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Research Article

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Taming wall teichoic acid multi drug resistance in gram positive pathogens

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ABSTRACT

Wall teichoic acid (WTA) plays important roles in cell growth, division, morphology and as a virulence factor. TagG and TagH are involved in the translocation of the teichoic acids on to the membrane surface. Present study was aimed to evaluate the effect of drugs at their half of MIC on tagG and tagH mRNA expressions through semi quantitative reverse transcription-polymerase chain reaction (RT-PCR). In the current investigation, twenty six methicillin-resistant Staphylococcus aureus (MRSA) were used to identify the occurrence of tagG and tagH genes using the previously reported primers. Susceptibility study in the selected isolates for drugs was carried out with broth dilution method according to the Clinical and Laboratory Standards Institute guidelines. To explore the effect of drugs on expression of tagG and tagH, TagG and TagH positive MRSA strains cultures were treated with different drugs, Vancoplus, ceftriaxone, vancomycin, linezolid, teicoplanin and daptomycin, at half of MIC for 24 h. Our results revealed that all the isolates were found to be TagG and TagH positive. Susceptibility results revealed that Vancoplus was found to be more susceptible against these isolates with MIC values 0.25 to 4 µg/ml. Second most active agent was linezolid with MIC values 1 to 32 µg/ml. The remaining drugs were resistant to these strains with MICs of ceftriaxone, teicoplanin, vancomycin, daptomycin, were in the ranges of 256 to 512 µg/ml. Vancoplus showed respectively 79.2±8.3 and 85.6±8.6% down-regulation in tagG and tagH genes down-regulation when treated with its half of MIC whereas linezolid produced 68.3±7.2 and 73.2±7.9% down-regulation in tagG and tagH genes, respectively. The other comparator drugs produced only 30 to 45 % down-regulation in both of the genes. From the above results, it can be seen that Vancoplus not only found to be more active against these isolates but also significantly down-regulate the expression of tagG and tagH genes which are actively involved in the translocation of teichoic acid and thus can be helpful tool in taming MDR gram positive resistance due to WTA.

Keywords: Clinical isolates, resistance, susceptibility, Vancoplus.

INTRODUCTION

Gram positive pathogens including Methicillin-resistant *Staphylococcus aureus* (MRSA) are responsible for both hospital and community acquired infections causing a wide range of diseases, including endocarditis, osteomyelitis, toxic-shock syndrome, pneumonia, food poisoning, carbuncles [1-5]. A number of *S. aureus* strains including MRSA are resistant to most of the β-lactams, the most effective antibiotics [6]. Mechanistically, beta-lactams acetylate an essential transpeptidation activity which is common to a family of penicillin binding proteins (PBPs), inhibitspeptidoglycan crosslinking and integrity of the cell wall [7].

It has also been reported that some methicillin-resistant *S. aureus* (MRSA) strains have begun to acquire resistance to vancomycin, the drug of last resort [8]. Emerging resistance to commonly using antibiotics is now a concern [9-10]. Thus, The increasing vancomycin resistance in MRSA has led to a clear requirement of a new targets for antimicrobials agents. The WTA (wall teichoic acids) biosynthetic pathway is a possible target since *S. aureus* strains lacking WTAs are unable to colonize host tissue and exhibit a greatly diminished capacity to establish infections in animal models [11-13]. WTA is a Gram-positive-specific anionic glycophosphate cell wall polymer of roughly equal abundance to peptidoglycan (PG). WTA plays important role in cell shape, cell division, biofilm

formation, phage infectivity, and pathogenesis [14-15].
The genes involved in the synthesis of these WTAs are known as tag genes (for teichoic acid glycerol) [16-18]. *S. aureus* WTA is assembled on a bactoprenol carrier embedded in the cytoplasmic membrane by the sequential addition of two sugar residues (by TarO and TarA), two to three glycerol 3-phosphate units (by TarB and TarF), and then finally the poly(ribitol-phosphate) repeat (by TarL) [19]. WTAs are then exported through the membrane by an ABC (ATP binding cassette) transporter complex (TarGH), and the polymer is transferred from the bactoprenol carrier to peptidoglycan by an unidentified transferase [16].

Interestingly, WTA biosynthetic pathway showed a mixed gene dispensability pattern [18]. The first two genes in the pathway, tarO and tarA, are not required for in vitro growth; however, WTA deletion strains are non pathogenic due to defects in host adhesion and dis regulated cell division [19]. Hence, these early steps are proposed targets for anti-virulence factor agents. In contrast, most of the downstream genes cannot be deleted unless flux into the biosynthetic pathway is prevented (e.g., by deleting tarO) [19]. The conditional essentiality of the late acting genes is proposed to result from toxicity of accumulated intermediates and/or depletion of the bactoprenol-phosphate carrier lipid, which is also used for PG biosynthesis [15]. Therefore, the late, essential steps in the WTA biosynthetic pathway have been suggested as novel targets for antibiotics.

Down regulating the expression of *tarG* and *tar H* genes involved in WTA pathway, sensitizes gra positive pathogens including MRSA to β-lactams and other drug like daptomycin. S. aureus WTAs are covalently attached to PG and consist of a poly(ribitol phosphate) [poly(RboP)] backbone containing three tailoring modifications: Dalanylation, α-O-GlcNAcylation, and β-O-GlcNAcylation [13].

Here, we described the impact of a sub-lethal concentration of various drugs on gene expression of *tagG* and *tagH* mainly responsible for transport of teichoic acid on to the membrane surface of antibiotics i.e. Vancoplus, daptomycin, teicoplanin, vancomycin, linezolid and ceftriaxone.

EXPERIMENTAL SECTION

Clinical isolate collection and screening

In the current investigation, twenty six MRSA strains were collected from Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGI), Lucknow, India. These isolates were further confirmed by Gram staining, catalase, oxidase, coagulase, growth characteristics on mannitol-salt agar and presence of mecA gene [20-21]. The prevalence of tagG and *tagH* genes was detected using the previously reported primers.

Antibacterial agents

The following antibiotics were used in this study: a novel antibiotic adjuvant entity of ceftriaxone sodium and vancomycin hydrocloride with VRP1020 (Vancoplus; Venus Remedies Limited, India), teicoplanin (T-planin, Glenmark, India), linezolid (Linospan injection, Cipla Limited, Mumbai, India), daptomycin (cubicin; Novartis Pharmaceuticals, United Kingdom) ceftriaxone (Rocephin, Genentech, USA) and vancomycin (vancocin-CP, Astra Zeneca Pharma India Limited, Banglore, India).

MIC testing

MIC of each drug was determined by the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [22]. 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 hrs.

Screening for *tagG* and *tagH* genes

All of the clinical isolates were processed for screening of *tagG* and *tagH* genes.

Deoxyribonucleic acid (DNA) isolation

DNA from all MRSA strains was isolated as following procedures: five milli liter 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 re suspended 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 into each tube and centrifuged at 14000 rpm for 3 min at 4°C.

Transferred the supernatant (800 µl) containing the DNA to a clean 2.0 ml tube containing 800µl of ice-cold ethanol (70 % v/v). Mixed the DNA solution 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 gel was photographed for analysis of integrity of extracted DNA.

Polymerase chain reaction (PCR)

The *tagG* and *tagH* genes were detected using gene specific primers with PCR. Primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India. Primers used for tagG-5'-F-TTGTGGCTACCAAAGAACCA-3' and tagG-5'-R-TGCAAAGAAAATGGCAACAA-3' that amplify a fragment of about 177 bp and for tagH-5'-F-5'-GAAGATCCTGAAGCGTTTGC-3' and tagH-5'-R-'-CTTGCCCTTTGCATTACCAT-3' that amplify a fragment of about 177 bp.

For PCR amplifications, about 1µl (50-100 ng) of DNA 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 (Banglore Genei) in 10x PCR buffer. Amplification reaction was carried out in 25 µL volume, under the following conditions: initial denaturation at 92°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 30 seconds, followed by final extension at 72°C for 3 min.

Agarose gel electrophoresis

The PCR products were analyzed in 1% (w/v) agarose gel containing 25 mg of ethidium bromide in Tris-EDTA buffer and the gel was photographed under ultraviolet illumination using gel documentation system (Bio-Rad, USA). After electrophoresis, optical density of each amplified band was measured by Image J software.

Effect of drugs on *tagG* and *tagH* genes expression

Of twenty six MRSA isolates, five isolates were used for the genes expression study. The selected isolates were treated with various drugs at their half of MIC.

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 [23]. 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 ^{0}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 ^{0}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 -20^0 C for 4 hours and then centrifuged at 12000 rpm for 10 min at 4^0 C. The pellet was air dried for 5 min and re-dissolved in 50µl 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 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 the mixture was incubated at 65 ^{0}C for 5 mins. 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 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 ^{0}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 *tagG* and *tagH* genes were the same as mentioned above. β-actin was used as a internal control and following sequences were used: β-actin-F-5'-GAAGCATTTGCGGTGGACCAT-3' and β-actin-R-5'-TCCTGTGGCATCCACCAAACT-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 (Banglore Genei) in 1x PCR buffer. Amplification was performed in an Eppendorf thermocycler (Germany) with the cycling parameters of initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 2 minute, followed by final extension at 72°C for 3 min. PCR products were then electrophoresed on a 1.0 % agarose gel containing ethidium bromide and optical density of each amplified band was calculated using image J software.

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 AND DISCUSSION

WTAs not only play an important role in controlling cell division in *S. aureus* and are but also involved in maintaining resistance to β-lactams in MRSA strains [24]. While most of the intracellular WTA biosynthetic steps seem to have been elucidated by now, understanding of the last steps is still in its infancy and represents a challenge for future studies. Our data showed that 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 hours 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 PCR confirmed to be MRSA. Further, our results revealed that all the isolates were found to be TagG and TagH positive. Inhibitors of the essential late stage WTA biosynthetic enzymes TarG through TarH should have lethal effects on bacterial cells, and thus would be a kin to traditional antibiotics. Literature showed that TarG and TarH are highly druggable targets [19,25]. Evidence for a role of TagG and TagH in WTA translocation to the outer leaflet of the cytoplasmic membrane is only indirect [16,26-27].

Table 1. MIC of drugs against TagG and TagH positive isolates

Drugs	MIC (µg/ml)	Half of MIC (µg/ml)
Vancoplus	0.25 to 4	0.125 to 2
Linezolid	1 to 32	0.5 to 16
Teicoplanin	256 to 512	128 to 256
Vancomycin	256 to 512	128 to 256
Daptomycin	256 to 512	128 to 256
Ceftriaxone	256 to 512	128 to 256

Figure 1. Percentage of tagG and tagH genes down-regulation after drugs treatments

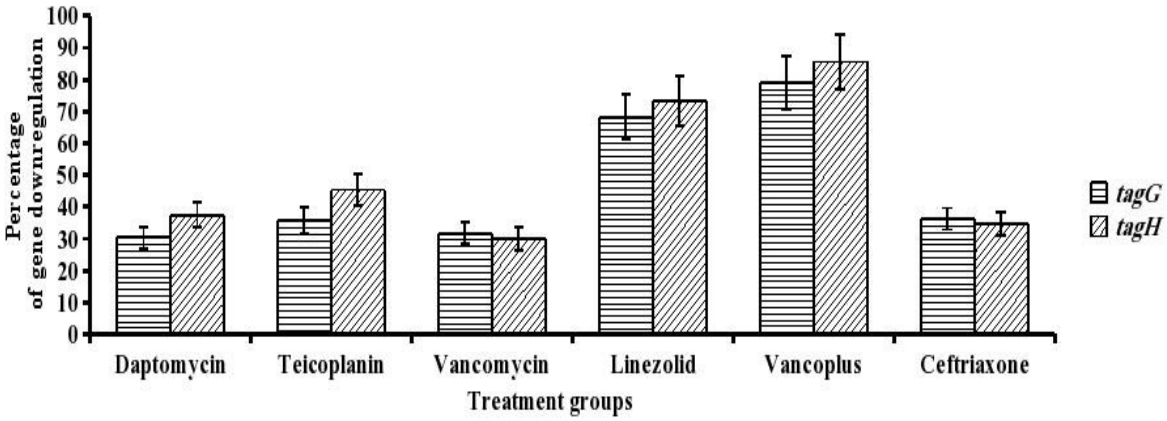
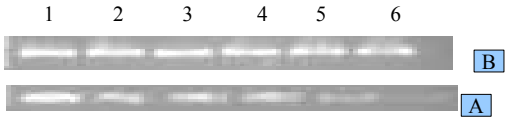


Figure 2A: Effect of drugs on *tagH* gene expression



Agarose gel electrophoresis of amplified tagH gene. In group A; 1= treated with ceftriaxone 2 = treated with daptomycin; 3 = treated with teicoplanin; 4= treated with vancomycin; 5 = treated with linezolid; 6 = treated with Vancoplus. In group B= 1 to 6 β-actin used as internal control.

Figure 2B. Effect of drugs on *tagG* gene expression

Agarose gel electrophoresis of amplified tagH gene. In group A; 1= treated with ceftriaxone 2 = treated with daptomycin; 3 = treated with teicoplanin; 4= treated with vancomycin; 5 = treated with linezolid; 6 = treated with Vancoplus. In group B= 1 to 6 β-actin used as internal control.

Our results showed that Vancoplus (combination of vancomycin plus ceftriaxone along with VRP1020) was found to be more active against MRSA (MIC 0.125 to 4 µg/ml). The enhanced activity of Vancoplus to MRSA may be due to synergistic action of ceftriaxone, vancomycin and VRP1020 (a non antibiotic adjuvant which prevents degradation of antibiotics after reconstitution with solvent provided with pack). Another probable reason for enhanced activity may be due to the penetration of antibiotics into the peptidoglycan layer through removal of ions required for its strength [28]. The MIC of linezolid was ranged 1-32 µg/ml, respectively. The MICs of vancomycin, teicoplanin, ceftriaxone and daptomycin were 256-512 µg/ml (Table 1).

Further we studied the gene expression of *tagG* and *tagH* genes half-MIC drugs. Earlier study has shown that overexpression of *tarG* and *tarH* weaken the antimicrobial activity of 1835F03 against *S. aureus* [29].Our data showed that Vancoplus significantly down-regulate the expression of both of the genes (*tarG* and *tarH) resulting in enhanced susceptibility of it towards MRSA. Vancoplus produced* 79.2±8.3 and 85.6±8.6% down-regulation in *tagG* and *tagH* genes respectively when treated with its half of MIC whereas linezolid produced 68.3±7.2 and 73.2±7.9% down-regulation in tagG and tagH genes, respectively. The other comparator drugs, vancomycin, teicoplanin, ceftriaxone and daptomycin produced almost equal effect ranging 30 to 45 % down-regulation in both of the genes (Figure 1; Figure 2A and B).

It was also previously observed that blocking the expression of WTA by inhibiting TarO sensitizes MRSA strains to b-lactams, even though the beta-lactam-resistant transpeptidase, PBP2A, is still expressed. This suggests a functional connection between ongoing WTA expression and localization of proteins involved in peptidoglycan assembly in *S. aureus* It has been suggested that the identification of several different inhibitors against TarG indicates that this is a drugable target. It is likely that WTA precursor export is a rate-limiting step in the pathway, and that TagG and TagH may also be more accessible to inhibitors than most other WTA targets since they spans the membrane.

CONCLUSION

From the above results, it can be seen that Vancoplus not only found to be more active against these isolates but also significantly down-regulate the expression of *tagG* and *tagH* genes which are actively involved in the translocation of teichoic acid. WTA gene down regulation by Vancoplus makes it suitable antibacterial product for MDR gram positive resistance.

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