Research Article

Prevalence and leukotoxic profile of biofilm forming Staphylococcal strains isolated from a tertiary care hospital in Mysore

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Abstract

Background: Staphylococci causes chronic skin infections, device associated infections, ear infections, bone infections and post-operative infections. It secretes toxins such as Pantone-Valentine Leukocidin which prolongs the infection in hospitalized patients. **Aim:** To investigate the prevalence of *Staphylococcal* biofilm associated strains and to understand the correlation between biofilm production, multi-drug resistance and leukotoxicity. **Methods:** 280 *Staphylococcal* clinical isolates obtained from biofilm associated infections are collected from a tertiary care hospital in Mysore. They were screened for the production of biofilm, multi-drug resistance and leukotoxicity. They were also screened for *icaAB* and PVL *luk F/S* genes by PCR. Correlation between leuktoxicity and biofilm production was established by finding the leukotoxic potential of biofilm positive strains. **Findings:** Among 280 *Staphylococcal* clinical isolates, 34.6% showed biofilm production. Out of these isolates, 25% showed the presence of *icaAB* genes and 29% showed the presence of PVL *luk F/S* genes. Presence of *icaAB* gene was found to be higher in isolates obtained from implant associated infections. But the prevalence of PVL *luk F/S* genes was higher in chronic skin infections. 78% of the isolates displayed multi-drug resistance and methicillin resistant strains were comparitively high in device associated infections (77%). Strong biofilm producing PVL positive isolates from chronic skin infections showed increased leucotoxicity compared to PVL negative, weak and moderate biofilm producers. **Conclusion:** Statistical analysis revealed that PVL in association with biofilms can promote higher leukotoxicity compared to non biofilm producers. This study foresee the essential role of biofilm production, multi-drug resistance and toxins for the increased *Staphylococcal* pathogenesis.

Keywords: Biofilm, Device-associated infections, *Staphylococcus*, Pantone-Valentine Leukocidin, *ica* operon

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Introduction

Staphylococci are common bacterial colonizers of the skin and mucous membranes of humans and other mammals. It is an important pathogen in both community and hospital acquired infections and produces a wide spectrum of diseases, ranging from minor skin infections to fatal necrotizing pneumonia [1]. Biofilm formation is considered as major virulence factor for the increased pathogenesis in hospitalized patients [2]. *Staphylococcus* is known to form biofilm on indwelling devices such as urinary catheters, peripheral venous catheters, cardiac prosthetic valves, contact lens, and orthopaedic joint prosthesis and on surgical sites [2-6]. Among *Staphylococci, Staphylococcus aureus* and *Staphylococcus epidermidis* are frequently encountered in hospital acquired infections and they are the leading cause of biofilm associated infections [6-8]. *Staphylococcal* biofilms on indwelling devices are difficult to eradicate as they exhibit higher drug resistance compared to its planktonic counterparts [9,10]. When these devices are inserted to the patients, they are rapidly coated with host derived extracellular matrix components, plasma proteins and function as adhesin molecules for microbial attachment. In regions devoid of circulation, *Staphylococci* are free to grow, spread and form a resistant biofilm structure. Production of biofilm in *Staphylococcus* is primarily mediated by the intercellular adhesin operon (*ica* operon) consisting of four genes *icaA, icaD, icaB* and *icaC* [11]. *ica* operon is

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frequently reported in device associated infections leading to therapeutic failure and increased hospitalization [12]. Biofilm production by *Staphylococcus* has also been reported in surgical site infections [13, 14], chronic skin infections such as wounds [15], abscess [16], bone infections [17], respiratory infections [18], ear infections [19] and urinary tract infections [20]. But, many reports have shown that biofilm producing *Staphylococci* occurs frequently on medical implants [3, 21, 22].

Occurrence of multi-drug resistant *Staphylococci* are considered as a major problem in health-care set up. These isolates are implicated in increased morbidity, mortality, length of stay and expenditure of hospitalized patients 23. Studies have shown that the biofilm production in *Staphylococci* strongly correlates with the multi-drug resistance, higher MIC values and the presence of methicillin resistance gene cassette [24-26]. Besides, the presence of PVL toxin in *Staphylococci* can further increase the pathogenicity in patients [27]. The ability of this toxin to form pores in the membrane of host immune cells such as neutrophils can further progress the infection cycle [28]. PVL comprises of two secretory proteins *Luk F*-PV and *Luk S-PV* encoded by a prophage present in the bacterial genome [29]. PVL is also considered as a marker for community-acquired Methicillin Resistant *Staphylococci* (CA-MRSA) 30 and less frequently it has been reported in biofilm associated infections [18]. The present study was carried out to understand the synergistic effect of biofilm strength, presence of *ica* gene cluster, multi-drug resistance and PVL toxins for the increased pathogenesis of clinical *Staphylococcal* strains.

Materials and Methods

Sample collection- 35 clinical *Staphylococcal* strains from each infection sites are collected from the clinical microbiology department of a tertiary care hospital in Mysore. A total of 280 samples were collected from this hospital. All the strains were confirmed by biochemical tests such as mannitol salt fermentation test, catalase test, DNase test and coagulase test. Based on the results obtained in Mannitol salt fermentation test and coagulase test, strains were classified into coagulase positive *Staphylococci* (CoPS) and coagulase negative *Staphylococci* (CoNS).

Quantitative analysis of biofilm production- Quantitative analysis of biofilm production in *Staphylococcal* isolates was performed using a modified protocol as described by Ziebuhr 12 and Stepanovic [31]. Single colony of the *Staphylococcal* isolate was inoculated to 25 ml trypticase soy broth (TSB) and incubated at 37oC for 8-10 hours until 0.5 OD is obtained. After incubation, cells were centrifuged and washed thrice with phosphate buffer saline and diluted to 1000 times in TSB to get an OD of 0.1. Cell suspension of 200 µl was inoculated to 96 wells flat bottom microtitre plate (Tarsons) in triplicates and incubated at 37oC for 48 hours. *S. epidermidis* (RP62A) ATCC 35984 was used as a positive control and 3 wells with TSB alone were used as negative control. After incubation, plates were washed thrice with 0.15 M sterile PBS and stained with 1% crystal violet (HiMedia Labs Mumbai, India) for 5 minutes at room temperature. Stained microtitre plates are again washed thrice with sterile distilled water and dried in hot air oven at 45oC. The bound dye was resolubilized by adding 160 µl of 33% (v/v) glacial acetic acid to each well and kept for 30 minutes at room temperature. The resolubilized dye was transferred to a new microtitre plate and OD was measured at 570 nm in a spectrophotometer (Thermo-Fischer Scientific). Based on the obtained OD values, quantification of biofilm strength was performed using a formula as described by Stepanovic 30. At first the cut off OD (ODc) was calculated using the formula

ODc = Average OD of negative control + 3 × SD of negative control.

The ODc was compared with the OD of samples (ODs) and the strength of biofilm production observed in each isolate was assessed using the following formula

If $ODs \leq ODc$ then Non-adherent
If $ODc < ODs \leq 2 \times ODc$ then Weakly adherent
If $2 \times ODc < ODs \leq 4 \times ODc$ then Moderately adherent
If $4 \times ODc < ODs < ODc$ then Strongly adherent

Identification of multi-drug resistance in biofilm positive isolates- Susceptibilities to antibiotics such as cefoxitin, ciprofloxacin, clindamycin, erythromycin, gentamicin, rifampicin, tetracycline, cotrimoxazole and linezolid are determined by the Kirby-Bauer agar disc diffusion method [32]. Interpretation criteria for susceptibility tests are carried out as per the CLSI guidelines [33]. Isolates which showed resistance to more than three classes of antibiotics and cefoxitin are considered as multi-drug resistant and methicillin resistant isolates respectively.

Detection of intercellular adhesin (*ica*) gene cluster and PVL gene by PCR

DNA isolation by heat lysis method- DNA from all the isolates is obtained by heat lysis method. Single colony of each isolate was transferred to 100 µl of sterile distilled water taken in 500 µl eppendorf tube. Tubes were placed in dry bath at 100oC for 10 minutes. Later, the tubes were spinned briefly for 1 minute at 2000 RPM and the lysate obtained was used for PCR.

PCR conditions- All the reagents for PCR were procured from HiMedia Labs Mumbai. PCR for all the isolates was carried out in a 25 µl reaction containing 5 µl of cell lysate and 20 µl of master mix consisting of PCR grade water, 1.5 mM MgCl2, 10X PCR buffer, 4 pico-moles of respective forward and reverse primers, 0.4 mM dNTP mix and 0.04 U Taq polymerase. For the detection of *ica* gene cluster the primers for *icaAB* genes designed by Frebourg 34 was used. PCR programme was set with an initial denaturation at 94°C for 5 minutes followed by 35 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C and 60 seconds of extension at 72°C. Final extension at 72°C for 10 minutes was set as the last step of PCR programme. PCR was carried out in Eppendorf Master Cycler PCR machine Germany. To detect the presence of PVL-*lukF/S* genes the primers designed by Lina was used [35]. The programme was set similar to *icaAB* detection but the annealing temperature was 52°C for 45 seconds. The sequence of primers used for molecular detection of *icaAB* and PVL-*lukF/S* genes are mentioned in **Table 1**. 10 µl of amplified PCR products along with 1KB DNA marker were analyzed on 2% agarose gel electrophoresis and captured in a gel documentation system.

Table-1: Sequence of primers used for the detection of *icaAB* and PVL *lukF/S* genes

Name of the gene	Primer sequence	Reference
icaAB	icaAB - F 5' – TCTTGCAGCCGATGTCATTA – 3' icaAB - R 5' – ATGCTTGCGAGCGCTAAGTT – 3'	[34]
PVL lukF/S	Luk PV-F 5-ATCATTAGGTAAAATGTCTGGACATGATCCA-3 Luk PV-R 5-GCATCAAGTGTATTGGATAGCAAAAGC-3	[35]

Leukotoxicity assay-The leukotoxic potential of PVL positive isolates using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) was performed by a modified protocol as described by Chung [36] and Padmaja [37].

Preparation of leukotoxin-Single colony of PVL positive isolates were initially grown on 20 ml of TSB at 37°C until it reached the late exponential phase. Further, cells were centrifuged at 8000 rpm and resuspended in RPMI 1640 (Sigma-Aldrich) medium supplemented with 5% fetal bovine serum (Gibco Laboratories). This suspension was incubated for 75 minutes in a shaker incubator at 37°C. Centrifugation was repeated at 8000 rpm to remove the cells. The supernatant obtained was filtered using a 0.45 µm and 0.22 µm membrane filters. 2 folds dilution of this filtrate was used for the leukotoxicity assay.

Preparation of neutrophils- Neutrophils separated from whole blood of a healthy individual were kindly provided by the Department of Pathology, JSS Medical College, Mysore and used for the assay. Neutrophils were resuspended in PBS supplemented with 0.5 mM glucose and 1% fetal bovine serum. This suspension was diluted to get 12.5×10^{4} cells per well which was equal to 0.5 OD.

MTT assay- 100 µl of neutrophils were transferred to flat bottom sterile microtitre wells in triplicates and 2 folds dilution of 50 µl PVL suspension was added. Plates were incubated at 37°C for 1 hour. PVL untreated wells with 200 µl of neutrophils suspension alone was used as the control for the assay. After incubation 20 µl of 5mg/ml (w/v) of MTT was added to PVL treated and untreated wells and incubated for 4 hours at 37°C. Neutrophils which were killed after PVL treatment showed diminished electron transport system and could not reduce the tetrazolium salt to a water soluble purple formazan crystal. But, viable neutrophils formed this crystal by reduction. The formazan crystal was resolubilized by adding 200 µl of DMSO to all the treated and untreated wells and incubated for 15 minutes at room temperature. OD was measured at 570 nm in a visible spectrophotometer.

Determination of toxic potential of PVL positive isolates- The percent leukotoxicity was calculated using the formula

Percent leukotoxicity = 1- (OD of cell treated with PVL/ OD of untreated cells) × 100- A 2 folds dilution of leukotoxin preparation which can kill 50% of human neutrophils under the above mentioned conditions gives the toxic unit of strains. This was calculated by linear regression of the percent cytotoxicity and the corresponding 2 folds dilution of the prepared cytotoxin as described by Chung [35]. Isolates which showed higher toxic units are considered as toxic strains.

Results

Isolation and confirmation of Staphylococci- 35 *Staphylococcal* strains from each infected site or medical implant were collected from a tertiary care hospital in Mysore to get a total of 280 samples. DNase test, Catalase test and mannitol salt fermentation test further confirmed that the isolates belong to *Staphylococci.* Based on the coagulase test and mannitol salt fermentation test 62.5% of the isolates were found to be *S. aureus* and remaining 37.5% were CoNS. The prevalence of *S. aureus* was comparatively more in chronic skin infections and abscess. Whereas CoNS were found to be higher in device associated infections and bone infections. **Table 2** shows the prevalence of *S. aureus* and CoNS isolated from the infected sites and medical implants.

Table-2: Prevalence of *S. aureus* and CoNS isolated from infected sites.

Isolation sites	Number of *S. aureus*	Number of CoNS
Post-operative infections	21	14
Chronic skin infections	29	6
Abscess	31	4
Implant associated infections	6	29
Bone infections	18	17
Otitis media	32	3
Respiratory infections	27	8
Urinary tract infections	11	24
Total	**175 (62.5%)**	**105 (37.5%)**

Table-3: Number of biofilm positive isolates and their characteristics.

Site of isolation	Total no. of biofilm positive isolates	No. of strong biofilm producers	No. of moderate biofilm producers	No. of weak biofilm producers	No. of *icaAB* positive isolates	No. of PVL *luk F/S* positive isolates
Post-operative infections	7	3	2	2	3	2
Chronic skin infections	33	23	6	4	6	8
Abscess	16	5	2	9	3	3
Implant associated infections	30	7	4	19	7	4
Bone infections	6	6	0	0	2	2
Otitis media	2	0	1	1	0	1
Respiratory infections	4	4	0	0	1	2
Urinary tract infections	2	1	0	1	2	2
Total	**97**	**49**	**15**	**36**	**24**	**28**

Quantitative analysis of biofilm production- By using the formula as described above, isolates were classified into non-adherent, weakly adherent, moderately adherent and strongly adherent. Out of 280 samples, 97 isolates (34.6%) showed biofilm production. It was prominent in isolates obtained from chronic skin infections and device associated infections. However, strong biofilm producers were comparatively high in isolates obtained from chronic skin infections and bone infections. Site of infection, number of biofilm positive isolates and their respective strength are shown in **Table 3**.

Antibiogram of biofilm positive isolates- By using Kirby Bauer agar disc diffusion method, 97 biofilm positive isolates were checked for antimicrobial susceptibility. 76 isolates were found to be multi-drug resistant and the percentage was higher in isolates obtained from device associated infections followed by chronic skin infections. Methicillin resistant isolates were found to be more in device associated infections when compared to other isolates. Interestingly, 4 isolates from chronic skin infections also showed resistance to Linezolid. **Table 4** shows the number of isolates showing resistance to the screened antibiotics.

Table-4: Number of biofilm positive isolates and their resistance to screened antibiotics.

Site of isolation	No. of biofilm positive isolates	Number of resistant isolates								
		Cefoxitin	Ciprofloxacin	Erythromycin	Clindamycin	Gentamicin	Tetracycline	Rifampicin	Cotrimoxazole	Linezolid
Post-operative infections	7	4	2	5	2	3	1	1	1	0
Chronic skin infections	33	23	25	28	12	27	8	3	20	4
Abscess	16	10	7	14	8	14	5	3	13	0
Implant associated infections	30	27	20	24	6	21	7	5	23	0
Bone infections	6	3	1	4	0	3	0	0	4	0
Otitis media	2	1	0	2	0	2	0	0	1	0
Respiratory infections	4	3	0	1	0	2	1	0	1	0
Urinary tract infections	2	1	0	0	1	1	1	0	1	0

Molecular screening of *icaAB* and PVL *lukF/S* genes- Out of 97 biofilm positive isolates, 24 showed the presence of *icaAB* genes. These genes were found to be higher in device associated infections followed by chronic skin infections. A total of 28 *S. aureus* isolates out of 97 biofilm positive strains showed the presence of PVL *lukF/S* genes. The prevalence was higher in isolates obtained from chronic skin infections followed by abscess. Isolated sites and the prevalence of *icaAB* and PVL *lukF/*S genes are shown in **Table 3. Figure 2 and 3** shows the agarose gel with amplified PCR products of *icaAB* and PVL *lukF/S* genes.

Figure-1: Molecular screening of *icaAB* genes

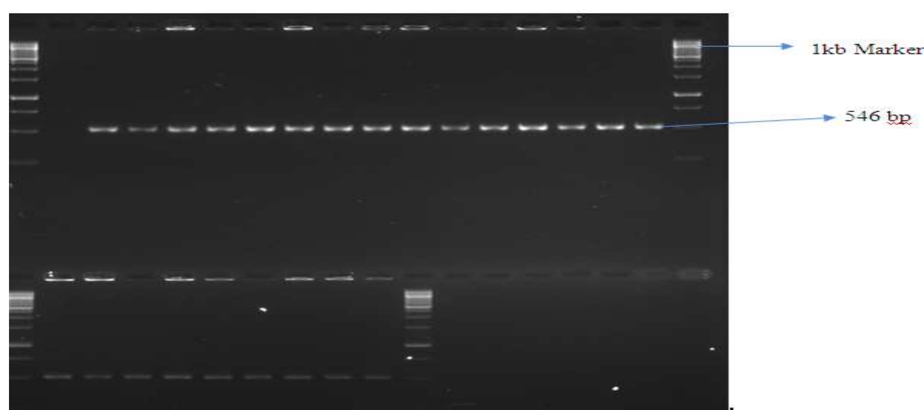
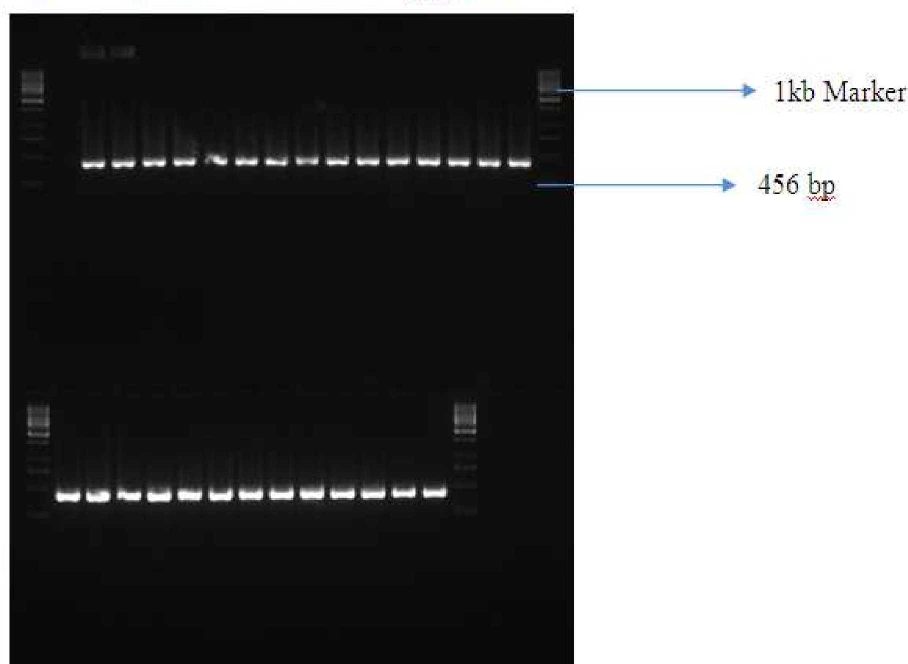


Figure-2: Molecular screening of PVL *lukF/S* genes



Leukotoxic potential of PVL positive isolates- Isolates which showed 50% leucotoxicity in MTT assay are considered as toxigenic strains. Percent leucotoxicity, dilution factor and toxic units of these strains are shown in **Table 5**. Biofilm producing PVL positive strains isolated from chronic skin infections were comparatively high with an average toxic unit of 477.71. Out of 28 PVL positive isolates 13 showed higher toxic units, *icaAB* genes, produced strong biofilms and displayed multi-drug resistance. **Table 6** shows the characteristic features and clinical symptoms associated with these isolates.

Table-5: Leukotoxic potential of biofilm positive isolates.

Site of isolation	No of PVL positive and biofilm producing strains	Mean dilution factor	Mean leukotoxic units
Post operative infections	2	24.9	29.85
Chronic skin infections	8	28.9	477.71
Abscess	3	25.3	39.39
Implant associated infections	4	28.3	315.17
Bone infections	2	24.8	27.85
Otitis media	1	25.8	55.71
Respiratory infections	2	23.9	14.92
Urinary tract infections	2	24.6	24.25

Statistical analysis-To understand the synergistic effect of biofilm production, PVL toxin and multidrug resistance on *Staphylococcal* pathogenesis, we carried out Fischer's exact test using the SPSS software version 14.0. Two-tailed p-value was found to be lesser than 0.05 and thus our results are statistically significant.

Our findings indicate that there is correlation between strong biofilm production (*ica*+), presence of PVL (*lukF/S* +) and high leukotoxic potential of the strains.

Table-6: Characteristics of 13 Strong biofilm producing, PVL +, *icaAB* + and Multidrug resistant strains

Sample no.	Source of isolation	Clinical symptoms	Leukotoxic potential in toxic units
S14	Cellulitis	Severe inflammation with pain	315.17
S18	Supparative lesion	Fever with pain	362.03
S43	Peripheral venous catheter	Early onset Septicaemia	294.06
S47	Peripheral venous catheter	Late onset septicaemia	386.24
S53	Urinary catheter	Burning micturition, fever	274.37
S55	Joint prosthesis	Fever, inflammation and pain in the implanted site	194.01
S56	Post-operative infection	Fever, pain with septicaemia	315.17
S63	Furuncle	Severe pain in the site of infection, fever and fatigue	238.85
S66	Carbuncle	Severe pain in the site of infection and fever	256
S75	Impetigo	Itchy skin, swollen lymph nodes	388.02
S79	Otitis media	Severe pain in the middle ear, increased nasal discharge	227.54
S86	Post plastic surgery infection	Severe pain and inflammation in the operated site	119.42
S93	Bronchiectasis	Dilated bronchi with inflammation and difficult to breath	256

Discussion

Biofilm formation is considered as a major problem in hospitals. Infection caused by biofilm forming *Staphylococci* can prolong the treatment strategies of patients and length of their stay in hospitals. Emergence of multi-drug resistant *Staphylococci* can further complicate the infection leading to increased morbidity and mortality of patients. Recent prevalence report from a two different tertiary care hospital in Mysore has shown that methicillin resistant CoNS are higher in neonates [38] and nearly 79% of health care personnel carry methicillin resistant *Staphylococci* [39]. The occurrence of linezolid resistant isolates is also reported from Mysore [40]. A study from Istanbul, Turkey showed higher biofilm production and PVL production by staphylococcal strains isolated from cystic fibrosis patients [18]. None of these findings have explained the importance of biofilm production, multidrug resistance and PVL genes in the increased pathogenesis of *Staphylococci*. Earlier studies have shown that *Staphylococcal* isolates obtained from device-associated infections often form strong biofilms [2]. But our results shows that biofilm formation can be observed even in surgical site infections, otitis media, bone infections, abscess, respiratory infections and chronic skin infections. Out of 280 samples isolated from different infection sites, 97 showed biofilm production. The percentage of biofilm producing strains was higher in chronic skin infections (97%) when compared to implant associated infections (86%). Our results supports the earlier findings wherein biofilm production need not be always associated with device associated infections, but can be observed even in non-device associated infections [13-20]. The antibiogram of biofilm positive isolates revealed that 78% of the biofilm positive isolates were multi-drug resistant and majority of these isolates were obtained from device associated infections. When we assessed the prevalence of methicillin resistance, it was higher in device associated infections. Our findings confirmed that multi-drug resistant and methicillin resistant strains have higher tendency to develop biofilms on medical

devices [25]. *ica* operon promotes strong biofilm production in *Staphylococci*. Out of 97 isolates only 24 showed the presence of *icaAB* genes. This finding opines that, biofilm production need not be always *ica* dependent. However, the increased strength of biofilm production is mediated by the presence of *ica* operon. PVL is considered as a marker for the severity of *Staphylococcal* infections [27] as well as a marker for CA-MRSA infections [30]. To correlate the significance of PVL production, biofilm production and multi-drug resistance, all the biofilm positive isolates were initially screened for the presence of PVL *lukF/S* genes. Our data revealed that 28.86% of biofilm positive isolates were PVL positive and displayed a varied leukotoxic potential in MTT assay. High leucotoxicity was observed in the isolates obtained from chronic skin infections followed by implant associated infections. These data supports the earlier findings that PVL is very active in chronic skin infections and can prolong the treatment with high leucotoxicity [35]. To understand the significance of biofilm production, multidrug resistance and high leucotoxicity, statistical analysis was carried out using Fischer's exact test. It revealed a strong correlation between PVL production, biofilm and multi-drug resistance. Our overall results suggest that identifying the drug resistance pattern, strength of biofilm production and leukotoxic potential of biofilm forming clinical strains is crucial to give an effective treatment for the patients suffering from biofilm associated infections in hospitals.

Conclusion

Our study represents a three-way diagnostic model for the detection of virulent biofilm forming staphylococcal clinical isolates. We were able to identify strong correlation in PVL toxin production and biofilm formation in *S. aureus* strains. CoNS strains displayed strong biofilm phenotype and they were also identified as multidrug resistant strains. Most of the studies stressed on identifying biofilms in device-associated strains. But here we mainly focused on strains obtained from non-device associated infections. Interestingly, these strains also displayed biofilm production and showed the presence of icaAB genes. Thus our overall study represents a unique tripartite diagnostic model for understanding the virulence factors in clinical staphylococcal strains.

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