: All strains of *S.aureus* were sensitive towards Linezolid, Teicoplanin

Table 1: Percentage distribution of hemolysis types among various strains of S.aureus.

	MRSA	MSSA	Total
α hemolysin	26(18.18%)	16(211.62%)	42(19.35%)
β hemolysin	56(39.16%)	28(37.84%)	84(38.70%)
α and β hemolysin	61(42.66%)	30(40.54%)	91(41.93%)
Total	143(65.89%)	74(34.10%)	217(100%)

Table 2: Biofilm produced by *S.aureus* on tissue culture plate.

Biofilm producer	Type	MRSA	MSSA	Total
Negative	No Biofilm	10(6.99%)	03(4.05%)	13(5.99%)
Positive	Weak	46(32.17%)	16(21.62%)	62(28.57%)
	Moderate	45(31.47%)	39(52.80%)	84(38.70%)
	Strong	42(29.37%)	16(21.62%)	57(57.26%)
Total		143(65.89%)	74(34.10%)	217(100%)

Table 3: Percentage of Qualitative detection of staphyloxanthin pigment production of *Staphylococcus aureus* isolates

Staphyloxanthin production	MRSA	MSSA	Total
Positive	33(23.08%)	16(21.62%)	49(22.58%)
Negative	110(76.92%)	58(78.38%)	168(77.41%)
Total	143(65.89%)	74(34.10%)	217(100%)

and vancomycin. On the other hand strains showed high resistance towards Cefoxitin (65.89%). Also, Penicillin was found to be 100% resistant.

Discussion

In this study, the determination of virulence factors like Coagulase activity, hemolysis, biofilm formation, staphyloxanthin production and drug resistance of the *Staphylococcus aureus* clinical isolates was studied [28,29]. In this study it was also found that the prevalence of *S.aureus* infection was more common in case of age group 41-60 yrs (31.34%) and in IPD patients which is usually hospital acquired.

Coagulase enzyme production is used for differentiating the pathogenic *S. aureus* from other strains or species of *staphylococci* In our study all 217 of the isolates were coagulase positive Staphylococcus spp. (CoPS) which represent pathogenic *S. aureus* [19].

In this study, it was that 19.35% *S.aureus* strains had the ability to cause alpha hemolysis on blood agar and 38.70% strains had the ability to cause beta hemolysis and 41.93% had ability to show both alpha and beta hemolysis which shows that most of the strains had capability to produce both alpha and beta hemolysis. There was no statistical difference in hemolysin production between MRSA and MSSA isolates ($p > 0.05$). In a similar study by Franco J.C. et. Al [30]. Hemolysin production was detected in 78% of the *S. aureus* isolates. Fourteen isolates (12%) were alpha hemolysin, thirty-four (29%) beta hemolysin, and forty-four (37%) showed both hemolysins. In another study by V. Pereira et al [31] 81% were demonstrated to be β–hemolytic & 8% were α-hemolytic.

In our study 93.54% of *S.aureus* strains had the ability to produce biofilm, 26.26% of these isolates strong biofilm producers, 38.70% moderate and 28.57% weak biofilm producers Also biofilm production was seen more so in MRSA strains. In a similar study by Khoramian B et al [32] approximately 70% of 215 isolates produced biofilm. Among these, 59.3% were producers of weakly adherent biofilms while 34.8% and 5.8% produced moderate and strong biofilms, respectively.

In this study, 22.58% of *S.aureus* strains had the ability to produce staphyloxanthin. However there was no statistical difference in staphyloxanthin production between MRSA and MSSA isolates ($p > 0.05$). In a study done by Al-Kazaz et. al [33] , 72.1% isolates of *S.aureus* produced the pigment staphyloxanthin.

In our study (65.89%) of *S.aureus* strains were resistant to Cefoxitin. Also Penicillin was found to be 100% resistant. On the other hand Vancomycin, linezolid, Teicoplanin were found 100% sensitive, which indicate that antibiotic vancomycin, linezolid & Teicoplanin could be used as antibiotic treatment for MRSA infection, with a recommendation of investigating an alternative therapeutic agents to avoid the multidrug resistance.

In a study by Emmanuel Onwubiko Nwankwo et al [34] the sensitivity pattern of *S. aureus* to the following antibiotics; Gentamicin, Amoxycillin/clavulanate, Streptomycin, Cloxacillin, Erythromycin, Chloramphenicol, Cotrimoxazole, Tetracycline, Penicillin, Ciprofloxacin, Ofloxacin, Levofloxacin, Ceftriaxone, Amoxycillin and vancomycin were 92.4%, 63.0%, 44.2%, 35.8%, 52.4%, 61.9%, 15.5%, 31.2%, 7.1%, 78.9%, 76.6%, 100%, 71.4%, 30.7% and 100% respectively.

Conclusion

Infection rates due to *Staphylococcus aureus* was higher in elderly age groups and most of the isolates were isolated from IPD suggesting higher rates of infection in hospital setting. Further, higher prevalence of MRSA was seen in IPD settings suggesting an urgent need of infection control practises. Furthermore most of these isolates produced various virulence factors like coagulase, hemolysin production and staphyloxanthin pigment production, which were more common in MRSA than in MSSA. Also, biofilm production was more common in MRSA isolates (65%) suggesting that infection control policies should be adhered positively because biofilm production leads to antibiotic resistance.

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