

Battling the Methicillin-Resistant *Staphylococcus aureus* Biofilm Challenge with Vancoplus

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Abstract

The present study was conducted with the aim to find the prevalence of biofilm formation ability among methicillin resistant *Staphylococcus aureus* (MRSA) isolates and to assess the activities of commonly used drugs against biofilm producing MRSA. Evaluation of prevalence of genes involved in MRSA biofilm production and their gene expression was also studied.

Of 55 MRSA, 47 isolates were biofilm producers and 8 isolates were non-biofilm producers. Of 47 biofilm producers, 24 (51.0%), 14 (29.8%) and 9 (19.1%) produced strong ($OD_{570} \geq 0.5$), medium ($OD_{570} \geq 0.2$ to <0.5), weak ($OD_{570} 0$ to <0.2) biofilm, respectively. Of the 47 isolates, eight determinants (genes) (*eno*, *hla*, *hlyB*, *clfA*, *fnaA*, *icaA*, *agrII* and *sar*) were found predominantly among 70 to 80% isolates whereas *cna* was observed only in 21.3%, *finbB* in 10.6% and *ebps* in 32% isolates. Amongst the strong biofilm producers (51%), the lowest MIC values obtained with Vancoplus (2-4 $\mu\text{g/ml}$) >linezolid (128 to 256 $\mu\text{g/ml}$) >daptomycin, clindamycin and teicoplanin (256 to 512 $\mu\text{g/ml}$). Biofilms eradication rate was also observed in the same order with Vancoplus (87%) >linezolid (51.8%) >clindamycin (31.9%) >daptomycin (27.5%) >teicoplanin (26.5%). Our results showed that the percentage of *finbA*, *hla*, *eno*, *clfA* and *fib* genes expressions down-regulation after Vancoplus treatment was 64.0 ± 5.9 , 63.8 ± 5.8 , 73.0 ± 7.4 , 72.8 ± 7.8 and $71.9 \pm 7.8\%$, respectively as compared to the control among strong biofilm producing MRSA whereas teicoplanin produced only 30.3 ± 2.7 to $34.5 \pm 3.8\%$ down regulation in *fnaA*, *hla*, *eno*, *clfA* and *fib* genes expression. The other comparator drugs, vancomycin, linezolid and daptomycin, demonstrated variable effects on these genes varying from 4.9 ± 3.9 to $30.3 \pm 2.7\%$.

Our data showed that Vancoplus has significantly enhanced activity against MRSA biofilm producing isolates as compared to other drugs. Therefore, use of this antibiotic should be considered to treat the infections caused by biofilm producing MRSA.

Keywords: Biofilm; Clinical isolate; MIC; MBEC; Vancoplus

Introduction

Staphylococcus aureus is among the 5 most common pathogens that constitutes normal skin and nasal flora in at least 25 to 30% of healthy humans and is associated with large proportions of hospital-acquired and community acquired infections ranging from superficial wound infections to life-threatening deep infections such as septicemia, endocarditis and toxic shock syndrome [1-3]. Phenomenon of *S. aureus* infection is becoming more worrisome with the emergence of antibiotic resistant strain in particular methicillin-resistant *S. aureus* (MRSA) [4]. *S. aureus* has been reported to form biofilms on various surfaces, including medical devices and host tissues [5]. The *S. aureus* with biofilm forming ability is capable of causing a number of diseases, including infective endocarditis, osteomyelitis, foreign body-related infections, catheters associated Urinary Tract Infections (UTIs), and ventilator associated pneumonia and blood stream infections closely related to intravascular devices [5-8].

Biofilms, surface associated sessile bacterial communities, are formed when planktonic cells colonize to a surface, aggregate and grow into multicellular colonies, and embed themselves in an exopolysaccharide [9]. In addition to a large number of cell surface associated proteins, secreted proteins, Polysaccharide Intercellular Adhesin (PIA) and intracellular adhesin A, D, B and C (*icaA*, *icaD*, *icaB* and *icaC*) which are synthesized by products of the intercellular adhesin A, D, B and C (*icaA*, *icaD*, *icaB* and *icaC*) operon are also required for biofilm formation in staphylococci [5-8].

The cell surface associated proteins allow *S. aureus* to interact

with host extracellular ligands, such as elastin binding protein (EbpS), laminin binding protein (Eno), collagen binding protein (Cna), fibronectin binding proteins A and B (FinbA, FinbB), fibrinogen binding protein (Fib), clumping factors A and B (ClfA, ClfB) [7,10-12]. The secreted proteins include tissue degrading enzymes and toxins [13]. Recently, α -toxin (Hla) has been shown to play integral role in biofilm formation [14]. Production of these factors in *S. aureus* which are responsible for adherence, colonization and biofilm formation is controlled by accessory gene regulators including Agr, Sar and others [15]. Based on the agr variations, *S. aureus* strains can be divided into 4 groups menstrual Toxic Shock Syndrome (TSS) strains belong to agr groups I [16], all the strains causing leucocidin induced necrotizing pneumonia belong to agr group III [17], most intermediate level glycopeptide resistance strains belong to agr group II [18] and most efoliatin producing strains belong to agr group II [19].

According to Del P et al. [20] more than 65% of hospital-acquired

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infections are caused by the organisms that have the capacity of producing biofilms. A study that was carried out in China, also reported 66% prevalence of biofilm-forming MRSA. The increasing incidence of biofilm producing MRSA in clinical infections has received increasing interest due to characterization of genes involved in biofilm formation [21,22]. The antibiotics such as methicillin, oxacillin and nafcillin, macrolides, tetracycline and aminoglycosides are being used to treat the infections caused by biofilm producing MRSA but they are getting resistant [23]. Now, glycopeptide antibiotic vancomycin is the remaining effective therapy. However, 76% treatment failure rate with vancomycin has been reported [24]. The emerging resistance towards vancomycin has limited the use of vancomycin and imposed a major concern to the global health community and reinforced the critical need for new methods of control and treatment of biofilm infections. In view of the growing consequences of biofilm producing MRSA and their resistance to commonly used drug, Venus Remedies Limited, India has developed a combination of ceftriaxone and vancomycin named as Vancoplus (US patent no:7960337, Japan patent no: 4918502). The current study was designed to investigate the biofilm formation ability among MRSA isolates and to assess the activities of commonly used drugs against biofilm producing MRSA. Evaluation of prevalence of genes involved in MRSA biofilm production and their gene expression by semi-quantitative PCR would be more advantageous in determining their relation.

Materials and Methods

Antibacterial agents

The following antibiotics were used in this study: a novel antibiotic adjuvant entity ceftriaxone sodium and vancomycin hydrochloride with VRP1020 (Vancoplus), teicoplanin, linezolid, daptomycin and clindamycin. All the drugs were reconstituted in water for injection except Vancoplus which was reconstituted in solvent provided with the pack as per manufacturer's instructions. Working solutions were prepared using Mueller Hinton broth (MHB, Himedia, Mumbai, India), and serial two fold dilutions were made using Cation-Adjusted Mueller-Hinton broth (CAMH, Himedia, Bombay, India) in wells of 96-well plate.

Bacterial isolates collection and their identification

A total of 70 clinical isolates of *S. aureus* were collected from various hospitals of North India including Vijayanagara Institute of Medical Sciences, Bareilly, Uttar Pradesh, India; Sanjay Gandhi Postgraduate Institute, Lucknow, Uttar Pradesh, India and Government Medical College and Hospital, Chandigarh, India. Initial inoculum of these isolates was made on blood agar plates and incubated at 37°C for 24 h. Identification of the *S. aureus* was confirmed by Gram staining, catalase, oxidase, coagulase and growth characteristics on mannitol-salt agar. MRSA isolates were detected by the presence of the *mecA* gene as described earlier [25].

Antimicrobial susceptibility testing

Minimum Inhibitory Concentration (MIC) of each drug was determined by the broth dilution method according to the Clinical and Laboratory Standards Institute [26]. The MICs of the drugs represent the lowest concentration at which bacteria fail to grow.

In vitro biofilm formation assay

Biofilm forming ability of MRSA was investigated according to the method described earlier [27]. Briefly, 250 µl of each MRSA isolate diluted 1:1000 in medium were inoculated in 96 wells polystyrene microtitre plates and incubated for 94 h at 37°C without shaking. After

development of biofilm, non-adherent bacteria cells were removed and washed twice with 200 µl of sterile phosphate buffer saline (PBS; pH 7.0), dried in an inverted position at room temperature under laminar air flow and stained with 300 µl of crystal violet (2%) for 45 min. It is then washed 3 times with distilled water to remove excess stain. For destaining, 300 µl of ethanol: acetic acid (95:5 v/v) was added to each well. Hundred microlitre of this solution was transferred to another 96 wells plate and absorbance was measured at 570 nm using Elisa reader (Merck, USA). As a control, uninoculated medium was used. The mean OD570 value from control wells subtracted from the mean OD570 value of tested wells. The degree of biofilm production was classified in three categories: strong (OD570 ≥ 0.5), medium (OD570 ≥ 0.2 to <0.5) and weak (OD570 0 to <0.2) [28]. Each test was done in triplicate.

Minimum biofilm eradication concentrations (MBECs) determination

MBECs of drugs were determined as described earlier [29]. Briefly, after development of biofilms in wells, the wells were washed with phosphate buffer saline (PBS; pH 7.0) to remove non-adherent bacterial cells and added 200 µl of each antibiotic of 2-fold dilutions (from 2 to 4096 µg/ml) and incubated for 24 h at 37°C. The MBECs values represent the lowest dilution at which bacteria embedded in biofilm failed to regrow. All samples were run in duplicate and one lane served as a positive control and another lane with media served as negative control.

Effect of drugs on biofilm

To study the effect of drugs, after development of biofilms, the biofilms were treated with half concentrations of MBECs of drugs for 12 hrs. After treatment, assessment of drug efficacy was performed by two independent methods: counting of bacterial cells before and after treatment with different antibiotics and crystal violet staining.

For counting of bacteria embedded in biofilm, free floating bacterial cells from the wells after 12 hrs of drug treatment were completely removed and 200 µl of PBS were added to each well to remove remaining biofilm from the wells by ultrasonic disruption for 5 min. The viable counts were determined on MHA plates. The same procedure was used for control plates to calculate the numbers of cells/well.

For staining of biofilm, after drug treatment and incubation, the antibiotics were removed from wells carefully without disturbing the cells in biofilms and stained as mentioned above.

DNA isolation

DNA from selected clinical isolates was isolated by using the method explained below. Five ml of each MRSA overnight grown culture in soybean casein digest medium (SCDM; Hi-Media, Mumbai, India) was centrifuged at 5000 rpm for 5 min at 25°C, washed twice in phosphate buffer saline (pH 7.2) and transferred to a 2 ml micro-centrifuge tube. Then 0.2 ml of the solution containing 25 mM Tris buffer (pH 8), 10 mM EDTA (pH 8) and 50 mM glucose was added. To this, 0.4 ml of solution containing 1% sodium dodecyl sulfate (SDS) and 0.2 N NaOH was added. The tubes were then gently inverted for 2-3 times and allowed to stand for 5 min at 28 ± 2°C. Finally, 0.3 ml of chilled solution of 3 M potassium acetate and 5 M glacial acetic acid were added and allowed to stand on ice for 10 minutes. After centrifugation (14000 rpm, 2 min) pellet was dissolved in 0.5 ml of 0.05 M Tris-EDTA buffer (pH 8.0), incubated at 65°C for 2 min and 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1) was added and shaken thoroughly for 10 min and then centrifuged (14000 rpm, 3 min). The DNA was precipitated by adding 1 ml of ice-cold 70% ethanol to the supernatant. The quality of

DNA was checked on 1.0% agarose gel electrophoresis. DNA purity and concentration were assayed in a spectrophotometer (260/280).

Polymerase Chain Reaction (PCR) assay

PCR assays were carried out to detect the various genes in the biofilm producing MRSA isolates. The primers used in this study are summarized in Table 1. All the respective primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India. For PCR amplifications, about 200 pg of DNA was added to 20 µl mixture containing 0.5 mM of dNTPs, 1.25 µM of each primer and 0.5 µl/unit of Taq polymerase (Bangalore Genei) in 1x PCR buffer. Amplification was performed in an Eppendorf thermal cycler (Germany). The amplified products were separated in 1.0% agarose gel containing 2.5 µl of 10 mg/ml ethidium bromide. The gel was run at 70 volt for 1 h. The gel images were taken under ultraviolet light using gel documentation system (Bio-Rad, USA). A 100 bp ladder (Bangalore Genie) was used to measure the molecular weights of amplified products.

RNA isolation

Total RNA from untreated and treated MRSA strains with various drugs at their half of MIC was extracted using the method described elsewhere [30]. Briefly, two milliliter of overnight grown MRSA strains was centrifuged at 5000 rpm for 5 min at 4°C and pellet was washed with 1 X TE buffer (pH 8.0), suspended in 1 ml of TE buffer containing 0.2% Triton X-100. The suspension was incubated at 100°C for 10 min and thereafter immediately placed on an ice bath. After incubation, an equal volume of chloroform : methanol (2:1) mixture was added, mixed thoroughly and centrifuged at 12000 rpm at 4°C for 10 min. This step was repeated twice. Finally, RNA was precipitated by addition of 2 volumes of pre-chilled 100% ethanol into the supernatant and mixture was incubated at -20°C for 4 hour and then centrifuged at 12000 rpm for 10 min at 4°C. The pellet was air dried for 5 min and re dissolved in 50 µl of Diethyl pyrocarbonate (DEPC) water. The purity of RNA was confirmed by measuring the absorbance at 260/280 ratio which came approximately 1.9 as indicator of pure RNA. RNA was stored at -70°C until use.

cDNA synthesis

Total RNA (2 µg) was then converted to first strand cDNA as

follows: two microgram of RNA was combined with 1 µl of oligodT primer and 9.2 µl of water and then mixture was incubated at 65°C for 5 mins. After incubation, following reagents were added sequentially: 4.0 µl of 5X RT buffer, 1.0 µl of 0.1 M DTT, 0.5 µl of 10 mM dNTP and 0.3 µl of 20 U/µl Moloney Murine Leukemia Virus Reverse Transcriptase (MMLVRT) mixed well and the mixture was subsequently incubated at 37°C for 60 min. The reaction was stopped by heating at 70°C for 10 min. The resultant solution was cDNA which can be used for further study.

RT-PCR analysis

RT-PCR was performed using the cDNA. The primers for the selected genes were the same as mentioned in Table 1. β-actin primer was used as internal control and following sequences were used: β-actin-F-5'-GAAGCATTGCGGTGGACCAT-3' and β-actin-R-5'-TCCTGTGGCATCCACCAA-3'. For PCR amplifications, about 3 µl of cDNA was added to 20 µl mixture containing 0.5 mM of dNTPs, 1.25 µM of each primer and 3.0 U of Taq polymerase (Bangalore Genei) in 1x PCR buffer. Amplification was performed in an Eppendorf thermocycler (Germany) with the same cycling parameters: 2 min at 94°C and 25 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and one cycle for 10 min at 72°C. PCR products were then electrophoresed on a 1.0% agarose gel containing ethidium bromide. After electrophoresis, density of PCR products was measured using image J software.

Effect of drugs on genes *fnbA*, *fib*, *clfA*, *eno* and *hla* expression

To evaluate the effects of drugs on expressions of *fnbA*, *fib*, *clfA*, *eno* and *hla* genes, MRSA strains were treated with all the selected drugs, Vancoplus, clindamycin, linezolid, teicoplanin and daptomycin, at their half of MIC for 24 h. Following treatment, first strand cDNA from RNA was synthesized as mentioned above. First strand cDNA was synthesized as mentioned above. The same strain without drug treatment served as a control. All experiments were carried out in triplicate and representative data are presented.

Results

Screening of MRSA

Out of 70 clinical isolates, 55 (78.6%) isolates were identified to be MRSA as confirmed by amplification of *mecA* gene. The prevalence of

Gene	Nucleotide sequence	Amplicon size (bp)	References
<i>eno</i>	5'-ACGTGCAGCAGCTGACT-3'	302	Tristan et al. (2003)
	5'-CAACAGCATYCTTCAGTACCTTC-3'		
<i>ebpS</i>	5'-CATCCAGAACCAATCGAAGAC-3'	186	Mariana et al., 2009
	5'-CTTAACAGTTACATCATCATGTTTATCTTTG-3'		
<i>fnbA</i>	5'-GTGAAGTTTTAGAAGGTGAAAGATTAG-3'	643	Tristan et al., 2009
	5'-GCTCTTGTAAAGACCATTTTCTTCAC-3'		
<i>fnbB</i>	5'-GTAACAGCTAATGGTGAATTGATACT-3'	524	Tristan et al. (2003)
	5'-CAAGTTCGATAGGAGTACTATGTTTC-3'		
<i>fib</i>	5'-CTACAACACTACAATTGCCGTAACAG-3'	404	Tristan et al. (2003)
	5'-GCTCTTGTAAAGACCATTTTCTTCAC-3'		
<i>clfA</i>	5'-ATTGGCGTGGCTTCAGTGCT-3'	292	Tristan et al. (2003)
	5'-CGTTTCTCCGTAGTTGCATTG-3'		
<i>clfB</i>	5'-ACATCAGTAATAGTAGGGGGCAAC-3'	205	Tristan et al. (2003)
	5'-TTCGCACTGTTTGTGTTTGCAC-3'		
<i>hla</i>	5'-CTGGCCTTCAGCCTTTAAGG-3'	455	Ando et al. (2004)
	5'-CTGTAGCGAAGTCTGGTGAAA-3'		
<i>cna</i>	5'-GTCAAGCAGTTATTAACACCAGAC-3'	423	Tristan et al. (2003)
	5'-AATCAGTAATTGCACCTTTGTCCACTG-3'		

Table 1: Nucleotide sequences and anticipated amplicon sizes for the *S. aureus* gene-specific oligonucleotide primers.

MRSA was maximum in pus (86.2%) followed by urine (75%), blood (73.3%) and wound swab (71.45%).

Biofilm formation assay

Of 55 MRSA, 47 isolates were biofilm producers and 8 isolates were non-biofilm producers. Of 47 biofilm producers, 24 (51.0%), 14 (29.8%) and 9 (19.1%) produced strong (OD570 \geq 0.5), medium (OD570 \geq 0.2 to <0.5), weak (OD570 0 to <0.2) biofilm, respectively. The highest number of strong biofilm producer was recovered from pus 58.3% (14/24) followed by urine 20.8% (5/24), blood 12.5% (3/24) and wound swab 8.3% (2/24). The strong biofilm producers MRSA strains were used for further study.

Analysis of genes encoding cell surface proteins and toxins

Of the 47 isolates, eight determinants (genes) (*eno*, *hla*, *hly*, *clfA*, *fnaA*, *icaA*, *agrII* and *sar*) were found predominantly among 70 to 80% isolates whereas *cna* was observed only in 21.3%, *finbB* in 10.6% and *ebps* in 32% isolates (Figure 1).

Relationship between biofilm formation and virulence determinants

The *icaA* gene was excluded from the evaluation as all the isolates possessed this gene. As shown in Table 2, the mean OD570 value was higher in *eno*, *hla*, *clfA*, *finbA* and *fib* positive isolates compared to *eno*, *hla*, *clfA*, *finbA* and *fib* isolates. The percentage of *hla*, *hly* and *fnaA* was 100% among strong biofilm producers whereas other determinants vary from 87 to 95%. The percentage of *hla* was the highest in medium biofilm producing MRSA (78.6%) while other ranged from 33 to 77%. None of the isolates was positive with *hly*. The percentage of *hly* and *fnaA* in weak biofilm producing MRSA was 100% and 33.3% while none

of the isolates was positive with *hla*. These results suggest that MRSA isolates possessing *hla*, *hly*, *fnaA* and *clfA* genes had greater capacities for biofilm formation than those of lacking these 3 genes (Figure 2).

MIC

As shown in Table 3, Vancoplus emerged as the most effective antibacterial agent with MIC values 2 to 4, 0.5 to 1 and 0.125 to 0.25 μ g/ml in strong, medium and weak biofilm producing isolates, respectively. Second most active agent was linezolid followed by teicoplanin, daptomycin and clindamycin.

MBEC

The MBEC of Vancoplus for strong, medium and weak biofilm producers MRSA was approximately 2X, 3X and 2X of MIC, indicating that 2 to 3 times more Vancoplus is required to kill the bacteria in biofilms than that was required to inhibit planktonic bacterial cells. The MBEC of linezolid varied from 1024 to 2048, 256 to 512 and 64 to 128 μ g/ml for strong, medium and weak biofilm producing MRSA, respectively. The MBECs of daptomycin, teicoplanin and clindamycin were >2048, 1024 to 2048 and 128 to 512 μ g/ml for strong, medium and weak biofilm producing MRSA, respectively which is 3 to 4 times higher than their respective MIC values (Table 4).

Biofilm breaking

Drugs were treated with half MBEC on preformed biofilms of selected strong biofilm producing strains, the data (Table 5) shows that only Vancoplus significantly reduced viable counts of bacterial cells embedded in biofilm of MRSA with log reduction value 5.31. Linezolid was the second most effective drugs with log reduction value 3.47. The other drugs showed only 2.24 to 2.25 logs reduction in bacteria after

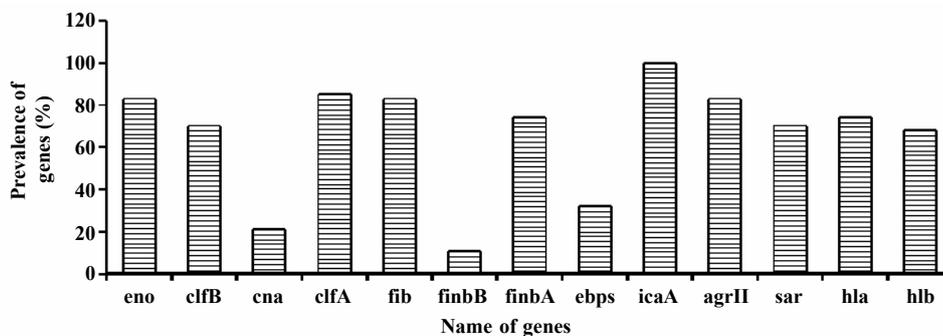


Figure 1: Prevalence of genes producing cell surface proteins and toxins required for biofilm formation among MRSA isolates.

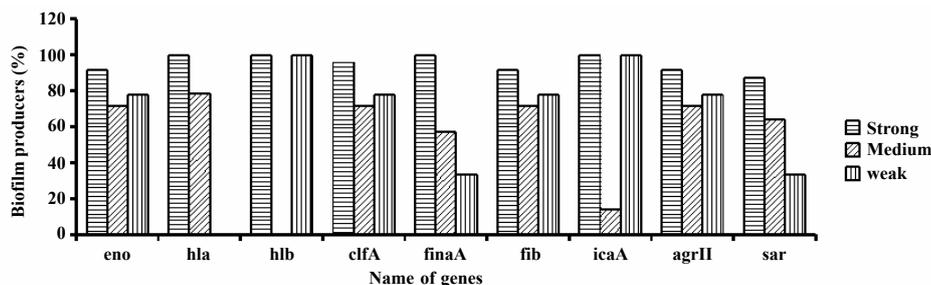


Figure 2: Percentage of *eno*, *hla*, *hly*, *clfA*, *fib*, *finbA*, *icaA*, *agrII* and *sar* positive isolates among MRSA isolates that belong to strong, medium and weak biofilm producer.

Virulence determinants	Total no. of isolates (47)	OD ₅₇₀ (mean ± SD)
eno-positive	39	0.721 ± 0.081
eno-negative	8	0.301 ± 0.089
hla-positive	35	0.465 ± 0.063
hla-negative	2	0.185 ± 0.020
clfA-positive	40	0.694 ± 0.060
clfA-negative	7	0.169 ± 0.043
clfB-positive	33	0.321 ± 0.068
clfB-negative	5	0.258 ± 0.098
finB-positive	35	0.612 ± 0.084
finB-negative	12	0.235 ± 0.069
agrII-positive	39	0.264 ± 0.049
agrII-negative	8	0.231 ± 0.035
sar-positive	33	0.284 ± 0.087
sar-negative	14	0.268 ± 0.065
fib-positive	39	0.635 ± 0.075
fib-negative	8	0.168 ± 0.035

Table 2: Corelation between biofilm forming capacities and cell surface proteins and toxins.

Drug Name	MIC (µg/ml)		
	Strong	Medium	Weak
Daptomycin	256 to 512	64 to 128	16 to 32
Teicoplanin	256 to 512	64 to 128	32 to 64
Linezolid	128 to 256	32 to 64	8 to 16
Vancoplus	2 to 4	0.5 to 1	0.125 to 0.25
Clindamycin	256 to 512	64 to 128	16 to 32

Table 3: Minimum inhibitory concentration of drugs against biofilm producing MRSA.

Drug Name	MIC (µg/ml)		
	Strong	Medium	Weak
Daptomycine	>2048	1024 to 2048	128 to 256
Teicoplanin	>2048	1024 to 2048	128 to 256
Linezolid	1024-2048	256 to 512	64 to 128
Vancoplus	8 to 16	4 to 8	0.5 to 1
Clindamycin	>2048	1024 to 2048	128 to 256

Table 4: Minimum biofilm eradication concentration of drugs against strong, medium and weak biofilm producing MRSA.

treatment. The staining of wells following treatment with half of MBEC of drugs also supports these results. The wells treated with Vancoplus showed 87% eradication of biofilm followed by linezolid (51.8% eradication), clindamycin (31.9% eradication), daptomycin (27.5% eradication) and teicoplanin (26.5% eradication).

Effects of drugs on *finB*, *fib*, *clfA*, *eno* and *hla* gene expression

For gene expression study, we selected those genes which were present predominantly among strong and medium biofilm producers strains. Our results showed that the percentage of *finB*, *hla*, *eno*, *clfA* and *fib* genes expression down-regulation after Vancoplus treatment was 64.0 ± 5.9 , 63.8 ± 5.8 , 73.0 ± 7.4 , 72.8 ± 7.8 and $71.9 \pm 7.8\%$, respectively as compared to the control among strong biofilm producers MRSA whereas teicoplanin produced only 34.5 ± 3.8 , 25.09 ± 2.3 , 25.6 ± 2.5 , 28.9 ± 2.5 and $30.3 \pm 2.7\%$ down regulation in *finB*, *hla*, *eno*, *clfA* and *fib* genes expression. The other comparator drugs, clindamycin, linezolid and daptomycin, demonstrated variable effects on these genes varying from 4.9 ± 3.9 to $30.3 \pm 2.7\%$ (Figure 3). Overall, Vancoplus was found to be significantly down regulating the genes required for biofilm formation hence controlling the biofilm formation.

Discussion

Over the past several decades, the incidence of resistant gram-positive organisms has risen throughout the world. Among these, MRSA are predominant pathogens known to form biofilm on various surface [28] and believed to have an enormous impact on healthcare and are estimated to be associated with 65% of nosocomial infections [31].

In this study, 85.4% MRSA isolates were confirmed to be biofilm producers of which approximately 51% (24/47) were strong biofilm producers, 19.1% and 14.5% of MRSA were weak biofilm and non-biofilm producers, respectively which is in agreement with previous studies [32,33]. *S. aureus* is capable of adhering to a large variety of surfaces which is frequently mediated by protein adhesins of the family MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules). The collagen binding proteins, fibronectin binding proteins, clumping factors and fibrinogen binding proteins belong to this family [7,10]. In the current investigation, we observed that >70% isolates possessed *clfA*, *eno*, *fib*, *hla* and *finB* which were responsible for strong biofilm production whereas the prevalence of other cell surface proteins were less than 40%. Of the studied determinants, *clfA*, *eno*, *fib*, *hla* and *finB* were predominants in those isolates that had significant greater capacities for biofilm formation than *clfA*, *eno*, *fib*, *hla* and *finB* negative isolates.

We also observed that *icaA* gene which is known to be involved in Polysaccharide Intracellular Adhesin (PIA) synthesis of staphylococci plays important role in cell to cell interaction during biofilm formation [5] and is present in all biofilm producers.

As universally reported, planktonic cells were found to be more susceptible than cells embedded in biofilm. This is because, once biofilm is formed, the bacteria undergo phenotypic changes that include increased production of extracellular polysaccharide, decreased metabolic rates and decreased multiplication. The decreased multiplication of bacteria is less susceptible to antibiotics by virtue of their reduced growth rates [34,35].

Vancomycin is a time dependent antibiotic and its clinical efficacy depends on various factors including inoculum size, tissue distribution and protein binding effects [36,37]. Our results revealed that among the tested drugs, Vancoplus (combination of vancomycin plus ceftriaxone along with VRP1020) was found to be more active against planktonic bacteria (MIC 0.125 to 4 µg/ml) as well as bacteria embedded in biofilm (MBECs 0.5 to 16 µg/ml).

The enhanced activity of Vancoplus to planktonic bacteria may be due to synergistic action of ceftriaxone, vancomycin and VRP1020 (a non-antibiotic adjuvant which prevents degradation of antibiotics). Ceftriaxone inhibits bacterial cell wall synthesis by means of binding to the penicillin-binding proteins [38]. The MBECs values for Vancoplus is less compared to previous study where vancomycin MBEC was reported >512 µg/ml against *S. aureus* [39]. This is probably may be due to VRP1020 which enhanced the penetration of antibiotics into the biofilms by breaking the exopolysaccharide structure through removal of Ca²⁺ ions required for biofilm structure [40]. Further, Vancoplus reduced 5.3 logs in bacterial count in bacterial cells embedded in biofilm with 87% biofilm eradication. As in previous studies [41,42], we also observed that teicoplanin and clindamycin are not much effective in bacterial cells embedded in biofilm. Contrary to previous study, our data demonstrated reduced activity of daptomycin and linezolid at high inoculum of bacterial populations [37].

Drug Name	Time (h)	Biofilm concentration (cfu/peg) ^a before treatment (A)	Biofilm concentration (cfu/peg) ^b after treatment (B)	Log reduction (logA-logB)
		MRSA	MRSA	MRSA
Daptomycin	12	4.3x10 ⁸ (8.63) ^a	2.4x10 ⁶ (6.38) ^a	2.25
Teicoplanin	12	6.2x10 ⁸ (8.79) ^a	3.6x10 ⁶ (6.55) ^a	2.24
Linezolid	12	5.3x10 ⁸ (8.72) ^a	1.8x10 ⁵ (5.25) ^a	3.47
Vancoplus	12	6.4x10 ⁸ (8.80) ^a	3.2x10 ³ (3.50) ^a	5.3
Clindamycin	12	4.9x10 ⁸ (8.69) ^a	2.8x10 ⁶ (6.44) ^a	2.25

This study was conducted only on strong biofilm producer strains.

Table 5: Effect of antibacterial agents on biofilm.



Figure 3: Downregulation of genes involved in biofilm formation.

It is interesting that Vancoplus demonstrated activity on these bacteria with MIC 0.125 to 4 µg/ml and MBECs of 0.5 to 16 µg/ml. Since 29% of the Vancomycin is protein bound [43], the calculated maximum concentration of unbound drug in serum is 34 µg/ml [44]. Thus clinical doses of Vancoplus (free drug) will exceed the MBEC and should have activity against bacteria embedded in biofilm. The clinical achievable concentration of other comparator drugs are less compared to their MBECs hence these drugs may not be effective against the bacteria embedded in biofilm [44,45]. The clinical data study supports the use of Vancoplus in the treatment of lower respiratory tract infections, endocarditis, meningitis and bone infections (unpublished data). The animal model data suggest efficacy in the treatment of intra abdominal infection and meningitis [46,47].

Several previous studies have demonstrated the influences of sub-inhibitory concentrations of antimicrobial agents on the expression of various virulence factors which are produced by *S. aureus* and required for biofilm formation [48-50]. In our study, expression of all the selected genes was down regulated when MRSA positive for these genes were treated with different drugs. Hla is important for *S. aureus* biofilm formation and deficiency in Hla caused defects in biofilm formation [51]. Howden et al. [52] reported that on treatment of VISA clinical isolates with vancomycin drug significantly down-regulated the expression of cell surface adhesion molecules and a number of genes involved in pathogenesis and toxin production (spa, finbA, finbB, efb). Furthermore, Schröder et al. [53] reported that on treatment of *S. aureus* isolate with novobiocin antibiotic showed decreased expression of finbA when analyzed with microarray and northern blot hybridization. In accordance with our study, Koszczol et al. [54] demonstrated that enolase (eno) was down-regulated following drug treatment. Clumping factor A (ClfA) was shown to be critical in mediating direct *S. aureus* platelet adhesion and in indirect binding via exogenously added fibrinogen [55]. During the early growth phase, ClfA was identified as the dominant staphylococcal adhesion receptor in both the presence and absence of exogenously added fibrinogen [55]. Numerous studies have demonstrated that treatment of *S. aureus* with

temporin L, ovispirin-1 and dermaseptin K4-S4 peptides caused down-regulation of several virulence factors and their regulators (saeRS and agr) including clfA and clfB. Beenken et al. [3] reported that fib gene showed down-regulation when treated with vancomycin in JH9 strain of *S. aureus*, these results are also in accordance with our study where we reported that fib gene get downregulated when culture of *S. aureus* treated with different antibiotics. These findings strongly suggest that changes in the regulation of transcription of these particular set of virulence determinants may represent an attractive therapeutic targets.

Conclusion

In conclusion, this work demonstrates combining ceftriaxone with vancomycin in presence of VPR1020 significantly reduces the MIC and MBEC values against strong biofilm producing MRSA isolates. Exposure to a sub inhibitory concentration of the Vancoplus significantly down regulated levels of expression of several genes encoding biofilm associated proteins, it was observed that the regulation and expression of certain virulence factors involved in pathogenesis in *S. aureus* are markedly downregulated in the presence of the Vancoplus, which is encouraging. Although Linezolid appeared to be the second best option after Vancoplus, but failed to eradicate biofilm effectively. This work also conclude the maximum biofilm eradication efficiency of Vancoplus and thus Vancoplus could be one of the best choice to eradicate the biofilm caused by these organisms and an effective therapeutic option for the treatment of biofilm producing MRSA strains.

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