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Phenotypic determination of specific virulence factors in *Staphylococcus aureus* circulating at Hyderabad, Pakistan

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Abstract: Understanding of virulence characteristics which help *Staphylococcus aureus* strains to survive in clinical and non-clinical settings is crucial for antibacterial strategies. This study is designed to investigate some phenotypic virulence traits if they relate *S. aureus* to defined environment. For this purpose fifty-three *S. aureus* cultures were isolated from clinical (n=22) and non-clinical (n=31) environments and processed for the determination of selected virulence factors i.e., Staphylokinase, lipase and haemolysins on specific media plates. The results showed that 39.6% (n=21) of total isolates were staphylokinase producers. Categorically the frequency of Staphylokinase producing isolates was higher among the clinical isolates (50%) than the non-clinical isolates (32.2%). Overall 81% (n=43) isolates were lipase producers. Unequivocally, the frequency of lipase production was higher among clinical isolates (86.3%) than the non-clinical isolates (77.4%). The haemolytic potential was observed in all tested *S. aureus* isolates. Beta-haemolytic pattern was most frequent observation among both clinical and non-clinical isolates. Interestingly, 81% of Staphylokinase producers showed beta-hemolytic pattern. In conclusion, the current data suggest that *S. aureus* in clinical setting are more virulent than non-clinical isolates circulating at Hyderabad region

Keywords: *Staphylococcus aureus*, virulence factors, staphylokinase, lipase

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a versatile pathogen normally residing as commensal onskin andinner nares in about 30% humanpopulations without any apparent infection (JanKluytmans, 1997; Williams, 1963).

*S. aureus*besides colonizing the epithelial surfacesalso could lead to severe infections including serious invasive diseases (Lowy, 1998). The pathogenicity of *S. aureus* is attributed to variety of immune modulating surface adhered andsecreted virulence factors which includes secretedtoxins, cofactors for activating host zymogens, and exoenzymes. All strains of *S. aureus*produce several extracellular enzymes which help them in nutrient acquisition,injuryto host tissues andinactivation of hostdefense mechanisms. These exoenzymes comprise of lipases, lecithinase, nucleases, proteases, hyaluronidase, and staphylokinase e*t al.*, 2019).

Glycerol ester hydrolases (EC 3.1.1.3) commonly called as lipases whichhydrolyzes fattyacidandlipids. Lipase enzymes activity results inthe release of considerate amounts of fatty acids whichcontribute to virulence by embeddingthe bacterial cells to survive inthe fatty secretions by mammalian skin (Ralf Rosenstein, 2000).

Staphylokinase (SAK) is 16kDa pro-fibrinolytic extracellular proteins produced by lysogenic *S. aureus* strains (Nedaeinia *et al.*, 2020). SAK is a bacterial plasminogen activator protein which activates plasminogen to active plasmin, which digests the fibrin clots (Rajamohan, e*t al.*, 2002). SAKis one of important secreted enzyme by*S. aureus*, which enhances bacterial invasiveness (Bokarewa, *et al.*, 2006). SAK from the *S. aureus* is known to cause fibrin-specific thrombolysis in human plasma. Several studies suggest the thrombolytic potential of recombinant SAK which is involve in myocardial infiltration andin platelet rich clots(Nedaeinia *et al.*, 2020; Otto, 2014).

S. aureus causesmany diseases by producing exotoxins that results in host cell damage like haemolysins which are classified into four types as: α-toxin (*hla*) , β-toxin (*hlb*) , γ-toxin (*hlg*) and δ-toxin (*hld*). These toxins has a cytolytic effect on many types of cells mainly erythrocytes, platelets, monocytes, neutrophils, endothelial and epithelial cells (Otto, 2014). Alpha-toxin is secreted by 90% *S. aureus* strains And thought to form heptameric β-barrel pores in target

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cell membrane which results into leaking of cytoplasmic proteins and cell lysis of many types of mammalian cells. It is dermonecrotic and neurotoxic (Otto, 2014).

Beta-toxin is anMg^{+}dependent sphingomyelinase enzyme which is produced by most of *S. aureus*strains. It degrades sphingomyelin in the outer phospholipid layer of erythrocytes membrane. The hydrolysis product of membrane collapses when cells are place at 4°Candclear lysis could be observed around the growth of bacteria on blood agar plates. Hence the β-toxin is also known as a "hot-cold haemolysin"(Low, Freer, Arbuthnott, Möllby, andWadström, 1974). Delta toxin is a ~3KDa peptide produced by 97% of*S. aureus*isolates. It produces a detergent like action on cell membrane resulting in leaky membrane andcell lysis. Delta-toxin gene *hld* is chunk of coding sequence of RNAIII, which is a main component of *agr* regulatory system in staphylococci (Janzon andArvidson, 1990).Gamma-haemolysin is a two component toxin part of main genome of *S. aureus*. The toxin could lyse many varieties of mammalian erythrocytes andalso toxic for leukocytes.Due to inhibitory effect of agar, the toxin activity is not visible on blood agar plates so, it is detected genotypically(Prévost *et al.,* 1995; Rana El-baz, 2016).

*S. aureus*isolates from various animal species abides some characteristics anddiffer from each other. Different virulence factor laid the foundation of biotyping schemes by various researchers (Hájek and Marsálek, 1971). Previous studies have explored the importance of *S.aureus*clonality for specific disease andassociations between different virulence genes and*S. aureus*diseases(Gill *et al.,* 2011; Rasmussen, Monecke, Ehricht, andSoderquist, 2013).

The aim of this work was to evaluate that what kind of virulence factors are most commonly present in local isolatesandwhether bacterial virulence isassociated with defined types of environment. Since the normal bacterial flora results in effective immune system andmaintain it for a rapid response against invading pathogen, the determination of certain virulence factor could lead to an idea of herd immunity of local region.

2. MATERIALS ANDMETHODS

Analytical grade media were used for this study. The nutrient brothandagar (#CM0003), mannitol salt agar (#CM0085) were purchased from Oxoid UK. The sterile cotton swabs were purchased from Local scientific store.The glassware andmedia were sterilized with standard protocol as 15Ib/inch sq for 20 min in autoclave.

Clinical Isolates: The clinical *S. aureus*isolates in this study were obtained from Diagnostic andResearch Laboratory, LUMHSat Hyderabad. These obtained isolates were isolated from clinical specimens i.e. blood, pusandnose. A total of 22 identified *S. aureus* isolates were collected From August 2019 to December 2019. The cultures were obtained on mannitol salt agar platesandidentified through standard methods using gram-staining andcoagulase test.

Non-clinical Isolates: The non-clinical *S. aureus*cultures were obtained from healthy volunteers. The isolation andidentification of the cultures was performed in the HEC funded Molecular MicrobiologyandGenetics Laboratory at the Institute of Microbiology, University of Sindh, Jamshoro. The sterile cotton swabs soaked in sterile normal saline were inserted andrubbed in the inner wall of nares of the volunteers and immediately inoculated on the Mannitol Salt Agar. After 24 hours incubation at 37°C, the suspected*S. aureus*colonies were sub-cultured on mannitol salt agar andfurther identified through microscopic examination of gram stainingandcoagulase test.

Determination of Haemolysin production

The determination of haemolysin production by isolates was performed using Blood agar media (Niederstebruch, *et al.,* 2017) (Puspitasari andTurista, 2019). The nutrient agar media (Oxoid UK) was prepared according to the manufacturer's guidelines. After sterilization andcooling down to 47°C, the fresh human blood (type A+ from a female healthy adult) was added to make 5% blood agar plates. The test isolates were streak inoculated andkept at 37°C for 24 hours. Next day theplates were kept at 4°C overnight to get clear zones. The zone of clearance around the growth were observed for determination of different types of RBCs lysisandclassified as alpha, beta andgamma-haemolysis.

Determination of Staphylokinase production

The determination of staphylokinase production by isolates was performed using plasma agar plates as described earlier with little modifications(Devries, 1984; Rana El-baz, 2016). The human citrated blood plasma 15 ml is added to 85 ml molten nutrient agar (Tryptone 0.5%, Yeast extract 0.3%, NaCl 0.5%, agar 2% pH 7.4 +/- 0.2 Oxoid. UK)andheated at 55°C for 10 minutes. Human fresh plasma was added immediately in nutrient agar to make 0.5% in medium before pouring the plates as plasminogen source. Control plates were devoid of fresh plasma. The isolates were inoculated on plasma agar plates andresults were read after 24 and 48hours at 37°C.

Determination of Lipase production

The determination of lipase production by the isolates was performed using lipase agar plates with

some modification (Khoramnia *et al.*, 2010). The nutrient agar media (Oxoid UK) supplemented with 1mM $CaCl_2$ prepared according to the manufacturer's guidelines. After sterilization andcooling down to 50°C, olive oil was added to make 2% lipase test agar plates. The test isolates were streak inoculated andkept at 37°C for 24-48 hours. The zone of white precipitates around the growth wereperceived for determination of lipase production (Nguyen *et al.*, 2018; RalfRosenstein, 2000).

Statistical analysis

Data was collected, andanalyzed descriptively using Microsoft Excel Worksheet andexpressed inpercentages andcross tabulated. Virulence factors among the clinical andnon-clinical isolates were presented by frequency andpercentage. Pearson's chi-square test was applied to determine relationships between two categorical variables. We also determined the odds ratios (OR) and95% confidence intervals (95%CI) for categorical data. P-values <0.05 were considered statistically significant.

3. RESULTS

Fifty three(n=53)*S. aureus*culturesof various clinical andnon-clinical natures were processed for the determination of a set of selected virulence factors.In total,41.5% (n=22) of *S. aureus* isolates were of clinical origin recovered from various clinical specimens while 58.5 % (n=31)of the isolates were fromnon-clinical origin isolated from the nares of healthy volunteers **(Fig.. 1).**

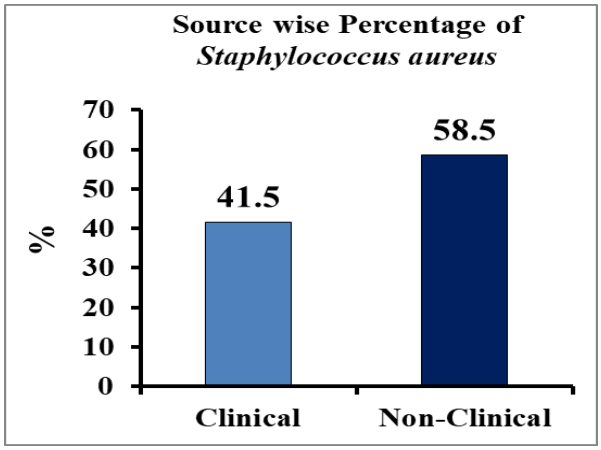

Fig. 1: Distribution of Samples(*S. aureus* isolates) from clinical andnon-clinical origin.

All of the 53 *S. aureus*cultureswere processed for the determination of potentialvirulence factors such as staphylokinase, lipase and haemolysins (**Fig.2**), using specifically designed standard techniques. Overall 39.6 % (n=21) of the *S. aureus* isolates were staphyllokinase producers and81.1 % (n=43) were lipase producers, while 100% of the isolates were different types of haemolysin producers (**Fig.3**).

Categorically among the clinical category 50% of the isolates were staphylokinase producers while 32.2% of the non-clinical isolates were staphylokinase producers. The percentage of lipase producers among the category of clinical isolates was observed to be 86.3% while 77.4% of non-clinical isolates were lipase producers (**Fig. 4**).

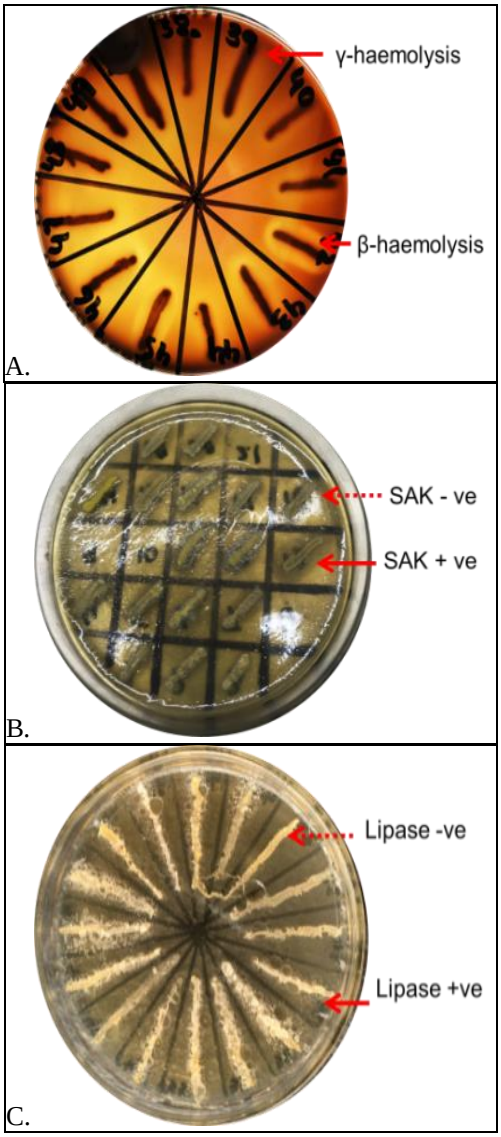

Fig..2:Determination of some extracellular virulence factors of *S. aureus*. The representative agar plates showing the haemolysis (5% blood agar plate), staphylokinase (15% plasma agar plate) andlipase production(2% olive oil,$CaCl_2$ agar plates) around the growth of *S. aureus* isolatesat 37°C. (SAK=*S. aureus*staphylokinase)

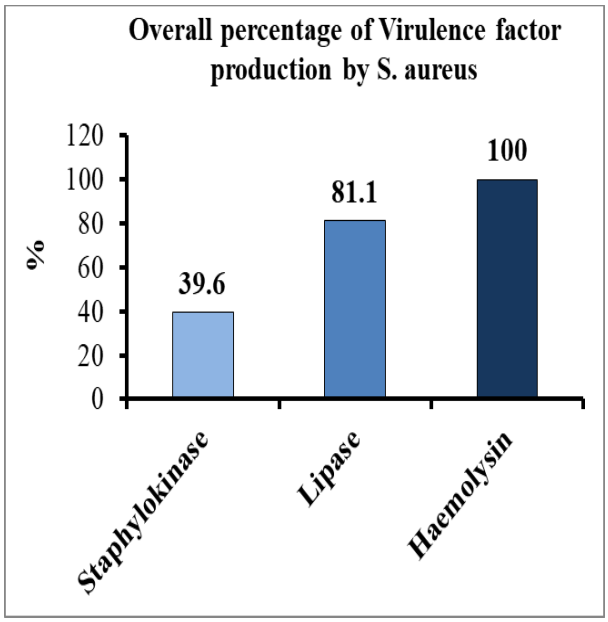

Fig..3: Production of various virulence factors. The graph showing the overall percentage of staphylokinase, lipase and haemolysin production by total number of isolates (n = 53).

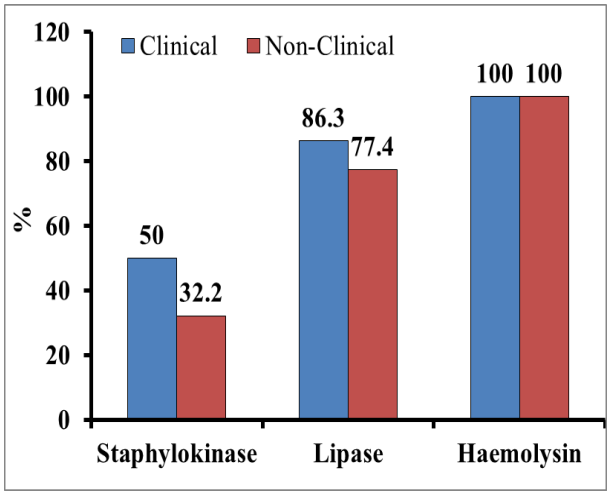

Fig. 4: Production of various virulence factors among *S. aureus* isolates. The percentage bar di*agra*m showing the staphylokinase, lipase and haemolysin production by clinical andnon-clinical *S. aureus* isolates.

Although all the isolates tested were haemolysin producers whereas the type of haemolysin production varied based on the clinical or non-clinical nature of the isolates. 36.3%of the clinical isolates were α-haemolysin producers while among the category of non-clinical isolates 45.1% were α-haemolysin producers. Comparatively the production of β-haemolysin was more (63.6%) among the clinical category of isolates than non-clinical category (45.1%) of isolates. Interestingly none of the clinical *S. aureus* isolate was observed to produce the γ-haemolysin whereas only 9.6% of non-clinical isolates were γ-heamolysin producer (**Fig. 5**).

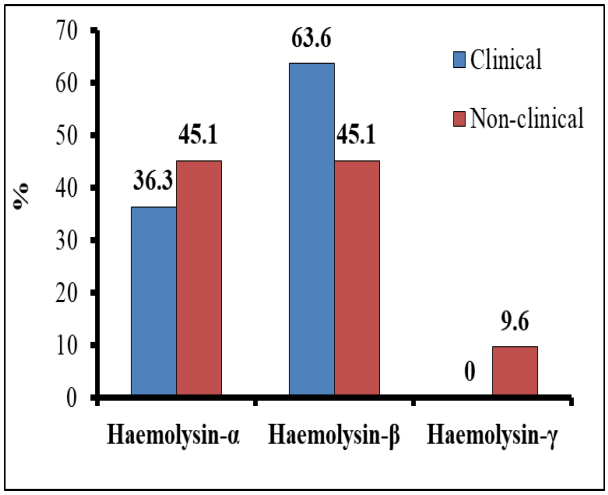

Fig. 5: Production of various haemolysins. The bar diagram showing percentage of α, β andγ haemolysis by clinical andnon-clinical *S. aureus* isolates.

To probe the differences in virulence potential between clinical andnon-clinical isolates, we determined the percentage difference for each category of virulence factor tested in this study. The highest percentage of difference (33.3%) was seen for β-haemolysin production between clinical andnon-clinical isolates (Table 1). To further evaluate the differences statistical analyses were performed. We calculated OR at 95% CI andapplied chi square test to determine the *p*-values in each category. The values are given in **(Table 1).** Intriguingly in each case non-significant ($p < 0.05$) differences were observed. We predicted the effect by increasing the original frequencies 10x times in each case (The calculations not shown) *p*-values less than 0.05 were observed, suggesting the significant differences for various virulence factors (i.e. Staphylokinase, Lipase and Beta-haemolysins) between clinical and non-clinical *S. aureus* isolates at Hyderabad.

Table 1: Virulence factor production of *S. aureus* isolated from both clinical (n=22) and non-clinical (n=31) sources. Absolute and relative values along with percentage of difference, OR, CI and p-values are expressed

Virulence factor	Source	Profile	No.	%	% of differ-rence b/w producers	OR	CI (95%]	*p*-value
Staphylokinase	Clinical	Producers	11	50	43.3	2.1	0.68 – 6.47	0.19318
		Non-producers	11	50				
	Non-Clinical	Producers	10	32.2				
		Non-producers	21	67.8				
Lipase	Clinical	Producers	19	86.3	10.8	1.85	0.42 – 8.12	0.412187
		Non-producers	3	13.7				
	Non-Clinical	Producers	24	77.4				
		Non-producers	7	22.6				
Haemolysin	Clinical	Producers	22	100	00	NA	NA	NA
		Non-producers	0	00				
	Non-Clinical	Producers	31	100				
		Non-producers	0	00				
Haemolysin α	Clinical	Producers	8	36	22.22	0.69	0.23 – 2.13	0.521861
		Non-producers	14	64				
	Non-Clinical	Producers	14	45				
		Non-producers	17	55				
Haemolysin β	Clinical	Producers	14	63	33.33	2.13	0.69 – 6.51	0.18431
		Non-producers	8	37				
	Non-Clinical	Producers	14	45				
		Non-producers	17	55				
Haemolysin γ	Clinical	Producers	0	0	NA	0	0 - NA	NA
		Non-producers	22	100				
	Non-Clinical	Producers	3	9.6				
		Non-producers	28	90.4				

4. DISCUSSION

With increase in multiple drug resistance among clinical and commensal *S. aureus* strains, there is a dire need to set up a prompt characterization of the pathogen to determine its virulence potential and invasive ability. Toxins andenterotoxins are known to work as super-antigen, pyrogenicandcytotoxic substances in disease development (Dinges *et al.*, 2000).

Various studies are conducted to determine the relationship of a set of virulence factors in *S. aureus* with severity and type of infection (Gill *et al.*, 2011). On the other handsome researcher focused on the virulence pattern with the clinical andnon-clinical isolates to relate them to some environment andhence to develop any biocontrol strategy if needed (Tuchscherr *et al.*, 2019).

Various bacterial lipases are able to hydrolyze triglycerol lipids for nutrient acquisition. Staphylococcal lipases could hydrolyze various triglyceride molecules such as ester bond in oleic acid, palmiticacid andstearic acid which are abundantly present in human sebum (Simons *et al.*, 1996). In our study we have used 2% olive oil as substrate to determine the lipase activity because olive oil is primarily composed of triacylglycerols (~99%) (Boskou, (Tuchscherr *et al.* 2006).

In many studies, *S. aureus* isolates were known to producevarious hydrolytic enzymes andmost were lipase producer. From peritonitis, 97.1% *S. aureus* isolates were lipase positive(Barretti, *et al.*, 2009), while 65.6% of the isolates from acne lesions were lipase positive (Saising, (Tuchscherr *et al..*, 2012). The production of hydrolytic enzymes in isolates from oral infection showed that 77% were lipase and59% were beta haemolytic among the tested isolates (Merghni, *et al.*, 2014).

In our results we found that 86.3% clinical and77.4% non-clinical *S. aureus*isolates were lipase positive which is reasonably similar to previous studies.All staphylococcal lipases are known to stabilized andfunction efficiently in the presence of Ca^{2+} ions.Direct relationship of lipase with disease is still not clear (Ralf Rosenstein, 2000) however lipase known to play a role in*S. aureus* invasionandin disease (Hu *et al.*, 2012; 2019). Lipase knock-out mutant showed less biofilm formation anddefective abscesses formation in mouse model (Hu *et al.*, 2012; Nguyen *et al.*, 2018) reduced granulocyte function and killing (Rollof, *et al.*, 1988).

Highlipase expressing *S. aureus* were found from clinical samples(Rana El-baz, 2016), further,eye isolates expressing higher lipasewere known to cause acne rosacea andseborrheic blepharitis (SaxenaandGomber, 2010). The antimicrobial minocycline is known to help inthe treatment of patients with acne rosacea or blepharitis by inhibiting the expression of aseries of lipases produced by these organisms (Ta *et al.,* 2003).

Staphylokinase (SAK) is a cofactor protein which binds with host plasmin andactivatesplasminogen for the breakdown of fibrin clots resulting in bacterial dissemination. The fibrin-bound complexes SAK-plasmin also cleave IgG and human C3b complement component. SAK also play a role in biofilm detachmentanddissemination of bacterial cells andin establishment of infection (Nedaeinia *et al.,* 2020; Otto, 2014; Tam and Torres, 2019). Rana El. B., studies showed that SAK was the lowest detected factor (41.15 %) in clinical *S.aureus*isolates (Rana El-baz, 2016). Tuchscherr L., elaborated that 66.7%nasal *S. aureus* isolates were SAK positive while clinical isolates from different infections were 100% SAK positive (Tuchscherr *et al.,* 2019). While 50% environmental *S. aureus* isolates were SAK positive (Shah, 2019).

In our study we found almost similar results and 39.6 % of the isolates were staphylokinase positive. Emphatically the frequency of staphylokinase producing isolates was higher among the clinical isolates (50%) than the non-clinical isolates (32.2 %).

Secreted toxins (exotoxins) represent approximately 10% of the total *S. aureus*secretome. Cytotoxins (α, β, γ and δ-toxin)act on the host cell membranes, resulting in lysis of the cells and inflammation. They can modulate the host immune system andare critical for *S. aureus* infections (Tam and Torres, 2019). The alpha-toxin is known to have role in endocarditis, skin infection, pneumonia and in murine mammary gland infection model (Tam and Torres, 2019). *S. aureus*isolates producing beta-toxin resulted in larger lesions in rabbit endocarditis andpneumonia models andinduce injuries in ocular keratitis in murine also enhances colonization of the skin (Tam andTorres, 2019). Gama-haemolysin is important factor in establishment of various infection and diseases by *S. aureus*isolates. Gama-haemolysin producing isolates are known to cause murine septic arthritis, endophthalmitis in animal models when compared with *hlg* knock-out isolates (Dinges *et al.,* 2000). Delta-toxin belongs to amphipathic peptide toxins which can lyse the eukaryotic cells in a receptor independent manner through targeting the cell membranes (Wang *et al.,* 2007).

All the haemolysinsecreted by *S. aureus* could produce clear zone (beta-haemolysis) around the bacterial growth on blood agar plates depending on the type of red cells, amount of receptors, composition of membrane lipids andculture media components.

However, alpha (partial lysis), beta (clear zone) andgamma (no haemolysis) type of haemolysisformed on blood agar plates by different *S. aureus*isolates is due to variable amount of different toxins produced andthe genotype of the isolate(s)(Herbert *et al.,* 2010; Zhang *et al.,* 2016).

In our study, we presumed andconsidered the clear zone around the *S. aureus* culture on blood agar plates after cold-shock is due to one or combination of α-, β- and δ-toxin. The partial lysis (not clear zone of haemolysis) around the growth is possibly due to factors described in previous paragraph (It is to be noted that partial haemolysis should not be confused with alpha-haemolysis by streptococcus species producing greenish zone). No change around the bacterial growth on blood agar plate was taken as gamma-haemolysisandpresumed to be a mutant strain(s) or non-producer of alpha-,beta-anddelta-toxin.

Săndulescu *et.al.,* described not much difference in lipase production but variable andsignificant difference in haemolysin production was observed in clinical (72.5%) and commensal (58.3%) *S. aureus*isolates (Săndulescu *et al.,* 2017). One study suggested positive andsignificant correlation exists between cytotoxicity as well ashaemolysis in clinical isolates (Tuchscherr *et al.,* 2019). Another study shows that *hld* gene was the most prevalent one (88.2%) followed by *hlg* gene (81.1%), *hlb* gene(70.5%) and *hla* gene (30.5%), respectively in different clinical isolates(Rana El-baz, 2016). Shah N. R., showed that 36%*S. aureus*isolates from environment were beta-haemolytic (Shah 2019), while another study suggested 100% nasal carriage isolates are beta-haemolytic (Deinhardt-Emmer *et al.,* 2018). However one group showed no difference between the clinical isolates andthe nasal carriage groupand82% isolates of both groups were haemolyticisolates(Rasmussen *et al.,* 2013). In our study we found out that 86.3% clinical isolates and77.4% non-clinical *S. aureus* isolates were haemolyticisolates which complement the previous studies.

5. CONCLUSION

Virulence factors are powerful predictors of pathogenic potential of isolates. A direct relationship between bacterial virulence factors andsource of isolation did not emerge in our study. However, the present study shows the virulence markers in *S. aureus* isolated from infections are more prevalent versus to the *S. aureus* isolated from asymptomatic carriers. The understanding of this relationship between the host andthe pathogen in infectiousandin non-infectious environment will help in evaluating the prominent

virulence factors of this pathogen in the particular region of Hyderabad.

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Conflict of interest
The author declares that there is no conflict of interest.

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