GENETIC CHANGES IN LOCAL METHICILIN RESISTANT Staphylococcus aureus LEADING TO HETRO-VANCOMYCIN SUSCEPTIBILITY

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ABSTRACT

Methicillin-resistant Staphylococcus aureus (MRSA), which emerged as a major nosocomial pathogen, is now spreading rapidly worldwide. Moreover, treatment of S. aureus infections is becoming more complicated. The emergence of glycopeptide resistance in S. aureus is considered to be a serious threat around the world, since current treatment of serious infections caused by MRSA relies mainly upon the administration of glycopeptide antibiotics. To understand the mechanism of vancomycin resistance, resulting from multiple mutations, in clinically isolated S. aureus. Complete sequences of multiple genes, including two-component vancomycin histidine kinase sensors (VraS), vancomycin response regulator (VraR), teacoplanin resistant gene (trf AB) were compared with those of their susceptible strain. Further genetic analysis was performed on 9 vancomycin- resistance S. aureus (VRSA) revealed that a single point mutations leading to amino acid substitutions in a gene: vraS, encoding a two-component histidine kinase sensor. The accumulation of the mutation in proteins VraR regulator, correlated with further increases in the glycopeptide MIC. Genetic analysis of 9 VRSA isolates also identified the predominant amino acid substitutions in VraS: 5 isolates revealed mutations in VraS gene, followed by 3 isolates with mutations in VraR gene. Finally the present research provides novel insights into genetic diversity of VraSR mutants among clinical S. aureus isolates with variable susceptibility to vancomycin.

Key words: Vancomycin, Sulaimani, Kurdistan, two-component Y/VraSR.

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INTRODUCTION

Glycopeptide antibiotics are first-line agents for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections, but there is growing concern about the emergence of glycopeptide-resistant isolates. Glycopeptides (vancomycin and teicoplanin) inhibit cell wall synthesis by binding to the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) residues of cell wall precursors and nascent peptidoglycan, which blocks the actions of glycosyltransferases and transpeptidases (1, 2). Four clinical isolates showing variable-level vancomycin resistance (MICs 1-8 µg/ml) have been identified in Sulaimani emergency hospital (3,4). The highly vancomycin-resistant phenotype is based on the acquisition of the exogenous *vanA* complex from *Enterococcus faecalis*. The *vanA*-associated mechanism of resistance in *E. faecalis* involves the production of cell wall precursor molecules with altered C-terminal D-Ala-D-Lac residues which are not recognized by vancomycin. Through the acquisition of the *vanA* gene complex, *S. aureus* is also capable of producing C-terminal D-Ala-D-Lac residues that may inhibit glycopeptide binding (5, 6). Since 1997, clinical isolates with low-level glycopeptide resistance (vancomycin MICs, ≥4 to <16 µg/ml) have been reported and are referred to as glycopeptide-intermediate *S. aureus* (GISA) isolates (7,8,9). The mechanism of resistance observed in GISA isolates is considered endogenous and results from multifactorial mutations that are gradually selected by exposure to glycopeptides (10,11). Several molecular studies have identified the genes involved in GISA resistance (12,13). Some of the genes revealed by transcriptomic analyses, genetic analysis to contribute to glycopeptide resistance (14,15). However, the functional links between these different pathways are still elusive, and no global model of the molecular mechanisms of glycopeptide resistance has been provided.

MATERIALS AND METHODS

**Bacterial strains**

*S. aureus* isolates were obtained from a patient at the Sulaimani emergency hospital. VRSA isolates were identified as *S. aureus* using traditional biochemical tests.

**Susceptibility testing**

The MIC determination was performed by agar dilution methods according to CLSI criteria (9). To detect small changes in susceptibility, linear sets of antibiotic concentrations with 1-mg/liter increments were adopted for the MIC determinations for vancomycin, teicoplanin, and daptomycin. Taking care not to miss a slow-growing resistant cell subpopulation of heterogeneous resistance expression, MIC was evaluated not only at 24 h but also at 48 h of incubation time (10).
Polymerase Chain Reaction (PCR)

The genomes of all the 4 VRSA isolates with variable susceptibility to vancomycin were tested by PCR technology for the presence of mutation in gra SR gene using primers. Oligonucleotide primers for the VraSR sequence were designed from gene sequence data published in NCBI.

VraSF(TCCATTTCTCGTTCTGTAAGC)(1885bp),VraSR(TTGCGACGGGTGAGGTTAGTG)(2202bp),VraRR(GTAAAGCGGTGCATAATACAG)(3645bp),VraRF(TGCAATCATTCATCAGCGTG)(1885bp), TecAF( CGCTAAACCAATCATAAATAC) (2136 bp), TecARGTAAGGCAAGTATTAAGCTCA(2136bp),TecBFTTAGCGAGAGATTA TCAAC(2102) and Tec BR TATGATGATGAAGAAGCGTG (2102bp).

The genes were amplified for 35 cycles 5 min. at 94 Cº for denaturation, 30 sec. at 50, 56, 58, 65 Cº for annealing and 30 sec. at 72 Cº for polymerization. Finally the last cycle continued by 30 sec. at 94 Cº for denaturation, 30 sec. at 50 Cº for annealing and 5 min. at 72 Cº for polymerization.

DNA sequencing

Sequence analysis of the graR and graS, PCR amplified product was performed in (cinaclon ) using automated DNA sequencer. The PCR products were used as template and individual up-or downstream primers were used in each partial sequencing reaction.

Sequence Alignment of the amplified genes

The amplified PCR products were sequenced on both strands. The raw sequences were concatenated and edited by using the EditSeq and MegAlign programs (DNASTAR). The amino acid sequences were deduced from the nucleotide sequences using the same MegAlign program (DNASTAR). Global identification of homologies between nucleotide and amino acid sequences of the VraR, VraS, TecA and Tec B genes and GenBank sequences was done using BLAST 2.0 and PSI- BLAST search programs online at (www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

Four isolates, with variable susceptibilities to vancomycin (MICs= 2-8), were examined for the presence of mutations in the genes coding for vraS, vraR, tcaA and tcaB table(2). Potentially important amino acid substitutions were identified by comparing the obtained amino acid sequence with the same sequence of the of S. aureus N315 and recorded in NCBI protein data base and also with the vancomycin and methicillin resistant isolates. The 4 isolates were classified into 4 patterns according to mutations in amino acid sequences of VraS, VraR, TcaA and TecB protein. Four isolates possessed mutations in VraS, which was the major pattern. The mutation patterns of VraS were diverse among the clinical isolates: 5 distinct changes in VraS (I46M, C160V for isolates 1 and 4, G88D for isolate no 2, V138M, V236A for isolate no 5.
Though rare mutations in VraR (V14I for isolate no 2 and I18T for isolate no 5), and no mutation in Trf A and Trf B were detected. Figures(1,2,3,4,5).

**Table(1): Distribution of mutations identified in 9 clinical VRSA isolates with different susceptibilities to vancomycin**

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>VraS</th>
<th>VraR</th>
<th>TrfA</th>
<th>Trf B</th>
<th>Van. MICs</th>
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<tbody>
<tr>
<td>1</td>
<td>I46M, C160V</td>
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<tr>
<td>2</td>
<td>G88D</td>
<td>V14I</td>
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<td>8</td>
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<td>4</td>
<td>I46M, C160V</td>
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<td>.</td>
<td>.</td>
<td>4</td>
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<tr>
<td>5</td>
<td>V138M, V236A</td>
<td>I18T</td>
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*aLocation of mutations in the S. aureus N315 strain sequence (GenBank accession no. BA000018). Dots, identical amino acids with the intact sequence of S. aureus N315.*

Figure(1): Isolate no 1 and 4 mutation in Vra S (I46M)

Figure(2): Isolate no 2 mutation in Vra S (G88D)
Many researches described the genetic mechanism of vancomycin resistance in VISA and still is not well understood. Several genes have been proposed as being involved in certain clinical VISA strains. mutation in graR caused remarkable cell wall thickening and an increase in the vancomycin MIC to the level seen for VISA strain, while the overexpression of graR in VSSA did not cause that significant effect on the level of vancomycin resistance or cell wall thickening, therefore, the mutated graR seems to confer the VISA phenotype only to cells that are producing cell wall peptidoglycan at an enhanced rate (16, 17, 18). The lack of a universal resistance marker common to all VRSA and VISA strains currently limits understanding the genetic mechanism of vancomycin resistance. Thus, the ability to detect and eliminate the development of vancomycin-resistant strains is severely restricted. The most remarkable finding obtained in the present study was the high prevalence of VraSR mutants among the clinical S. aureus isolates with variable susceptibility to vancomycin. Clearly, the present study was not designed to identify all of the mutations that correlate with increases in vancomycin MIC.
The mutations identified in this study may, however, act as useful diagnostic markers to detect risk factors associated with glycopeptide resistance, particularly vancomycin, in S. aureus. Mutations in several genetic loci other than TCRS, such as sigB (19) is known to contribute to glycopeptide resistance. Yoshihisa et al. 2009(19) showed that the selective pressure of growth at a lethal concentration of imipenem resulted in a single point mutation leading to amino acid substitutions in two proteins: VraS, a two-component histidine kinase sensor; and YvqF, a conserved hypothetical protein. Exposure to teicoplanin resulted in accumulation of mutations in two distinct proteins, a peptide methionine sulfoxide reductase regulator, and TcaA, a teicoplanin-resistance-associated protein—which correlated with a further increase in glycopeptide MIC. The appearance of resistant isolates may, in part, be owing to the continued widespread use of glycopeptide. However, it is important to note that mutations in VraSR occurred frequently at the lethal concentration of imipenem (20) β-Lactam antibiotics are thought to be implicated in the emergence of hetero-VISA, containing VISA cells within its small subpopulation that express a heterogeneous type of resistance to vancomycin (21). Our results imply that the use of β-lactam antibiotics to treat MRSA infections might be one of the risk factors for the emergence of hetero-VRSA. Indeed, this mechanism may sometimes involve mutation in VraSR. Of the 4 clinical isolates with variable vancomycin resistance, mutations in VraS were the most prevalent mutations. Furthermore, mutation patterns in VraSR in patients treated by cell-wall-active antibiotics, other than imipenem, remain to be proven. Further understanding of selective pressures in a clinical setting may help in establishing proper treatment protocols, thereby avoiding the development of antibiotic-resistant strains. Although more remains to be learned concerning the genetic basis of VRSA formation, we believe that our findings will help further understanding of the mechanism of glycopeptide resistance in S. aureus.

REFERENCES


