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# Hemolysins profile of clinical isolates of *S. aureus* from tertiary care hospitals in India

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**Abstract**
**Background:** *Staphylococcus aureus* is an important pathogenic bacterium causing both hospital and community acquired infections. Hemolysins are important virulence factors of *S. aureus* which help in the pathogenesis of diseases. Screening of hemolysins is important in infection control and prevention.
**Methods:** Aim of the study was to characterize hemolysins of *Staphylococcus aureus* (n=152) isolated from tertiary hospitals in India, by phenotypic and genotypic methods. Phenotypic characterization of hemolysins of *Staphylococcus aureus* was done on either sheep or rabbit blood agar plates. Genes encoding hemolysin were amplified with specific primers in a multiplex Polymerase Chain Reaction (PCR) and analyzed by agarose gel electrophoresis.
**Results:** Genes of all four hemolysins namely *hla, hlb, hlg* and *hld* were screened by multiplex PCR. Genotyping revealed that the isolates contained patterns of four, three or two hemolysin genes in multiple combinations. The most common profile comprised of all four hemolysin genes, namely *hla, hlb, hlg, hld* in a majority of isolates (47.3%, 72/152). Sixty one isolates (40.1%, 61/152) carried three hemolysin genes in three different patterns: 56 (37%) isolates contained *hla, hlb, hld* genes, second pattern combined 3 genes namely (1.9%) *hla, hlg, hld* and the third pattern was found in 2 (1.3%) isolates with *hlb, hlg, hld*. Five (3.28%) isolates in this study had only two hemolysin genes in three different combinations: 2 (1.3%) isolates with *hlb*:*hld*, 2 (1.3%) isolates with *hla*:*hld* pattern and 1 (0.7%) isolate with *hlg*:*hld* pattern. 14 (9.2%) isolates carried only *hld* gene. Phenotyping showed that all the isolates had α, β and δ activities and high activity of α, β and δ hemolysins were seen in isolates which were obtained from pus, blood and other body fluids. All these sites suffer enormous tissue damage in severe *S. aureus* infection. The genotype data correlated well with the phenotype analysis.
**Conclusions:** Four hemolysin genes were detected in all clinical isolates of *S. aureus*. We could identify the activity of α, β and δ hemolysins on blood agar media. We found out that the isolates displayed varying combination of the four hemolysin genes. These differences in genotype of four genes and the hemolysin activity may indicate a preference mandated by the host tissue or site during the pathogenesis of the disease since each hemolysin has different activity profile. Body fluids in general, pus, blood, sputum in particular carried *S. aureus* with higher hemolysin activities compared to isolates of specimen from other sites. The results of this study reinforce the importance of hemolysins in *S. aureus* diseases and suggest that the tissue damages and symptoms are related to the hemolysins activity during pathogenesis.

**Keywords:** *Staphylococcus aureus*, hemolysins, Virulence, PCR

## Introduction
*Staphylococcus aureus* is a commensal bacterium which doubles-up as one of the most common human pathogens causing both nosocomial and community acquired infections. It produces a variety of virulence factors depending on the site of infection ranging from minor skin infections to life threatening diseases such as osteomyelitis, endocarditis, skin and soft tissue infection, brain abscesses, meningitis, bacteremia and pneumonia [1].
A major pathogenic attribute of *S. aureus* is to escape both innate and adaptive immune responses wherein a number of virulence factors including surface components such as the capsule, coagulase, protein A, teichoic acid, polysaccharides and adhesins; enzymes such as esterases, lipases, fatty-acid modifying enzymes, various proteases, hydrolytic enzymes, catalase, β-lactamase and various toxins such as leukocidin, enterotoxins, TSST-1 and alpha, beta, gamma and delta hemolysins [2], play important roles. Interestingly, several of these secreted exotoxins are directed to damage the host cells' plasma membrane. Of the wide variety of *Staphylococcal* cytolytic exotoxins produced, the most prominent and well

characterized ones are the hemolysins, which play important role in the *Staphylococcal* disease pathogenesis and have the ability to kill variety of host cell populations including immune cells and help the bacteria to spread within the host [3]. The first report of hemolytic activity of *Staphylococci* recovered from human lesions appeared in 1894 and subsequently in 1900 [4].

Alpha toxins are especially hemolytic to rabbit erythrocytes though they act on a wide range of mammalian cells including human erythrocytes. This toxin is a heptamer, toxic to epithelial cells, dermonecrotic and neurotoxin in human host [5]. Currently, pore formation by α-toxin at the molecular level is being investigated. β-toxin is a magnesium dependent sphingomyelinase that is active on sheep erythrocytes [6]. γ-toxin is a two component exotoxin which comprises of six different combinations of proteins active against erythrocytes [5]. δ-toxin is a low molecular weight toxin with the ability to lyse many cell types [7]. Generally *S. aureus* infection is multifactorial due to the combined action of several virulence determinants. One exception is toxinoses, which are caused by toxic shock syndrome toxin, ex-foliative toxins A and B and different *Staphylococcal* enterotoxins [8]. This study was aimed at understanding the distribution of hemolysins in *S. aureus* isolated from different types of clinical specimen and their relevance to the disease process.

## METHODS

### Bacterial isolates

A total of 152 clinical isolates of *S. aureus* were obtained from: the Department of Microbiology of Gleneagles Global Hospital at Hyderabad, Bangalore and Chennai, AIIMS-New Delhi, and LV Prasad Eye Hospital Bhubaneshwar India. The biological source of these isolates included blood, body fluids, pus, skin, tissue, sputum and other body sites.

### AST profile MSSA and MRSA

Susceptibility of clinical isolates to antibiotics including Cefoxitin (30 µg) and Oxacillin (1 µg), was evaluated by the agar disk diffusion method on Muller Hinton Agar (MHA) plates as recommended by Clinical and Laboratory Standards Institute (CLSI) [9]. All the isolates confirmed as *S. aureus* on Mannitol Salt Agar (MSA) were screened using the Cefoxitin (30µg) and Oxacillin (1µg) disks to detect the methicillin resistance. The *S. aureus* ATCC 25923 (MSSA) and ATCC 43300 (MRSA) were used as controls. An inoculum was prepared for each isolate by inoculating single colonies into sterile MH broth and grown at 37°C till it reaches 0.5 McFarland, equivalent to $10^8$ cfu/mL. The bacterial suspension was spread evenly on the MHA plates using sterile swabs and left for 3-5 min prior to the introduction of antibiotics. The antibiotic disks were placed on the agar surface. Plates were incubated at 37 °C for 24h. The inhibition zone diameters were measured and results interpreted according to CLSI: a diameter of ≤ 21mm for Cefoxitin and ≤ 12mm for Oxacillin were considered as MRSA whereas the diameter ≥22mm for Cefoxitin and ≥ 12mm for Oxacillin indicates MSSA.

### Hemolysin Characterization

### Detection of hemolysins by Blood agar plate assay

Hemolysis of *S. aureus* was tested on 5% sheep blood agar medium. Clinical isolates were cultured on blood agar plates using Columbia blood agar medium supplemented with a) rabbit erythrocytes to detect α-hemolysin, b) sheep erythrocytes to detect β-hemolysins [10] c) γ- hemolysin activity is determined on rabbit erythrocytes in agarose plate instead of agar plate as agar inhibits the γ-hemolysin activity and d) delta hemolysin activity is detected and semi quantitated by cross streaking of test isolate perpendicular to *S. aureus* 25923 [11]. *S. aureus* 25923 is a reference strain which produces only β-hemolysin on sheep blood agar plates. The δ-hemolysin positive isolates produce a clear zone at the confluence of the two streaks (the area around the intersection) due to enhanced hemolysis. All the inoculated plates were incubated at 37 °C for 24h to detect the hemolysin activity

### DNA preparation

Single colonies were inoculated into Muller Hinton Broth (MHB) and incubated at 37 °C overnight (12-16h). Cultures were grown overnight to 0.5 McFarland, corresponding to $10^8$ CFU/mL, and used for DNA isolation by standard methods [12]. Briefly, the bacterial cell pellets were subjected to initial lysis with 50 µL of lysozyme (10 mg/mL) and incubated at 37 °C for 1h. 70µL of 10% Sodium Dodecyl Sulfate (SDS), 6µL of Proteinase K (20 mg/mL) was added to the bacterial suspension and incubated at 50 °C for 1h. 100µL 5M NaCl and 600µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1) were added to the lysate and vortexed gently for 10 sec. The lysate was centrifuged at 10000 rpm for 10 min. The DNA was precipitated from the supernatant by adding 450µL of chilled isopropanol and centrifuged at 12000rpm for 10min. The pellet was carefully washed with 70% ethanol and dissolved in Tris-EDTA buffer (10mM Tris-HCl, 1mM EDTA pH 8.0) and stored at -20 °C for further experiments. The DNA concentrations were checked in Nanodrop (Model No. ND2000, Thermo Scientific) and visualised in 0.8% agarose gel electrophoresis.

### Multiplex PCR assay for hemolysin profiling

The prevalence of hemolysin genes (*hla*, *hlb*, *hlg* and *hld*) among clinical isolates of *S. aureus* was examined by multiplex PCR with specific primers. Oligonucleotide primers for identification of hemolysin genes were selected from the published sequences of *S. aureus* (Table 1) [13] and the reaction was first optimized with the reference strain of *S. aureus* ATCC 43300 (MRSA).

Amplification of hemolysin genes was performed in a total of 25µL reaction volume consisting of: 1µL of template DNA, 2.5µL of 10X PCR buffer, 1.5µL of 25 mM $MgCl_2$, 2µL of 10mM dNTPs, 2µL of 10pmol of each primer, 0.2µL of Kappa Taq DNA polymerase (5 U/µL) made up to 25µL with molecular biology grade water (MBGW). Template DNA was initially denatured in a thermo cycler at 94 °C for 7 min followed by a total of 35 amplification cycles. Each cycle consists of 1 min denaturation at 94 °C, 1 min annealing at 58 °C and 1 min extension at 72 °C. The final extension was 7 min at 72 °C. The amplicons were analyzed in 2% agarose gel.

## Results

### Antibiotic sensitivity testing of clinical isolates (Kirby Bauer Disk Diffusion).

Clinical isolates were tested with the following antibiotics Vancomycin 5µg (VAN), Amikacin 30µg (AMK), Erythromycin 15µg (ERY), Clindamycin 2µg (CLI),

Linezolid 10µg (LZD), Teicoplannin 30µg (TEC), Cefoxitin 30µg (FOX), Mupirocin 200/30 (MUP), Tigicycline 15µg (TGC), Gentamycin 10 µg (GEN), and Penicillin 2 and 10µg (PEN) in the microbiology department for drug sensitivity profiling. The clinical isolates were re-tested in the molecular diagnostics department by Kirby Bauer disk diffusion assay with the antibiotic discs of (HiMedia, India) Cefoxitin (30µg) and Oxacillin (1µg) to reconfirm the sensitivity to methicillin. Isolates displaying inhibition zone < 22mm with Cefoxitin and <10mm with Oxacillin were considered resistant (Figure 1 A) and >23 with Cefoxitin and >13 with Oxacillin were sensitive (Figure1 B) as per CLSI guidelines. Among 152 *S. aureus* clinical isolates, 97 (63.81%) were methicillin resistant (MRSA) and 55 (36.18%) were methicillin sensitive (MSSA).

**Hemolysins Phenotype Assay**

Growth on blood agar medium decides whether the bacterial isolate is hemolytic or non-hemolytic. The hemolytic bacteria are generally pathogenic. The hemolytic activity of *S. aureus* was detected by its phenotypic expression of hemolysin on Columbia blood agar plates supplemented with rabbit (α-hemolysin screening) and sheep blood (β and δ hemolysin screening). The α, β and δ hemolysin activities were scored as 3+ for highest activity 2+ for Moderate activity and 1+ for Low activity (Figure 2). The α-hemolysis is associated with reduction of red cell hemoglobin, where the bacteria produce hydrogen peroxide which oxidizes hemoglobin to green met-hemoglobin. On the other hand, the complete destruction of red cells surrounding the colony indicates β-hemolysis. The δ-hemolysin activity is detected and semi-quantitated by cross-streaking of test isolate perpendicular to *S. aureus* 25923 which is a reference strain that produces only β-hemolysin on sheep blood agar plates. The δ-hemolysin positive isolates produce a clear zone at the confluence of the two streaks (the area around the intersection) due to enhanced hemolysis.

Almost all *S. aureus* isolates were positive for α, β, and δ hemolytic activity (96.05-100%) and showed different degrees of hemolysis on blood agar plate. Based on the extent of hemolysis produced on the blood agar, the isolates were grouped as high producers of hemolysin (30.9%-40%), moderate producers (20.3%-23.3%) and low producers (32.2%-44.7%). (Table 2, 3, 4) and (Figure 3).

**Prevalence and distribution of hemolysin genes in *S. aureus***

The isolates were screened for four hemolysin genes: *hla*, *hlb*, *hlg*, *hld*, by PCR. Nearly all the isolates contained single, two, three or four hemolysin genes though, in different combinations. Multiplex PCR (Figure 4) results showed that *hld* gene was omni present (152/152) in all followed by *hla* in 133 isolates (87.5%), *hlb* in 132 isolates (86.8%) and *hlg* in 77 isolates (50.6%) though there are reports of very high prevalence (99.5%)of *hlg* gene in human *S. aureus* [14, 15].

We identified different patterns of genomic distribution of hemolysin genes in our study. Presence or absence of all hemolysin genes and their percentages are given in the form of Bar diagram (Table 5 and Figure 5) All isolates were screened by multiplex PCR amplification for four hemolysin genes: *hla, hlb, hlg* and *hld*. The results of this study showed the most common gene profile was, a) all hemolysins: *hla*, *hlb*, *hlg*, *hld* - Pattern I found in 72 (47.3%) isolates and b) 57 (37.5%) of isolates carried three hemolysin genes: *hla*, *hlb*, *hld* - Pattern II or c) 3 (1.9%) *hla*, *hlg*, *hld* - Pattern IIa and d) Pattern IIb-1 (0.7%) with *hlb*, *hlg*, *hld*. Five (3.28%) isolates in this study had two hemolysin genes in 3 different patterns: Pattern III- 2 (1.3%) isolates with *hlb*, *hld*; Pattern IIIa- 2 (1.3%) isolates with *hla*, *hld* as and 1 (0.7%) isolates with *hlg*, *hld* as Pattern IIIb. 14 (9.2%) isolates carried only *hld* gene as Pattern IV.

**Table 1:** Primer sequences used for hemolysin PCR

| Gene | Primer sequence (5'-3') | Product size (bp) | Reference |
|---|---|---|---|
| *hla* | CTG ATT ACT ATC CAA GAA ATT CGA TTG CTT TCC AGC CTA CTT TTT TAT CAG T | 209 | Jarraud S *et al*. 2002 [13] |
| *hlb* | GTG CAC TTA CTG ACA ATA GTG C GTT GAT GAG TAG CTA CCT TCA GT | 309 | |
| *hlg* | GTC AYA GAG TCC ATA ATG CAT TTA A CAC CAA ATG TAT AGC CTA AAG TG | 535 | |
| *hld* | AAG AAT TTT TAT CTT AAT TAA GGA AGG AGT G TTA GTG AAT TTG TTC ACT GTG TCG A | 111 | |

**Table 2:** Beta hemolysin activity by blood agar plate assay

| S. No | Types of specimen | (n) | β-Hemolysin activity | | | | Positive | Negative (Non-producers) |
|---|---|---|---|---|---|---|---|---|
| | | | (+++) High | (++) Moderate | (+/±) Weak | (-) Non | | |
| 1 | Blood (38) | 38 | 12 | 10 | 8/7 | 1 | 37 | 1 |
| 2 | Body fluids (12) | 12 | 2 | 4 | 2/4 | - | 12 | 0 |
| 3 | Pus (72) | 72 | 27 | 11 | 15/16 | 3 | 69 | 3 |
| 4 | Skin (2) | 2 | - | 1 | 1/0 | - | 2 | 0 |
| 5 | Tissue (6) | 6 | 1 | 1 | 2/1 | 1 | 5 | 1 |
| 6 | Sputum (3) | 3 | 1 | - | 1/1 | - | 3 | 0 |
| 7 | Others (19) | 19 | 4 | 4 | 6/4 | 1 | 18 | 1 |
| | (n) | 152 | 47 | 31 | 35/33 | 6 | 146 | 06 |
| | As% | 100 | 30.9 | 20.3 | 23/21.7 | 3.9 | 96.05 | 3.94 |

**Table 3:** Delta hemolysin activity by blood agar plate assay

| S. No | Types of specimen | (n) | δ-Hemolysin activity | | | | Positive | Negative (Non-producers) |
|---|---|---|---|---|---|---|---|---|
| | | | (+++) High | (++) Moderate | (+/±) Weak | (-) Non | | |
| 1 | Blood (38) | 38 | 14 | 13 | 5/4 | 2 | 36 | 02 |
| 2 | Body fluids (12) | 12 | 2 | 5 | 2/3 | - | 12 | 0 |
| 3 | Pus (72) | 72 | 28 | 19 | 9/12 | 4 | 68 | 04 |
| 4 | Skin (2) | 2 | 1 | 1 | - | - | 2 | 0 |
| 5 | Tissue (6) | 6 | 1 | 1 | 2/2 | - | 6 | 0 |
| 6 | Sputum (3) | 3 | - | 1 | 1/1 | - | 3 | 0 |
| 7 | Others (19) | 19 | 8 | 3 | 5/3 | - | 19 | 0 |
| | (n) | 152 | 54 | 43 | 24/25 | 6 | 146 | 06 |
| | As% | 100 | 35.5 | 28.2 | 15.7/16.4 | 3.9 | 96.05 | 3.94 |

**Table 4:** Alpha hemolysin activity by blood agar plate assay

| S. No | Types of specimen | (n) | α-Hemolysin activity | | | | Positive | Negative (Non-producers) |
|---|---|---|---|---|---|---|---|---|
| | | | (+++) High | (++) Moderate | (+/±) Weak | (-) Non | | |
| 1 | Blood (11) | 11 | 5 | 3 | 3 | 0 | 11 | 0 |
| 2 | Body fluids (03) | 03 | 0 | 1 | 1/1 | 0 | 3 | 0 |
| 3 | Pus (09) | 09 | 03 | 02 | 3/1 | 0 | 9 | 0 |
| 4 | Skin (1) | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| 5 | Tissue (1) | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| 6 | Sputum (0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | Others (05) | 05 | 03 | 0 | 1/1 | 0 | 5 | 0 |
| | (n) | 30 | 12 | 7 | 11 | 0 | 30 | 0 |
| | As% | 100 | 40 | 23.3 | 36.6 | | 100 | 0 |

**Table 5:** Genotypic analysis of Hemolysin genes from different clinical specimen

| S. No | Types of specimen | (n) | Hemolysin Genotypic pattern | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | I | II | IIa | IIb | III | IIIa | IIIb | IV |
| 1 | Blood (38) | 38 | 22 | 12 | 1 | 0 | 1 | 0 | 1 | 1 |
| 2 | Body fluids (12) | 12 | 7 | 4 | 0 | 1 | 0 | 0 | 0 | 0 |
| 3 | Pus (72) | 72 | 35 | 24 | 2 | 0 | 1 | 1 | 0 | 9 |
| 4 | Skin (2) | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 5 | Tissue (6) | 6 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| 6 | Sputum (3) | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 7 | Others (19) | 19 | 1 | 16 | 0 | 0 | 0 | 1 | 0 | 1 |
| | Total (152) | 152 | 72 | 57 | 3 | 1 | 2 | 2 | 1 | 14 |

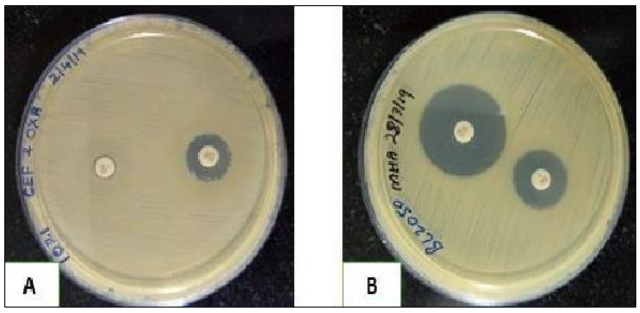


**Fig 1:** Kirby Bauer Disc Diffusion Test

Fig 1: Growth inhibition zone against Cefoxitin and Oxacillin; plate A) Methicillin resistant, MRSA (1021) and plate B) Methicillin sensitive, MSSA (BL 2050)

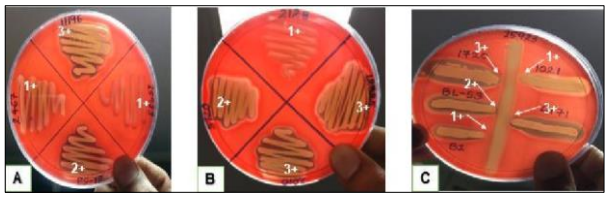


**Fig 2:** Hemolysins activity of *S. aureus* isolates on blood agar plates, A), Alpha hemolysis: score 3+ = high activity # 11196, 2+ = Moderate activity # BL-53, 1+ = Low activity # 2467, 25923, B), Beta hemolysis: score 3+ = high activity # 12325, 2010; 2+ = Moderate activity # 3720, 1+ = Low activity # 2128. C), Delta hemolysis: see the enhancement and extension of beta- hemolytic zone (arrow head) at the intersection of test isolates of *S. aureus*. The enhanced zone of activity which is due to delta hemolysin is scored as 3+ = High activity # 1720, 4171; 2+ = Moderate # BL-53; 1+ = Low activity # 1021, B1

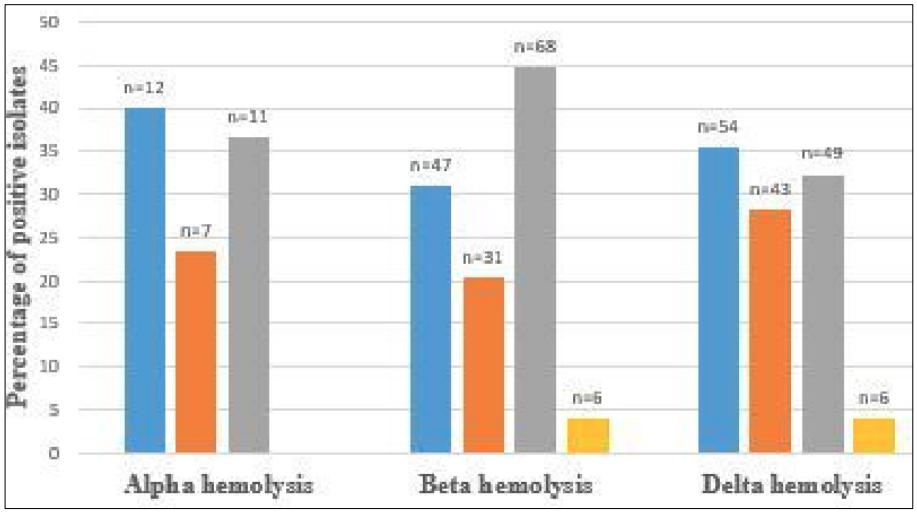


**Fig 3:** *Staphylococcus aureus* hemolysin activity assay on blood agar plates.

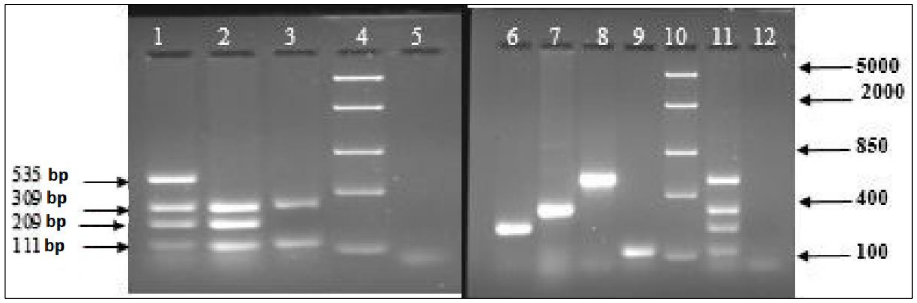


**Fig 4:** Hemolysin genes profile in clinical isolates of *S. aureus*

Multiplex PCR assays for the simultaneous detection of multiple hemolysin genes: Lane: 1- ATCC 43300 (*hla*, *hlb*, *hlg*, *hld*); 2-*S. aureus* BL-66 (*hla*, *hlb*, *hld*); 3-*S.aureus* 2886 (*hlb*, *hld*); 4- Low range ladder (100 bp - 5000 bp); 5-Negative control; 6-9 monoplex PCR of *hla* gene 209 b, *hlb* 309 bp, *hlg* 535 bp, *hld* 111 bp; 10- Low range ladder (100 bp -5000 bp); 11-P.C ATCC 43300 (*hla*, *hlb*, *hlg*, *hld*); 12-N.C.

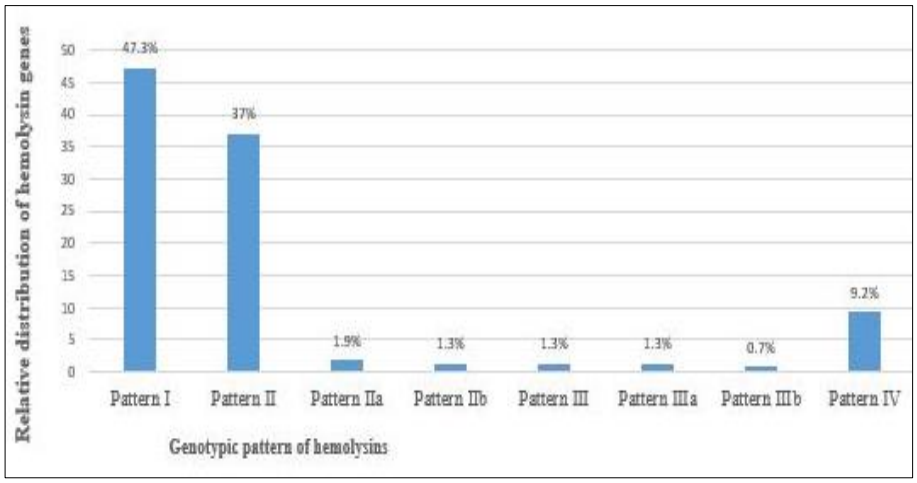


**Fig 5:** Distribution of hemolysin genes in clinical isolates of *S. aureus*

Genotyping of hemolysin genes from different clinical specimen. *hla*, *hlb*, *hlg* and *hld* genes were determined by multiplex PCR amplification and 8 different combinations of hemolysin genes were detected in different isolates depending on the specimen type. The results of this study showed the most common gene profile was: all hemolysins, *hla*, *hlb*, *hlg*, *hld* - Pattern I found in 72 (47.3%) isolates and 57 (37.5%) of isolates carried three hemolysin genes: *hla*, *hlb*, *hld* - Pattern II or 3 (1.9%) *hla*, *hlg*, *hld* - Pattern IIa and Pattern IIb-1 (0.7%) with *hlb*, *hlg*, *hld*. Only 5 (3.28%) isolates in this study have two hemolysin genes with 3 patterns: Pattern III- 2 (1.3%) isolates with *hlb*, *hld*; Pattern IIIa- 2 (1.3%) isolates with *hla*, *hld* as and 1 (0.7%) isolates with *hlg*, *hld* as Pattern IIIb. 14 (9.2%) isolates carried only *hld* gene as Pattern IV.

## Discussion

Multi drug resistance in general and MRSA in particular have become formidable problems in the management of nosocomial infections and also in the control of community

acquired infections [16]. The ability of *S. aureus* to withstand host immune responses and cause a wide range of diseases has been attributed to its ability to express multiple virulence determinants. The pathogenesis of *S. aureus* diseases depends on the production of surface proteins which mediates the bacterial adherence to host tissues, secretion of extracellular toxins and enzymes which destruct host cells and tissues and helps the growth and spread of bacteria in the host cells.

The common toxins secreted by *S. aureus* are hemolysins, leukotoxin, enterotoxin, exfoliative toxin and toxic shock syndrome toxin. Hemolysins are cytolytic and help the bacteria acquiring nutrients from the lysed cells. They lyse the host cells like Red Blood Cells (RBC), neutrophils and their action is usually receptor mediated. Hemolysins also contribute to biofilm formation [17]. There are four classes of hemolysins α, β, γ and δ. Remarkably, the role of γ hemolysin to pathophysiology is poorly understood and also the assay protocol is not simple. There is no simple blood agar plate assay. However, a functional approach would yield useful information regarding the role of γ-hemolysin as a virulence factor of *S. aureus* and its role in pathogenesis. Production of cytolysins including the hemolysins determines the pathogenicity in several *S. aureus* diseases [18]. Majority of *S. aureus* isolates are capable of producing hemolysins [19]. Our recent review on hemolysins of *S. aureus* describes in detail the background, biology, role in pathogenesis and potential role as targets for development of anti-virulence drugs [20].

Our multiplex PCR protocol for genotyping hemolysin genes is an improvement and more convenient than previous methods used for detecting staphylococcal toxin genes [21]. Hemolysins in clinical isolates of *S. aureus* have not been characterized or profiled in India. We are reporting for the first time the prevalence and distribution of the four hemolysin genes in the clinical isolates from a tertiary care hospital in India. It is remarkable that *hld* gene was detected in all the isolates (100%). Further, nearly 50% of the clinical isolates showed the presence of all hemolysin genes (*hla*, *hlb*, *hlg* and *hld*) which suggests that there is a correlation of these genes with the source of specimen.

Three hemolysin genes were found to be prevalent in three sets: *hla*, *hlb* and *hld*; *hla*, *hlg* and *hld*; and the third set was *hlb*, *hlg* and *hld*. A few isolates had only two hemolysin genes one of them being *hld* while the other gene was *hla*, *hlb* and *hlg*. These isolates interestingly lacked *hlb* the significance of this is not known. About 9.2% of the isolates had only δ-hemolysin gene. It will be interesting to investigate the pathogenesis of the diseases and host tissue involvement by these isolates which do not have *hlb* or have only *hld*. These differences in genotype of four genes and the hemolysin activity may indicate a preference mandated by the host tissue or site during the pathogenesis of the disease since each hemolysin has different biological activity profile. Body fluids in general, pus, blood, sputum in particular carried *S. aureus* with higher hemolysin activities compared to isolates of specimen from other sites. The results of this study reinforce the importance of hemolysins in *S. aureus* diseases and suggest that the tissue damages and symptoms are related to the hemolysins activity during pathogenesis. A better understanding of *S. aureus* epidemiology and pathogenesis is crucial for the development of potential therapeutic targets. Anti-virulence therapies which interfere with bacterial virulence factors or toxins and/or pathways that regulate virulence factor production, offer an attractive option to conventional antibiotics.

## Conclusion

Hemolysins are integral to the virulence weaponry of *S. aureus*. This study has shown that like in the rest of the world, clinical isolates of S. aureus from India also have a full range of hemolysins to help invade and survive during infections. Considering the low cost and much shorter time required to detect the hemolysin genes of *S. aureus* by multiplex PCR and/or by blood agar hemolysis test, we believe these to be powerful tools for studying the genotypes and in molecular epidemiology of *S. aureus* diseases outbreaks. These procedures easily fit into the daily work pattern of a routine clinical laboratory, since genotypic detection of drug resistance and the presence of toxin genes is becoming an important component of the diagnostic inventory of such laboratories.

Overall screening of hemolysins is an important step in understanding the virulence of the bacteria and developing strategies to prevent and treat *S. aureus* infections. Hemolysins play an important role in pathogenesis of all diseases due to *S. aureus*. They help to lyse the host cell membrane, maim or evade the immune system, release the nutrients for the pathogen's survival and progression of disease. Since *S. aureus* has become a super bug resistant to all available antibiotic, it is imperative that alternate targets are identified and drugs designed. Conventional antibiotics target the nucleic acid and protein biosynthesis in the microorganisms and it became inevitable for the microbe to protect itself and survive which lead to development of resistance through several mechanisms. Virulence factors like hemolysins offer attractive targets for anti-virulence drug development. The chances of resistance development to such anti-virulence drugs/therapeutics are very low, as these drugs do not threaten the survival of the microbe. As there are no new antibiotics under approval stage in any country, it is imperative that efforts are maintained to identify new targets and develop new drugs which would either limit the growth of these super bugs or at least reduce the severity of such infections through anti-virulence drugs.

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## Declarations

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