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Prevalence of Amino Acid Changes in the *yvqF*, *vraSR*, *graSR*, and *tcaRAB* Genes from Vancomycin Intermediate Resistant *Staphylococcus aureus*

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Vancomycin intermediate *Staphylococcus aureus* (VISA) strains are increasingly prevalent in the hospital setting, and are of major concern in the treatment of methicillin-resistant *S. aureus* infections. Multiple mutations in vancomycin-susceptible *S. aureus* (VSSA) strains likely led to the emergence of VISA, and point mutations in the *agr*, *orf1*, *yvqF*, *vraSR*, *graSR*, and *tcaRAB* genes of VISA strains have been shown to contribute to glycopeptide resistance. Therefore, we investigated point mutations in these genes from 87 VISA and 27 VSSA clinical strains isolated from Korean hospitals. All strains were assigned an *agr* type (I, II, or III) on the basis of multiplex PCR, with the majority of VISA strains belonging to *agr* groups I and II. Sequencing revealed amino acid changes in *vraS* from VISA strains which were not present in the VSSA strains. The E59D substitution in the *vraR* gene occurred in 36.3% of VSSA/*agr*I and 92.7% of VISA/*agr*I strains, suggesting that this mutation associated with emergence of VISA/*agr*I strains. VISA strains were classified into 31 mutation patterns according to mutations in the *yvqF*, *vraSR*, *graSR*, and *tcaRAB* genes. In addition, the mutation patterns were correlated with *agr* and sequence type (ST). The most prevalent pattern included *agr* type I (ST 72) strains with E59D (*vraR*), L26F and T224I (*graS*), D148Q (*graR*), and L218P, R283H and G312D (*tcaA*) amino acid substitutions. The minimum inhibitory concentration (MIC) range of mutation pattern 5 toward oxacillin and imipenem was much lower than that of patterns 6 and 24. These results improve our understanding of emergence of VISA strains.

***Keywords*:** amino acid substitution, point mutation, vancomycin intermediate *S. aureus*, multiplex PCR

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the major cause of nosocomial infections, and virulent MRSA strains are spreading rapidly through the community (Yamakawa *et al.*, 2012). Vancomycin is the main therapeutic agent for treatment of multiresistant MRSA strains (Deresinski *et al.*, 2005; Moellering *et al.*, 2005), but excessive use of vancomycin has led to the emergence of vancomycin-resistant strains. High-level vancomycin-resistance, encoded by the *vanA* operon transmitted from *Enterococci*, is extremely rare. In contrast to VRSA, the occurrence of vancomycin intermediate *S. aureus* (VISA) is being increasingly reported worldwide, and has become a problem in the treatment of MRSA infection since it was first described in 1997 (Hiramatsu *et al.*, 1997; Hiramatsu *et al.*, 2002; Tenover *et al.*, 2007). The underlying mechanisms of molecular resistance in these clinically important VISA strains remain to be fully elucidated. Usually, VISA is considered to have emerged from vancomycin susceptible *S. aureus* (VSSA) through various genetic changes. Recently, point mutations and amino acid changes in vancomycin intermediate resistance-related genes, including the teicoplanin resistance genes *yycF*, *yycG*, *tcaA*, and *ccpA*, were confirmed to contribute to glycopeptide (vancomycin, teicoplanin) resistance (Cui *et al.*, 2005; Seidl *et al.*, 2006; Jansen *et al.*, 2007; McCallum *et al.*, 2007). A point mutation causing defective accessory gene regulator (*agr*) function also influenced vancomycin resistance (Sakoulas *et al.*, 2002; Neoh *et al.*, 2008; Cui *et al.*, 2009). In recent years, genetic alterations in two-component regulatory system (TCRS) genes have been reported to be strongly related to the glycopeptide-resistance phenotype; these include point mutations in the *vraSR* and *graSR* TCRS genes (Kuroda *et al.*, 2003; Meehl *et al.*, 2007; Neoh *et al.*, 2008). Mutations in several genetic loci other than TCRS, such as *sigB* and *trfAB*, also contribute to glycopeptide resistance (Singh *et al.*, 2003; Maki *et al.*, 2004; Renzoni *et al.*, 2009). A previous study demonstrated that single base substitutions could be associated with the evolution of VISA from VSSA strains during persistent infection associated with vancomycin treatment failure (Kato *et al.*, 2010). In Korea, the first VISA strains were identified in 2000, and further VISA strains have been confirmed through the nationwide laboratory surveillance program for VISA/VRSA (Chung *et al.*, 2010). However, as yet, no studies on the point mutations and amino acid changes between clinical VSSA and VISA strains have been conducted. In this study, we sequenced the *orf1*, *yvqF*, *vraSR*, *graSR*, and *tcaRAB* genes from VISA and VSSA strains collected from hospitals to investigate the prevalence of amino acid changes, and to characterize mutation patterns of VISA strains.

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Table 1. Primers used in this study

Gene and primers	Sequence (5′→3′)	Reference
vraSR operon		
SA1700-3-F	TGCAATCATTCATCAGCGTAG	
SA1700-3-R	GTAAAGCGGTGCATAATACAG	
graSR		
SA0615-F	GATGAGTATGGAACTTGGCG	
SA0615-R	AAAATTGCCACTTTAACACTCC	Kato *et al.* (2010)
tcaRAB region		
SA2147-F	ACTTCGGGCAAGATTTCATAC	
SA2147-R	AGCTAACCACATAGACAAACC	
SA2416-F	CGCTAAACCAATCATAAACATC	
SA2416-R	GTAAGGCAAGTATTAGAAGTCA	
SA2415-F	TTAGCGAGAGATTACTATCAAC	
SA2415-R	TATGATGATGTAAAGAAGCGTG	

Materials and Methods

Bacterial strains

Eighty-seven VISA strains were collected from Korean hospitals participating in a nationwide laboratory surveillance program for VISA/VRSA, from 2000–2011. VISA was confirmed by agar dilution, broth dilution and E-test methods, as described below. Twenty-seven VSSA strains were also collected to compare point mutations and amino acid changes with VISA strains.

Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) for vancomycin was determined by the standardized agar dilution and broth dilution methods, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007). In brief, for the agar dilution method, bacterial suspensions were prepared from overnight cultures on blood agar and their turbidity was adjusted to be equivalent to that of a 0.5 McFarland standard (10^8 CFU/ml). This suspension was inoculated onto Mueller-Hinton agar containing serial dilutions of vancomycin (0.25–64 μg/ml). In addition, the vancomycin concentration in the broth dilution method was tested over the range of 0.25 to 64 μg/ml. The E-test (AB Biodisk, Sweden) was also performed with vancomycin, teicoplanin, daptomycin, imipenem, oxacillin, and rifampicin according to the manufacturer's instructions. For determination of the MIC for vancomycin, a bacterial suspension of a 0.5 McFarland standard inoculum in sterile water was spread on a Mueller-Hinton agar plate and the plates were incubated at 35°C for 24 h. The strains were classified as susceptible or resistant to vancomycin according to the breakpoints published by the CLSI.

Accessory gene regulator (*agr*)-type determination

To determine the *agr*-type of each strain, multiplex PCR was performed as described previously (Gilot *et al.*, 2002). Primers were designed from the reference sequences *agr*-1 to *agr*-4 (GenBank accession nos. *X52543*, *AF001782*, *AF001783*, and *AF288215*, respectively) to amplify specific *agr*-alleles. PCR products were analyzed by electrophoresis on 1% agarose gels.

Multilocus sequencing typing (MLST)

MLST was performed on VISA strains as previously described (Enright *et al.*, 2000). Seven housekeeping genes were sequenced to determine the allelic profile. To assign a sequence type (ST), the sequences obtained were compared with the sequences described on the MLST website (http: www.mlst.net/).

Nucleotide sequencing and mutation detection

To identify point mutations and amino acid changes in VISA and VSSA strains, the complete *orf1*, *vraSR* operon, *graSR*, *yvqF*, and *tcaRAB* gene sequences were analyzed. Genomic DNA was extracted from VSSA and VISA strains using a DNeasy tissue kit (Qiagen, USA) according to the manufacturer's instructions. PCR amplification from genomic DNA was performed with Taq DNA polymerase (TaKaRa Shuzo Co., Japan) using the primers listed in Table 1, according to the manufacturer's instructions. The cycling reactions were performed under the following conditions: 94°C for 5 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, followed by a final step at 72°C for 7 min.

PCR products were purified and sequences were analyzed

Table 2. Distribution of amino acid changes by point mutation in 87 VISA strains

Gene	Number of strains	Amino acid changes	agr type
yvqF	4	E156G, L86I, Q136H	II
	4	E156G	III
vraS	2	F243S, I317T, G88D	I
	5	F321L, K272I, L315M, L123H, S167N	II
vraR	38	E59D	I
	7	S164P	II
	22	A113V	II
graS	41	T224I, L26F	I
	5	N332K, N289Y, A153P, M29R, V301E, V304E, R14L	II
graR	27	D148Q	I
	4	F151L	III
tcaA	40	L218P, R283H, G312D	I
	5	N371I, T279I	II
	4	L218P, G312D, M202T	III
tcaB	10	I232L	I
	1	A91P	II
	1	W308G	III

Table 3. Distribution of amino acid changes by point mutation in 27 VSSA strains

Gene	Number of strains	Amino acid changes	*agr* type
yvqF	2	M109L, H95Q	I
	2	Q136H	II
vraR	4	E59D	I
	1	S26R	I
	1	F85L, I86L, E87K, R117H, R121S	II
	7	S164P	II
	5	A113V	II
graS	9	L26F, T224I, I59L, S303R, R325K, V676I	I
	1	N332K	II
	5	T224I	III
graR	7	S197G, D147E, D148Q, M90N, V135I, V136I	I
tcaA	11	L218P, Y237H, Y262S, R283H, G312D, I431V, T262S, K2E, N133I, M202T	I
	3	M202T, L218P, G312D	III
tcaB	5	S341N, V145F, H6Y, K396R, F207L, V360I	I
	1	H6Y, K396R	III

by the Macrogen Service Centre (Macrogen, Korea). The analyzed sequences were assembled using Lasergene version 5.0 (DNAStar, USA), with reference to the vancomycin susceptible *S. aureus* N315 genome sequence (GenBank accession no. *BA000018*). Nucleotide and amino acid sequence comparisons were performed using the NCBI BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

Results

agr types

agr-type was determined for each using a specific multiplex PCR. Forty-one (47.1%) of the VISA strains (87 strains) belonged to *agr* group I, 42 strains (48.3%) to *agr* group II and four strains (4.6%) to *agr* group III. Most of the VISA strains were assigned to two (I and II) major *agr* groups. The VSSA strains were assigned to *agr* group I (11 strains, 40.7%), *agr* group II (12 strains, 44.5%), and *agr* group III (four strains, 14.8%).

MLST

The 87 VISA strains were classified into 4 ST types. Forty-four (50.5%) strains showed ST type 5 and 27 (31%) strains showed ST 239. Furthermore, 12 (13.8%) strains were assigned to ST 72 and 4 (4.6%) strains ST1.

Point mutation and amino acid changes

DNA sequencing revealed point mutations corresponding to amino acid changes in *vraSR*, *yvqF*, *graSR*, and *tcaAB* (Table 2). Point mutations in *orf1* were also identified, but did not cause amino acid changes. Point mutations in *yvqF* caused the amino acid changes E156G, L86I, and Q136H, but only four VISA strains showed these mutations.

Eight distinct amino acid changes (F243S, I317T, G88D, F321L, K272I, L315M, L123H, and S167N) were observed in *vraS* from VISA strains, but no amino acid changes were found in VSSA strains. In *vraR*, three amino acid changes were observed in VISA strains, according to the *agr* group. E59D in *vraR* appeared only in the *agr*-type I group, while substitutions such as A113V or S164P were observed only in VISA *agr*-type II strains. Finally, no amino acid changes were found in *agr*-type III strains.

The point mutations in *graS* also differed according to the *agr*-type. All *agr*-type I VISA strains (41 strains) had L26F or T224I, but only five strains belong to the *agr*-type II strains showed amino acid changes in the *graS* gene (one or three amino acid changes). Moreover, the positions of these substitutions also differed from those in *agr*-type I strains.

In *graR*, only D148Q (*agr*-type I) or F151L (*agr*-type III) amino acid changes were found in VISA strains. However, various amino acid changes (from one to three amino acid changes), including the D148Q mutation, were found in VSSA strains, but the F151L mutation was not found in

Table 4. Major mutation patterns of amino acid changes in VISA strains

Pattern	Number of strains	Mutation[a]							*agr*	ST
		yvqF	*vraS*	*vraR*	*graS*	*graR*	*tcaA*	*tcaB*		
5	9	.	.	E59D	Sub[c]	.	Sub[d,e,f]	I232L	I	72
6	23	.	.	E59D	Sub[b,c]	D148Q	Sub[d,e,f]	.	I	239
19	2	.	.	S164P	.	.	.	.	II	5
24	18	.	.	A113V	.	.	.	.	II	5
29	3	E156G	.	.	Sub[c]	F151L	Sub[d,f,g]	.	III	1

[a] Location of mutations in the *S. aureus* N315 strain sequence (GenBank accession no. BA000018).
Dots indicate amino acids identical to the sequence of *S. aureus* N315.
[b] substitution in *graS* (L26F); [c] substitution in *graS* (T224I); [d] substitution in *tcaA* (L218P);
[e] substitution in *tcaA* (R283H); [f] substitution in *tcaA* (G312D); [g] substitution in *tcaA* (M202T).

Table 5. MICs of the three main mutation patterns out of 31 patterns (including no mutation pattern) identified in VISA

Strains	MIC (μg/ml)					
	Vancomycin	Teicoplanin	Daptomycin	Oxacillin	Imipenem	Rifampin
pattern 5						
v215	3	6	1	8	0.125	0.016
v231	4	12	3	6	0.125	0.008
v803	4	8	0.19	≥256	2	0.008
v804	4	4	0.75	192	1	0.012
v805	4	4	0.75	32	0.5	0.008
v809	4	16	1.5	4	0.25	0.012
v813	4	8	0.75	192	8	0.012
v815	4	4	0.38	64	0.5	0.012
v920	4	8	0.25	≥256	0.25	0.25
pattern 6						
v015	4	4	1	≥256	6	0.012
v018	4	4	1.5	≥256	2	0.004
v019	4	4	1.5	≥256	≥32	0.008
v023	4	8	0.75	≥256	≥32	1.5
v067	3	4	0.75	≥256	≥32	2
v209	3	6	1.5	≥256	≥32	0.008
v320	3	3	1.5	≥256	≥32	0.016
v331	4	4	0.75	≥256	≥32	32
v333	4	4	0.75	≥256	≥32	32
v414	4	6	0.5	≥256	≥32	≥32
v507	3	4	0.5	≥256	≥32	0.012
v517	4	8	1.5	≥256	≥32	0.008
v519	4	8	1	≥256	≥32	0.006
v520	4	8	1.5	≥256	≥32	0.006
v521	4	8	1	≥256	≥32	0.006
v522	4	8	1.5	≥256	≥32	0.008
v605	4	8	2	≥256	≥32	0.008
v705	8	4	0.75	≥256	1	0.012
v807	4	4	0.75	≥256	≥32	0.004
v808	4	4	0.5	≥256	8	0.006
v814	4	8	0.75	≥256	≥32	≥32
v945	4	8	0.75	≥256	≥32	0.25
v946	3	4	1.5	≥256	≥32	0.75
pattern 24						
v001	3	6	1	≥256	≥32	≥32
v020	3	6	0.75	≥256	≥32	0.016
v027	4	8	1	≥256	≥32	0.006
v202	4	4	0.75	≥256	≥32	0.008
v259	4	8	1	≥256	≥32	0.004
v412	3	6	0.38	≥256	≥32	0.006
v415	3	32	0.5	≥256	≥32	0.008
v701	4	4	1	≥256	≥32	0.006
v702	4	8	0.38	≥256	≥32	≥32
v703	4	8	0.38	≥256	≥32	≥32
v704	4	8	0.38	≥256	≥32	≥32
v706	4	2	0.19	≥256	≥32	0.004
v806	4	12	0.75	≥256	4	0.008
v810	4	12	1	≥256	≥32	0.006
v948	4	8	0.38	≥256	≥32	0.5
v949	4	4	0.25	≥256	≥32	0.38
v951	4	8	0.125	≥256	≥32	0.5
v955	4	8	1	≥256	≥32	0.38

VSSA strains (Table 3). No amino acid changes in *graR* were detected in *agr*-type II VISA strains.

In the *tcaRAB* gene cluster, no mutations were detected in *tcaR*. Nine strains had the I232L (*agr* type I) mutation in *tcaB*, while the A91P (*agr* type II) and W308G (*agr* type III) changes were detected in one VISA strain each. The combination of amino acid changes in *tcaA* differed according to the *agr* type of VISA or VSSA strains. In VISA strains, all *agr* type I strains, except one, had the L218P, R283H, and G312D changes, but among the *agr* type II strains (43 strains), just four strains had N371I changes. All of the *agr* type III strains (four strains) had the M202T, L218P, and G312D mutations. In VSSA strains, there was no amino acid change in *agr* type II strains, and L218P, Y237H, Y262S, R283H, and G312D were prevalent in *agr* type I strains.

Mutation patterns with antimicrobial resistance

The 87 VISA strains were classified into 31 mutation patterns, including a no mutation pattern according to mutations in the *yvqF*, *vraSR*, *graSR*, and *tcaRAB* genes. Among the mutation patterns, 50 strains (58%) were assigned to 1 of 3 major mutation patterns (i.e., mutation patterns 5, 6, and 24) (Table 5), whereas 7 VISA strains (8%) showed no amino acid changes in any of the sequenced genes. The most prevalent mutation pattern was pattern 6, which included 23 VISA strains. Pattern 6 strains were *agr* type I, ST 239 type and had E59D (*vraR*), L26F and T224I (*graS*), D148Q (*graR*), and L218P, R283H and G312D (*tcaA*) amino acid changes concurrently. Mutation pattern 24 (18 strains) was the next most prevalent mutation pattern, and had only the A113V mutation in *vraR* and strains of this mutation patterns showed *agr* II, ST 5 type. The VISA strains with mutation pattern 5 (*agr* I, ST 72 type) had E59D (*vraR*), T224I (*graS*), L218P, R283H, G312D (*tcaA*), and I232L (*tcaB*) amino acid changes concurrently. All strains containing mutation pattern 6 were susceptible to teicoplanin, with an MIC range from 4–8 μg/ml (Table 5). The strains with pattern 24 mutations had MIC values for daptomycin ranging from 0.125–1 μg/ml. The MIC range of strains belonging to mutation pattern 5 was 4–192 μg/ml to oxacillin (except 2 strains) and 0.125–8 μg/ml to imipenem. However, the MIC of major patterns 6 and 24 was ≥ 256 μg/ml to oxacillin and 1- ≥32 μg/ml, respectively. In particular, specially, 87% of strains of mutation patterns 6 and 24 showed an MIC of ≥ 32 μg/ml to imipenem.

Discussion

In this study, we compared point mutations and amino acid changes in the *orf1*, *vraSR*, *graSR*, *yvqF*, and *tcaRAB* genes between VISA and VSSA strains. The nucleotide substitutions in *vraS* and *graS* have previously been reported to be associated with the VISA phenotype (Kuroda *et al.*, 2003). Furthermore, an I5N substitution in *vraS* gene from a VISA Mu50 strain was also identified (Howden *et al.*, 2010), however no substitutions in the corresponding position (I5N) were identified in VISA strains in the current study. And the mutations in *yvqF* and *vraS* were mutually exclusive, so the presence of both mutations is, in fact, disadvantageous to the cell (Kato *et al.*, 2010). However, in this study, the simultaneous occurrence of mutations in *yvqF* (Q136H) and *vraS* (L123H) was observed, which indicates further functional studies of these mutations are required. The *agr* type I VISA strains had fewer amino acid changes in *graS*, *graR*, *tcaA*, and *tcaB* than VSSA strains. Substitutions in *vraR* (E59D, A113V, and S164P) seen in VISA strains were also observed in VSSA strains. However, the proportion of amino acid changes involving E59D was 44.4% in VSSA and 92.7% in VISA strains. The proportion of other substitutions in VSSA strains, specifically A113 and S164P, was 38.5% and 61.5%, respectively; however in VISA strains, these amino acid changes were present in 52.4% and 16.7% of strains, respectively. Although the number of VSSA strains was small compared with the number of VISA strains, the prevalence of mutations in *vraR* (E59D and A113V) of VISA strains was higher than that of VSSA strains. These results suggest that changes in the proportion in *vraR* substitutions may be accompanied by the evolution of the VISA strain from the VSSA strain through continuous exposure to vancomycin and genetic alteration.

Among the mutation patterns classified, most of the *agr* type I strains were classified into the mutation patterns 5 and 6, whereas most of the *agr* type II strains were included in mutation pattern 24. The ST types were also classified according to the mutation patterns. ST 72 strains showed mutation pattern 5, ST 239 strains pattern 6, and ST 5 patterns 19 and 24. With regard to strains of *agr* type III, ST 1 strains showed mutation pattern 29. These results strongly suggest that the point mutation patterns of VISA strains are associated with the *agr* and ST types. Strains with mutation pattern 5 had MIC range of 4–192 μg/ml to oxacillin. Among the VISA strains (87 strains), only 13 did not have an MIC ≥ 256 μg/ml against oxacillin. Of these 7 strains showed mutation pattern 5, and an imipenem MIC, ranging from 0.125–8 μg/ml. This is in contrast to most of the VISA strains (84%), which had MICs ≥ 32 μg/ml against imipenem. Most *agr* type I VISA strains had substitutions in *graS*, *graR*, and *tcaA*, but most *agr* type II VISA strains had either only mutations in *vraR* or no amino acid change.

D148Q in *graR* has been reported as a missense mutation, and a mutated *graR* impairs oxacillin-resistance (Neoh *et al.*, 2008). However, in this study, except for 2 strains with an MIC ≥ 256 μg/ml, all strains exhibiting mutation pattern 5, showed an MIC of 4–192 μg/ml for oxacillin. In addition, they had no mutation in *graR*, and just 1 substitution in *graS* (T224I), *tcaB* (I232L), and *vraR* (E59D), respectively. This finding indicates that this mutation combination correlates with low oxacillin and imipenem resistance. Further functional studies for these mutants are needed.

In conclusion, we observed various amino acid changes in the *vraSR*, *graSR*, and *tcaRAB* genes of VISA strains, which were mostly different from those seen in VSSA strains, especially in *vraS* and *tcaB*. In addition, high proportion of the E59D substitution in the *vraR* gene was characteristic in VISA/*agr*I strains. Moreover, the mutation patterns correlated with the *agr* and ST types. The *agr* type I group showed more substitutions than *agr* type II VISA strains in *graRS* and *tcaAB*. Mutation pattern 5 strains had MIC of 4–192 μg/ml to oxacillin (except two strains) and MIC of 0.125–8

μg/ml to imipenem, which was different from that of other VISA mutation pattern groups. Further studies are needed to elucidate the molecular effects of these amino acid changes, and, in particular, the relationship between the *agr*, and ST types of VISA strains and the prevalence of amino acid substitutions.

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Conflicts of Interests

None to disclose.

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