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Evaluation of Different Drugs in Down-Regulation of Efflux Pump Genes expression in Methicillin-Resistant *Staphylococcus aureus* Strains

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Abstract: Antimicrobial resistance caused by efflux systems is not widely studied in *Staphylococcus aureus*. In the present study, prevalence of NorA and NorB efflux pumps was studied in thirteen Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains using a pair of primers with Polymerase Chain Reaction (PCR). Antibiotic susceptibility study in NorA and NorB efflux positive isolates was carried out according to the Clinical and Laboratory Standards Institute guidelines. To evaluate the effects of various drugs at half of their MIC on NorA and NorB efflux pump, expression of NorA and NorB genes was measured through semi-quantitative PCR. Our results demonstrated that out of 13 MRSA strains, 8 strains were both NorA and NorB positive and 2 strains were NorA positive. Susceptibility data revealed that Vancoplus appeared to be the most active antibacterial against NorA and NorB efflux pumps positive MRSA strains with MIC values 0.25 to 0.5 μg mL^{-1}. The second most active antibacterial agent was teicoplanin with MIC values 0.5 to 1 μg mL^{-1}. The MICs of vancomycin and daptomycin ranged from 32 to 64 μg mL^{-1}. Treatment of MRSA strains possessing NorA and NorB efflux pumps with Vancoplus caused significant down-regulation of the expression of NorA and NorB genes ($P<0.001$) as compared to the control (without treatment) whereas other drugs such as vancomycin, linezolid, teicoplanin and daptomycin failed to produce any significant changes in the expression of these genes ($P>0.05$). From the above results, it can be concluded that Vancoplus (vancomycin+ceftriaxone+VRP1020) can be one of the best options to treat infections caused by MRSA possessing NorA and NorB efflux pumps.

Keywords: Clinical Isolate, Methicillin-Resistant *Staphylococcus aureus*, Vancoplus

Introduction

Resistance of microorganisms to antibiotics which is associated with several mechanisms such as enzymatic inactivation of the drugs (Sabatini *et al*., 2012), alteration of the drug target (s) (Ruiz *et al*., 2003), reduction of intracellular drug concentrations by changes in membrane permeability (Nikaido, 2003) and over expression of efflux pumps (Nikaido, 2003; Li, 2009) has become a great problem across the world. Nowadays, of these mechanisms, recently efflux pumps have been recognized as a major factor for resistance in both Gram-negative and Gram-positive bacteria (Jadwiga *et al*., 2013; Saiful *et al*., 2008). These are transporter proteins involved in the extrusion of toxic substrates including all classes of antibiotics from within cells to the external environment. Till date, four families of efflux pumps namely, MFS (major facilitator super family), Small Multidrug Resistance (SMR), ABC (ATP-binding cassette) and MATE (multidrug and toxic compound extrusion) have been reported in Gram-positive bacteria (Jarmula *et al*., 2011; Kaatz *et al*., 2005). Among these efflux pumps, MFS types of efflux pumps are predominantly present in Gram-positive bacteria such as NorA, NorB, MdeA, Tet38 (*Staphylococcus aureus*), LmrB, Bmr, Bmr3, Blt (*Bacillus subtilis*), MefA (*Streptococcus pyogenes*), MefE (*Streptococcus pneumoniae*) or CmlR (*Streptococcus coelicor*) (Jarmula *et al*., 2011; Borges-Walmsley *et al*., 2003; Poole, 2005; Markham and Neyfakh, 2001; Sabatini *et al*., 2012).

Methicillin-Resistant *S. aureus* (MRSA) is a major multidrug resistant Gram-positive bacterium that causes various types of Healthcare-Associated Infections (HAIs) resulting in a high death rate (Kallen *et al*., 2010).

Several studies have shown that increased resistance to antibiotics, biocides and dyes has been associated with NorA and NorB mediated efflux pumps (Handzlik *et al*., 2013; Sabatini *et al*., 2012; Truong-Bolduc *et al*., 2005; 2011; 2012; Ding *et al*., 2008). However, antimicrobial resistance associated with NorA and NorB efflux pumps is poorly characterized in *S. aureus* (Handzlik *et al*., 2013). NorA uses proton motive force to energize the transport of antimicrobial compounds across the cell membrane (Neyfakh *et al*., 1993). On the other hand, NorB efflux pump is structurally similar to the efflux pumps, Blt (41%) and Bmr (30%) of *B. subtilis*, as well as to the NorA (30%) and QacA (39%) of *S. aureus*. The wide specificities of multidrug efflux systems suggested their overexpression might result in the efflux of intracellular concentrations of many such agents, causing an impact on their clinical efficacy (Handzlik *et al*., 2013; Truong-Bolduc *et al*., 2011).

Efflux Pumps Inhibitors (EPIs), which are compounds targeting the efflux activity and/or pump components, have been identified as promising therapeutic agents, as they may restore the activity of standard antibiotics (Truong-Bolduc *et al*., 2011). The combination of antibiotics and efflux pump inhibitors such as Ethylene Diaminetetraacetic Acid (EDTA) is expected to increase the intracellular concentration of antibiotics, decrease the intrinsic bacterial resistance to antibiotics, reverse the acquired resistance associated with efflux pumps overexpression and reduce the frequency of the emergence of resistant mutant strains (Chaudhary *et al*., 2012). In the present investigation, prevalence of NorA and NorB efflux pumps among MRSA strains was evaluated and further effects of different drugs on NorA and NorB gene expression were studied.

Materials and Methods

Drugs

The following antibiotics were used in this study: A combination of ceftriaxone sodium and vancomycin hydrocloride with VRP1020 (Vancoplus; Venus Remedies Limited, Chandigarh, India), vancomycin (Vancocin-CP, Astra Zeneca Pharma India Limited, Banglore, India) teicoplanin (Ticocin, Cipla Limited, Mumbai, India), linezolid (Walibur, Novartis India, Limited, Mumbai, India) and daptomycin (Cubicin; Novartis Pharmaceuticals, United Kingdom).

Collection of S. aureus Strains

A total of 27 *Staphylococcus aureus* strains were obtained from Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Raebareli Road, Lucknow, India. Hundred microliter of glycerol stock culture of each of 27 *S. aureus* strains was transferred to 100 mL of autoclaved Soybean Casein Digest Medium (Hi-Media, Mumbai, India) and grown for 14-16 h at 37°C.

Growth of S. aureus Strains and their Identification

Mannitol salt agar (Hi-Media, Mumbai, India) was used as a selective medium for *S. aureus*. All the strains were re-inoculated onto mannitol salt agar plates and then incubated at 37°C for 24-48 h. Mannitol fermenting yellow colored colonies were selected, subcultured and subjected to re-identification based on morphological and biochemical studies (Akbar *et al*., 2013).

Screening for MRSA

A 0.5 McFarland standard suspension of each *S. aureus* strain was prepared in Mueller-Hinton Broth (MHB, Hi-Media, Mumbai, India) and plated on Mueller-Hinton Agar (MHA, Hi-Media, Mumbai, India) plates containing 2% NaCl. An oxacillin (1 µg) disk was placed on the surface of the plates and incubated at 35°C for 24 h. Zone diameter of <10 mm is considered as resistant, >13 mm as susceptible whereas 11-12 mm is considered as intermediate.

DNA Isolation

DNA from all MRSA strains was isolated as following: five milliliter of each overnight grown MRSA strain was centrifuged at 5000 rpm for 4 minutes at 25°C and pellets were washed once in phosphate buffer saline (PBS; 0.05 M; pH 7.2). After addition of 0.2 ml ice-cold solution 1 (25 mM Tris-HCl pH 8.0; 10 mM EDTA pH 8.0 and 50 mM glucose) and 0.4 ml of solution 2 (1% SDS; 0.2 N NaOH), Eppendorf tubes were inverted five times gently and allowed to stand at room temperature for 5 minutes. Subsequently, 0.3 mL ice-cold solution 3 (3 M potassium acetate and 5 M glacial acetic acid) was incorporated into each tube, inverted five times gently and allowed to stand on ice for 10 minutes. After centrifugation (14000 rpm, 2 min), pellets were resuspended in 0. 5 ml of TE (Tris-EDTA, 0.05 M, pH 8.0) and incubated for 5 min at 65°C. Following incubation, 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1) was added to each tube and centrifuged at 14000 rpm for 3 min at 4°C. The supernatant (≤800 µL) containing the DNA was transferred to a clean 2.0 ml tube containing 800 µL of ice-cold ethanol (70% v/v). The DNA solution was mixed with the ethanol by inverting the tubes at least 15 times and centrifuged at 14000 rpm for 2 min. The pellet was air dried for 5 min and re-dissolved in 100 µL of Tris-EDTA buffer. The

electrophoresis was run in 1.0% agarose gel. After electrophoresis at 70 volt for 55 minutes the gel was photographed for analysis of integrity of extracted DNA.

Minimum Inhibitory Concentration (MIC)

MIC of each drug was determined by the agar dilution method according to the (CLSI, 2013) guidelines. MIC was defined as the lowest concentration of a drug that inhibits the visible growth of a microorganism when incubated at 37°C for 18 h.

Identification of NorA *and* NorB *Genes in MRSA Strains*

All the MRSA strains were analyzed for presence of NorA and NorB genes using following primers with PCR. Primers were obtained from Sigma Aldrich Chemicals Private Limited, Banglore, India. The following primer sequences were used for NorA-F-5'-TTCACCAAGCCATCAAAAAG-3'; NorA-R-5'-CTTGCCTTTCTCCAGCAATA-3'; and NorB-F-5'-AGCGCGTTGTCTATCTTTCC-3, 5'-Nor-B-R-5'-GCAGGTGGTCTTGCTGATAA-3'. for PCR amplifications, approximately 100 ng of DNA was added to a 20 µL mixture containing 0.5 mM of dNTPs, 1.25 µM of each primer and 0.5 µL of 3.0 U μL^{1} of Taq polymerase (Banglore Genei). Amplification was performed in an Eppendorf thermocycler (Germany) with cycling parameters, consisting of initial denaturation at 94°C for 5 min followed by 30 cycles (30 seconds of denaturation at 94°C, 30 s of annealing at 45 °C for NorA and 53°C for NorB and 1 min of extension at 72°C) and final extension at 72°C for 1 min. The PCR products were analyzed on 1.0% agarose gel containing ethidium bromide. *Staphylococcus aureus* MTCC 737 was used as NorA-NorB negative strain.

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 (Sung *et al*., 2003). Briefly, two milliliters of overnight grown MRSA strains were centrifuged at 5000 rpm for 5 min at 4°C and pellet was washed with 1 X TE buffer (pH 8.0), suspended in 1ml of TE buffer containing 0.2% Triton X-100. The suspension was incubated at 100°C for 10 min and thereafter immediately placed to 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 to the supernatant and the mixture was kept at -20°C for 4 h 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 DEPC water. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as "pure" for RNA. RNA was stored at -70°C until use.

CDNA Synthesis

Total RNA (2 µg) was converted to first strand CDNA as follows: Two microgram of RNA was added to 1 μL^{-1} of oligodT primer and 9.2 µL of water and the mixture was incubated at 65°C for 5 min. After incubation, following reagents were added sequentially: 4.0 µL of 5X RT buffer, 1.0 µL of 0.1M DTT, 0.5 µL of 10mM dNTPs and 0.3 μL^{-1} of 20 U μL^{-1} 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 gene expression study by PCR.

PCR Analysis

PCR was performed using the CDNA as template. The primers for NorA and NorB genes were the same as mentioned above. 16S rDNA primers were used to amplify the 16S rDNA as internal control and following sequences were used: 16S-F-5'-AACTCTGTTATTAGGGAAGAACA-3' and 16S-R-5'CCACCTTCCTCCGGTTTGTCACC-3'. For PCR amplifications, about 3 µL of CDNA was added to a 20 µL mixture containing 0.5 mM of dNTPs, 1.25 µM of each primer and 3.0 U of Taq polymerase (Banglore Genei) in 1x PCR buffer. Amplification was performed in an Eppendorf thermocycler (Germany) with the cycling parameters mentioned in section 2.7. PCR products were then electrophoresed on a 1.0% agarose gel containing ethidium bromide and intensity of each amplified band was analyzed using the image J software.

Effect of Test and Comparator Drugs on NorA *and* NorB *Gene Expression*

To evaluate the effects of drugs on expression of NorA and NorB genes, NorA and NorB positive MRSA strains were treated with all the selected drugs, vancoplus, vancomycin, linezolid, teicoplanin and daptomycin, at their half of MIC for 24 h. Following treatment, total RNA was isolated as mentioned above. First strand CDNA was then synthesized from 2 µg total RNA using reverse transcriptase (Invitrogen). The same strain without drug treatment served as a control. All experiments were carried out in triplicate and representative data are presented.

Statistical Analysis

Data were analyzed using Graph Pad prism 5.01 and expressed as mean ± Standard Deviation (SD). The continuous variables were tested with one-way Analysis Of Variance (ANOVA) and Dunnet test. Values lower than 0.05 were considered statistically significant.

Results

Characterization of Clinical Isolates

All the isolates were confirmed to be *S. aureus* as all these appeared as yellow colonies with yellow zones on the mannitol salt agar media after 24 h of incubation at 37°C. Gram staining, catalase, oxidase and coagulase tests also confirmed that all the collected strains were *S. aureus*. Further screening of these isolates with methicillin disk confirmed approximately 48.1% (13/27) were MRSA. All the MRSA isolates produced a zone of inhibition <10 mm.

Identification of NorA *and* NorB *Genes in MRSA Strains*

Out of 13 MRSA strains, 8 strains were found to be positive for both NorA and NorB and 2 strains were positive only for NorA gene. All the strains positive for NorA and NorB genes were used for MIC and gene expression study.

MIC

As shown in Table 1, vancoplus appeared to be the most active antibacterial against NorA and NorB efflux pumps positive MRSA with MIC values of 0.25 to 0.5 µg mL^{-1}. The second most active antibacterial agent was teicoplanin with MIC values of 0.5 to 1 µg mL^{-1}. The MIC of vancomycin and daptomycin ranged from 32 to 64 µg mL^{-1}. The MIC of linezolid ranged from 4 to 8 µg mL^{-1}. For gene expression study half of MIC of drugs was used.

Effects of Drugs on NorA *and* NorB *Gene Expression*

Our results revealed that the expression of NorA and NorB in the vancoplus treated group was down regulated approximately 9.3 and 9.9 fold, respectively as compared to the control (P<0.001), whereas other drugs such as vancomycin, linezolid, teicoplanin and daptomycin produced 0.12, 0.36, 0.65 and 2.1 fold down regulation (P>0.05) of NorA gene, respectively and 0.16, 0.32, 0.74 and 2.4 fold down regulation (P>0.05) of *nor B* gene (Fig. 1A and B).

Table 1. Susceptibility study of drugs in NorA-NorB positive and NorA-NorB negative strains

	MIC (µg/mL)	
Drugs	NorA-NorB positive	NorA-NorB negative
Teicoplanin	0.5 to 1	0.13
Linezolid	4 to 8	0.25
Daptomycin	32 to 64	0.50
Vancomycin	32 to 64	1.00
Vancoplus	0.25 to 0.5	0.25

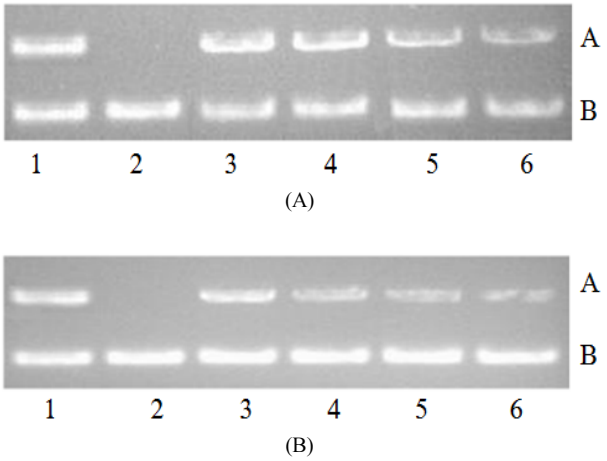

Fig.1. (A) Effect of drugs on NorA gene expression, in group A; 1 = control (without treatment) 2 = treated with Vancoplus; 3 = treated with vancomycin; 4 = treated with linezolid; 5 = treated with teicoplanin; 6 = treated with daptomycin. In group B; 1 to 6, 16S rDNA used as internal control (B) Effect of drugs on NorB gene expression, In group A; 1 = control (without treatment) 2 = treated with Vancoplus; 3 = treated with vancomycin; 4 = treated with linezolid; 5 = treated with teicoplanin; 6 = treated with daptomycin. In group B; 1 to 6, 16S rDNA used as internal control

Discussion

Efflux pumps are largely conserved in bacteria for self-defence and can be a potential target for effective antimicrobial therapy to treat infectious disease caused by multi drug resistant bacteria possessing efflux pumps. A number of methods have been used to identify the efflux systems in bacteria including use of radio labelled substrates, fluorometric assays or the determination of the MIC in the presence of efflux inhibitors (DeMarco *et al*., 2007; Patel *et al*., 2010; Kaatz *et al*., 2000). In our work from 13 clinical isolates only 8 isolates (61.5%) were found to be NorA and NorB efflux pumps positive. A previous work reported 49% prevalence of NorB type efflux pump among *S. aureus* clinical isolates recovered from blood (DeMarco *et al*., 2007). Another study also demonstrated occurrence of NorB, MdeA or MepA type efflux pumps in *S. aureus* (Costa *et al*., 2011).

It has been reported that efflux pumps contribute a varying degree of resistance to antibacterial agents (Costa *et al*., 2013). The efflux activity has been correlated with increased resistance to antibiotics. It has been established that efflux pump inhibition might be associated with disturbance of the regulatory pathway required for efflux pump expression, modification of the chemical structure of the antibiotic inhibiting its attachment to specific substrates, hindrance of assembly of efflux pump components, inhibition of antibiotic by completive or non-completive binding,

closing of the pores responsible for efflux of antimicrobial agents or disturbance of energy essential for pump activity (Poole and Lomovskaya, 2006; Pagés and Amaral, 2009). Our susceptibility data revealed that efflux pump positive isolates showed higher MIC compared to efflux pump deficient strains. Further, our data demonstrated that vancomycin+ceftriaxone+VRP1020 combination displayed a 4 fold reduction in MIC which might be due to synergistic activity of vancomycin+ceftriaxone+VRP1020. It has been suggested that efflux pumps belonging to the MFS, particularly NorA and NorB, utilize the proton motive force to energize the transport of antimicrobial compounds across the cell membrane, via an H+: Drug antiport mechanism (Costa *et al*., 2013; Martins *et al*., 2009).

It is thought that the effectiveness of an efflux pump inhibitor may be different when used with different antibiotics (Chevalier *et al*., 2010) and this may also depend on the level of expression of efflux pumps as well as the relative affinity of the antibiotic binding site for different antibiotics. Recently, the antimicrobial activity of tetracycline was enhanced by the addition of silver to bacterial cells that had been previously resistant to this antimicrobial agent (Morones-Ramirez *et al*., 2013).

Our study, when drugs at half MIC were studied on NorA and NorB gene expression coding NorA and NorB efflux pumps, revealed that vancoplus significantly ($P<0.001$) down regulated expression of both of these genes suggesting increased antibiotic accumulation within the bacteria.

Conclusion

Based on the observations, it is concluded that Vancoplus (vancomycin+ceftriaxone+VRP1020) can be one of the best choices to treat infections caused by MRSA possessing NorA and NorB efflux pumps.

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Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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