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Preliminary Analysis of *gra SR* for Vancomycin Resistance in *Staphylococcus aureus* Local Isolates

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Abstract: Methicillin-resistant *Staphylococcus aureus* has become a frequent cause of nosocomial infection. Extensive burn injuries are particularly susceptible to infection. Extended hospitalization and antibiotic therapy have been identified as additional risk factors for MRSA carriage and infection, since prolonged use of antibiotics change the bacterial response to the surrounding medium and increase the mutation rate especially to the targeted site and also to the bacterial signal transduction system leading to the occurrence of the new strain showing various resistant mechanisms to the old as well as new antibiotic such as vancomysin. The aim of this study was to analyze response regulator *graR* gene to determine if the mutation in the signal transduction system lead to the variable susceptibility to vancomycin. Ten VRSA isolated from burned patients in the Burns Unit at sulaimani emergency hospital in 2008. All the isolates were β- lactamase producers. The PCR based identification technique used in this study proved to be rapid and accurate for the detection the genes regulate the vancomysin availability (sensor kinase and response regulator *gra SR*). Comparison of *gra R* DNA sequence and translated nucleotide sequence with N315 DNA and translated nucleotide sequences showed substitution of T_{553} instead of C, C_{554} instead of G, G_{556} instead of T, C_{565} instead of T and G_{568} instead of A in N315 DNA sequence. This nucleotide substitution result in alteration of Gln_{548} instead of Asp and Asn_{717} instead of Ser amino acid in N315. No mutation was detected in *graS* gene.

Key word: Burn, mrsa, two component signal, vrsa

INTRODUCTION

Vancomycin-Intermediate *Staphylococcus aureus* (VISA), first described in 1997, has continuously been a worldwide problem in the treatment of methicillin-Resistant *S. aureus* (MRSA) hospital infection (Hiramatsu *et al*., 2002). VISA has been detected after a patient, initially infected with a vancomycin-susceptible strain and remained unwell despite vancomycin treatment. VISA does not directly emerge from vancomycin-susceptible MRSA. It emerges from hetero-VISA that expresses heterogeneous-type vancomycin resistance (Howden *et al*., 2006). Hetero-VISA spontaneously produces VISA cells within its cell population at a frequency of 10^{-6} or above (Hiramatsu *et al*., 1997). Phenotypically, hVISA/VISA strains have thickened cell walls and reduced autolytic activity (Hiramatsu *et al*., 2002; Sieradzki and Tomasz, 2003) Although the phenotypic changes have been well characterized, the genetic changes leading to the hVISA/VISA phenotype are poorly understood. Microarray data suggest that global regulators are involved in the expression of the hVISA/VISA phenotype, often leading to cell wall thickening (Kuroda *et al*., 2003; McAleese *et al*., 2006; Scherl *et al*., 2006). Attempts to define the mutations causing resistance by sequencing loci such as *vraSR*, *saeSR* and *agr* and *graSR* found to be involved in global regulation (Benjamin *et al*., 2008). A mutation in the response regulator *graR* was linked to a change in vancomycin resistance from hVISA to VISA status, suggesting that the mutation found in *graR* could not fully explain the resistant phenotype and additional mutations are required for resistance (Benjamin *et al*., 2008). This study was conducted to search for the second genetic alteration that promotes hetero-VISA-to-VISA phenotypic conversion.

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 (CLSI, 2008). To detect small changes in susceptibility, linear sets of antibiotic concentrations with 1 mg/L 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 (Hiramatsu *et al*., 2004).

Polymerase Chain Reaction (PCR): The genomes of all the 6 VRSA isolates and 4 VRSA isolates with variable susceptibility to vancomysin were tested by PCR technology for the presence of mutation in *gra SR* gene using primers. Oligonucleotide primers for the gra*SR se*quence were designed from gene sequence data published in NCBI. graS forward: GGA TGA TCA TGA AAT GGT ACG TAT AGG, graS reverse: GTG CAA GAT AGA ACA CAA GCT GTT ATC, gra R forward: TAC ATC TAT ACG ATT ATA TC, graR reverse: ACA TAT GAC TAA CAT CTA TC. The *gra SR* gene was amplified for 35 cycles 5 min. at 94ºC for denaturation, 30 sec. at 50, 56, 58, 65ºC for annealing and 30 sec. at 72ºC for polymerization. Finally the last cycle continued by 30 sec. at 94ºC for denaturation, 30 sec. at 50ºC for annealing and 5 min. at 72ºC 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 *graR* and *graS* genes: To specify the sequence of the *graR* and *graS* 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 *graR* and *graS* 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

The vancomycin MICs of six VRSA isolates was between 32-128 µg/mL. while the vancomycin MICs of other four hetro VISA isolates which considered to demonstrate variable resistance in the same growth condition was between 1-8 µg/mL. The reason for the variable vancomycin MIC may be related to the level of expression of the signal transduction system genes, For this the *graR* and *graS* genes PCR amplified producing 1200 bp (Fig. 1). The four hetero-VISA isolates compared the sequence of Mu50 (defined as VISA) and N315 (defined as MRSA) strain recorded in NCBI. The search also identified several GenBank entries that shared sequence homologies with the *graR* and *graS* genes and protein amino acid sequences of the four hVISA isolates. The result indicated that *gra S* DNA and its deduced amino acid sequences is the similar by 100% to the

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Hv7,8,9,10    GATAGTATACAAAAAGGTGATC-AGACGATTTTCCTGTCCAAAACAGAAATGATTATATT
              |||||||||||||||||||| | | |||||||| || ||||||||||||||||||||||||
Mu50          GATAGTATACAAAAAGGTGA-CGATACGATTTTTCTATCCAAAACAGAAATGATTATATT
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Fig. 1: *gra R* DNA sequence alignment of of isolate hv7, hv8, hv9 and hv10 with the DNA sequence of gra R of *Staphylococcus aureus* Mu50 BA000017.4

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Hv7,8,9,10     LYLSKRFDFFW**EFLI*SFLFWTGKSSDHLFVYYLLIDQRQHLAKSMYVFLQL*THRHD  459
               LYLSKRFDFFW**EFLI*SFLFW  KS  HLFVYYLLIDQRQHLAKSMYVFLQL*THRHD
Mu50           LYLSKRFDFFW**EFLI*SFLFWIEKSYRHLFVYYLLIDQRQHLAKSMYVFLQL*THRHD  732840
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Fig. 2: *gra R* translated nucleotide sequence alignment of of isolate hv7, hv8, hv9 and hv10 with the translated nucleotide of *Staphylococcus aureus* Mu50 BA000017.4 response regulator protein graR

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Hv7, hv8       AAAAGGTGATCAGACGATTTTCCTGTCCAAAACAGAAATGATTATATTAGAAATTCTTAT  603
               |||||||||  | |||||||| || ||||||||||||||||||||||||||||||||||
N315           AAAAGGTGACGATACGATTTTTCTATCCAAAACAGAAATGATTATATTAGAAATTCTTAT  708735
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Fig. 3: *gra* R DNA sequence alignment of of isolate hv7, hv8, hv9 and hv10 with the DNA sequence of gra R of Staphylococcus aureus N315 BA000018.3

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Hv7,8,9,10       KGDQTIFLSKTEMIILEILITKKNQIVSRDTIITALWDDEAFVSDNTLTVNVNRLRKKLS  724
                 KGD TIFLSKTEMIILEILITKKNQIVSRDTIITALWDDEAFVSDNTLTVNV+RLRKKLS
N315             KGDDTIFLSKTEMIILEILITKKNQIVSRDTIITALWDDEAFVSDNTLTVNVSRLRKKLS  733105
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Fig. 4: *gra* R translated nucleotide sequence alignment of of isolate hv7, hv8, hv9 and hv10 with the translated nucleotide of *Staphylococcus aureus* N315 BA000018.3 response regulator protein graR

previous recorded *gra S* DNA and protein amino acid sequences for sensor histidine kinase in multi drug resistant MRSA strain (N315) and VISA strain (Mu50). Comparison of *gra R* DNA and deduced amino acid sequences of isolates hv7, hv8, hv9 and hv10 with DNA sequences of N315 and Mu50 showed different deletion (G_{443}), addtion (T_{553}) and substitution of T_{445} to G, T_{454} to C and A_{457} to G) point mutations in DNA sequence of Mu50 (Fig. 1), resulted in an alteration of Asp_{489} to Arg, Ser_{490} to Tyr, Gly_{493} to Glu and Tyr_{494} to Ile in Mu50 VISA strain (Fig. 2).

Comparison of gra R DNA sequence and translated nucleotide sequence with N315 DNA and translated nucleotide sequences showed substitution of T_{553} instead of C, C_{554} instead of G, G_{556} instead of T, C_{565} instead of T and G $_{568}$ instead of A in N315 DNA sequence (Fig. 3), This nucleotide substitution result in alteration of Gln_{548} instead of Asp and Asn_{717} instead of Ser amino acid in N315 (Fig. 4).

Many researches decribed 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 (Kuroda *et al*., 2003; Maki *et al*., 2004; Jansen *et al*., 2007). The graR mutation replaces Met121 of graR with Val. May cause a conformational change of the GraR protein and result in a disrupted activation of its response regulator function without signal input from the signal transducer GraS. In this study, we identified a mutation in another two-component system, graSR mutation was located in the response regulator graR and its expression in hv7, 8hv, 9hv and 10hv may caused remarkable cell wall thickening and an increase in the vancomycin MIC to the level seen for hVISA strain Mu3 which used as standerd hVISA strain (Hui-min *et al*., 2008). The mutations in gra R may well be explained by the fact that the enzymes of cell wall synthesis, such as PBP2 and the sgtB and murZ products, etc., are more abundant in hVISA isolates than in MRSA isolates due to the activated vraSR system. Therefore, the mutated graR seems to confer the VISA phenotype only to cells that are producing cell wall peptidoglycan at an enhanced rate.

CONCLUSION

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, another different mutation in *graR* also seems to confer the VISA phenotype. Although detailed studies on the physiological function and activation mechanism of graR are necessary, the data presented in this study indicate that there is no mutation graS found in VISA isolates.

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