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Nationwide Surveillance Study of Vancomycin-Intermediate *Staphylococcus aureus* Strains in Korean Hospitals from 2001 to 2006

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We investigated the prevalence and the molecular characteristics of vancomycin-intermediate *Staphylococcus aureus* (VISA) among methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from clinical samples at tertiary or general hospitals participating in a nationwide surveillance program for VISA and vancomycin-resistant *Staphylococcus aureus* (VRSA) in Korea during an 8-week period in each year from 2001 to 2006. Of 41,639 MRSAs isolated, 37,856 were screened and 169 grew on brain heart infusion agar supplemented with 4 μg/ml vancomycin. A vancomycin MIC of 4 μg/ml was confirmed for 33 VISA isolates of the 169 isolates. Eighteen of the 33 isolates were classified as hetero-VISA (hVISA) by the population analysis profile (PAP) method. All VISA isolates were susceptible to linezolid, tigecycline, and quinupristin–dalfopristin. Most VISA isolates (MIC 4 μg/ml) showed a PFGE C pattern with *sec*, *seg*, and *sei* enterotoxin genes, including ST5-SCC*mec* type II, or a PFGE A pattern with *sea*, including ST239-SCC*mec* type III.

Keywords: *Staphylococcus aureus*, surveillance, vancomycin-intermediate

Staphylococcus aureus is one of the leading causes of life-threatening infections in hospital settings and is increasingly a cause of disease in the community. Glycopeptides, such as vancomycin, are effective against methicillin-resistant *S. aureus* (MRSA). Although vancomycin resistance was first reported for enterococci in 1988, the first glycopeptide-intermediate *S. aureus* strain was isolated in France in 1995 [27]. The first documented infection caused by vancomycin-intermediate *S. aureus* (VISA) was reported from Japan in 1996 [15]. The strain, known as Mu50, had a minimum inhibitory concentration (MIC) to vancomycin of 8 μg/ml. Since then, there have been further reports of VISA in the United States, Germany, and Korea [3, 4, 19, 32]. In addition to VISA and another type of vancomycin-resistant bacteria, a hetero-VISA (hVISA), known as Mu3, has been described [14]. This strain appears to be borderline susceptible to vancomycin (MIC 2–4 μg/ml) but exhibits low-level subpopulation, at a frequency of 10^{-6} or greater, and is able to grow at vancomycin concentrations of 4–8 μg/ml. hVISA may be a precursor of VISA [16, 33] and may be associated with treatment failure [11, 14, 25, 37]. Methicillin resistance is prevalent (about 70%) among *S. aureus* isolates from tertiary hospitals in Korea [21]. Therefore, vancomycin used for the treatment of MRSA infections is increasing. Moreover, with the revised Clinical and Laboratory Standards Institute (CLSI) guidelines [7], the isolates with MIC values for vancomycin of 4 μg/ml are classified as intermediate. Thus, many of the isolates originally described as vancomycin-susceptible *S. aureus* are now classified as VISA strains. The possibility that VISA/VRSA may be on the rise necessitates periodic nationwide surveillance for these *S. aureus* strains. In this study, we conducted a nationwide survey to investigate the prevalence and molecular characterization of VISA in Korea from 2001 to 2006.

Materials and Methods

Bacterial Isolates

During an 8-week period from 2001 to 2006, 58,501 *S. aureus* strains isolated from clinical samples at general and tertiary hospitals participating in a nationwide laboratory surveillance program for VISA/VRSA in Korea were collected. MRSAs were defined as *S. aureus* that grew on mannitol salt agar with 6 μg/ml oxacillin and confirmed by *mecA* gene PCR in our laboratory. *S. aureus* ATCC

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29213, Mu3, and Mu50 were used as controls for vancomycin-susceptible, hVISA, and VISA phenotypes.

Vancomycin Agar Screening Test

All isolates were screened for reduced susceptibility to vancomycin by plating 10 μl of 10^6 colony-forming units (CFU)/ml of a 0.5 McFarland suspension onto brain heart infusion agar supplemented with 4 μg/ml (BHI-V4 medium) and 6 μg/ml vancomycin. Plates were incubated for 24 h at 35°C, and growth was observed. If cell growth was observed within 24 h to 48 h, the isolate was considered as a possible VISA strain. VISAs were confirmed as the isolates with vancomycin MIC of 4 μg/ml by agar, broth dilution methods, or E-test.

Antimicrobial Susceptibility Testing

The MICs for vancomycin and teicoplanin were determined by the standardized agar dilution and broth dilution methods using Muller–Hinton broth and Muller–Hinton agar, according to CLSI guidelines [7]. An E-test was performed on Muller–Hinton agar with 0.5 McFarland inoculums according to the manufacturer's instructions (AB Biodisk). The susceptibilities for additional antibiotics were tested using the disk diffusion method for 13 antibiotics of oxacillin (OX), penicillin (P), ampicillin (AM), erythromycin (E), clindamycin (CC), cefazolin (CZ), ofloxacin (OFX), tetracycline (Te), rifampin (RA), gentamicin (GM), trimethoprim–sulfamethoxazole (SXT), linezolid (LINE), and quinupristin–dalfopristin (SYN). The susceptibilities of daptomycin (DAP) and tigecycline (TIGE) were measured using the E-test micromethod. *S. aureus* ATCC 29213 was used as the quality control strain.

Population Analysis Profiling

For the strains with MIC values for vancomycin of 4 μg/ml, a population analysis profile–area under the curve method (PAP–AUC) was performed as described previously [38]. Mu3 and Mu50 strains served as controls for every test sample. A ratio was then calculated by dividing the AUC of test strain by the AUC of Mu3. Vancomycin resistance was determined using PAP–AUC values. PAP–AUC ratios of ≤0.90 indicated vancomycin-susceptible *S. aureus*, 0.90~1.3 indicated hVISA, and ≥1.3 indicated VISA [38].

Detection of *vanA*, *vanB*, *vanC*, and Toxin Genes

Genomic DNA was extracted from *S. aureus* strains with a Wizard genomic DNA purification kit (Promega, Madison, WI, U.S.A.). All strains with a vancomycin MIC of 4 μg/ml were analyzed for the *vanA*, *vanB*, and *vanC* resistance genes according to a previous PCR method [6]. The multiplex PCR was performed in a 25-μl volume containing 10× PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), 2.5 mM of dNTP mixture, 2.5 U *Taq* DNA polymerase (TaKaRa Shuzo Co. Ltd., Shiga, Japan), and template DNA (10 ng) for toxin genotyping. Master mixtures of the appropriate toxin gene primers for each multiplex reaction were prepared as follows: aliquots (2.5 pmol) of set A (*sea*, *seb*, *sec*, *sed*, *see*, and 16s rRNA), set B (*seg*, *seh*, *sei*, *sej*, and 16s rRNA), and set C (*tst*, *eta*, *etb*, and 16s rRNA) were brought to 100 μl with PCR-quality water and stored at −20°C until use. DNA amplification was carried out in a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA, U.S.A.) instrument with an initial denaturation at 95°C for 2 min followed by 28 cycles of amplification (denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 2 min), ending with a final extension at 72°C for 5 min. Toxin primers have been described previously [2, 24].

Genotyping

All VISA strains were profiled by SCC*mec* using multiplex PCR and typed by PFGE after DNA digestion by *Sma*I as previously described [26, 34]. "PFGE patterns were" were analyzed with the GelCompar version 4.1 using Dice coefficient, and represented by the unweighted pair-grouping method using arithmetic averages (UPGMA) with a 0.48 tolerance and 0% optimization setting. Results were interpreted using a cut-off point of 80%. Multilocus sequence typing (MLST) was carried out on all isolates as previously described [26]. The three forward primers for *aroE* (aroE-F), *gmk* (gmk-F), and *yqi*L (yqiL-F) were designed using the sequences of highly conserved regions flanking more variable regions in this study and were as follows: aroE-F (5'-CTAAAGTAATCGTTCGTGTTCCT-3'), gmk-F (5'-AGCTTAGAGAGGTCGTAAGGCAT-3'), and yqiL-F (5'-AGTGGC GTATTGGTTCATTAGAC-3'). Data were compared with the database on the multilocus sequence typing Web site (http://saureus.mlst.net).

Results

Vancomycin Agar Screening

Among 58,501 *S. aureus* isolates, 41,639 MRSA strains were isolated. The annual rate of MRSA isolation ranged from 69.6–74.1% (data not shown). The 37,856 possible MRSAs that grew on mannitol salt agar with 6 μg/ml oxacillin were screened on BHI-V4 medium, where 169 (0.4%) exhibited cell growth. The positive rates for the screening test were 1.1%, 0.3%, 0.7%, 0.3%, 0.3%, and 0.2% for 2001 through 2006, respectively (Table 1).

Antimicrobial Susceptibilities

By E-test and dilution methods, 33 out of 169 screening-test positive isolates were identified as VISA strains. Among 33 VISA strains, the MIC values of vancomycin ranged from 2 to 4 μg/ml on agar dilution, and 1 to 4 μg/ml on broth dilution, and E-test values were 3 to 4 μg/ml, respectively (Table 2). These VISA isolates were collected from various sources: 30% from pus, 27% from wounds, 18% from sputum, 12% from urine, 9% from blood, and 3% from

Table 1. Screening of resistance to vancomycin of clinical MRSA isolates from 2001 to 2006 in Korea.

Year	2001	2002	2003	2004	2005	2006	Total
No. of participating hospitals	27	42	40	49	52	45	
No. of MRSA screened	3,764	5,057	5,506	7,372	7,748	8,409	37,856
No. of screening-test positive (%)	43 (1.1)	16 (0.3)	41 (0.7)	24 (0.3)	28 (0.3)	17 (0.2)	169 (0.4)

Table 2. MICs of vancomycin and teicoplanin against 33 VISA isolates.

PAP (No. of isolates)	MICs (μg/ml)					
	Agar dilution method		Broth dilution method		E-test	
	VAN[a]	TEC[b]	VAN	TEC	VAN	TEC
Range	2–4	1–16	1–4	0.5–16	3–4	2–24
MIC_{50}	4	4	4	4	4	6
MIC_{90}	4	8	4	16	4	24

[a]VAN; vancomycin, [b]TEC; teicoplanin.

body fluids. Their susceptibilities, by the disk diffusion or E-test, against 15 antibiotics are listed in Table 3. Most of the isolates were resistant to 10 antibiotics, at a range 21% to 100%. All the isolates were susceptible to linezolid, quinupristin–dalfopristin, and tigecycline. Some isolates (15/33, 45%) were nonsusceptible to daptomycin, as E-test results of daptomycin showed MICs of >1 μg/ml.

Population Analysis Profiling

Eighteen of the 33 VISA strains were identified as hVISA, and 15 were identified as VISA (Table 2). The PAP–AUC ratio of these isolates ranged from 0.9 to 1.2 (hVISA) and from 1.3 to 1.7 (VISA) (data not shown).

Table 3. The antimicrobial susceptibilities of 33 VISA strains isolated from 2001 to 2006.

Antibiotics[a]	No. of isolates (%)		
	Resistant	Intermediate	Susceptible
OX	33 (100)	0 (0)	0 (0)
AM	33 (100)	0 (0)	0 (0)
P	31 (94)	0 (0)	2 (6)
E	29 (88)	1 (3)	3 (9)
CZ	28 (85)	0 (0)	5 (15)
CC	25 (76)	1 (3)	7 (21)
Te	24 (73)	0 (0)	9 (27)
OFX	21 (64)	0 (0)	12 (36)
GM	21 (64)	0 (0)	12 (36)
SXT	19 (58)	1 (3)	13 (39)
RA	7 (21)	1 (3)	25 (76)
LINE	0 (0)	0 (0)	33 (100)
SYN	0 (0)	0 (0)	33 (100)
TIGE	0 (0)	0 (0)	33 (100)
DAP	15 (45)[b]		18 (55)

[a]OX, oxacillin; P, penicillin; AM, ampicillin; E, erythromycin; CC, clindamycin; CZ, cefazolin; OFX, ofloxacin; Te, tetracycline; RA, rifampin; GM, gentamicin; SXT, trimethoprim–sulfamethoxazole; LINE, linezolid; SYN, quinupristin–dalfopristin; the susceptibilities were measured by the disk-diffusion method. DAP, daptomycin; TIGE, tigecycline; the susceptibilities were measured by the E-test method (AB biodisk).
[b]Categorized as "nonsusceptible", as E-test results of daptomycin MICs of >1 μg/ml. *S. aureus* 29213 (control strain) was susceptible to daptomycin MIC of 0.5 μg/ml.

Contribution of Various Genotypes

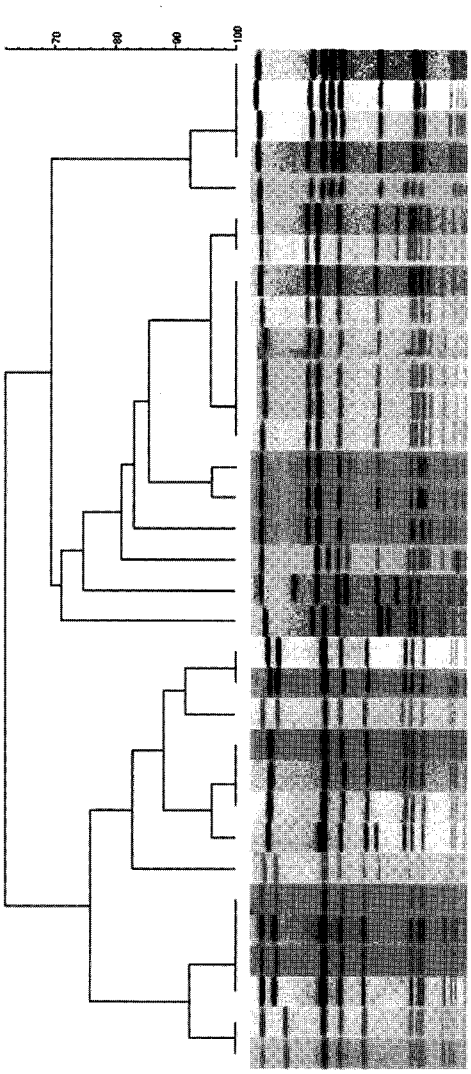
For 33 VISA isolates, detection of staphylococcal enterotoxin genes and typing of STs, SCC*mec*, PFGE were characterized, as shown in Fig. 1. All the strains were identified as follows: 14 isolates hold enterotoxin genes of *sec*, *seg*, and *sei* with SCC*mec* II and ST5; *sea* with SCC*mec* III or IIIa and ST239 (12 isolates); *seg* and *sei* with SCC*mec* IV and ST72 (5 isolates); and *seh* with SCC*mec* IV and ST1 (1 isolate). One VISA strain isolated in 2006 was identified as *sea* with SCC*mec* III and a new MLST (2-3-1-new-4-4-3), showing 99% similarity to the *gmk*-1 allele. Analysis of the PFGE band patterns for the 33 isolates revealed three different groups: pattern A with A1 to A5 subtypes, pattern B with B1 to B2 subtypes, and pattern C with C1 to C7 subtypes, with a Dice coefficient of 80% cut-off.

DISCUSSION

After the first VRSA appeared in 2002 in Michigan, U.S.A., an additional two cases occurred in 2007 and a total of nine cases with the *vanA* gene have been reported to date in the U.S.A. [5, 10]. VRSAs have also been reported in other countries such as India (5 VRSAs including 1 *vanA*-positive isolate) and Iran (2 VRSAs including 1 *vanA*-positive isolate) [1, 31, 35]. In Korea, *S. aureus* with reduced susceptibility to vancomycin was reported for the first time in 2000 [19], but VRSA has not appeared to date. Since its first appearance, some surveillance studies [18, 20] reported that numerous VISAs (MIC of 4 μg/ml) and hVISAs had been isolated from specimens from hospitalized patients. The incidence of VISAs and VRSAs is in an increasing trend globally, including in Korea, and thus the necessity of information sharing through an international surveillance system is increasing further.

During the period of this study (2001–2006), VISAs were identified in 33 out of a total of 37,856 MRSA isolates, with vancomycin MIC of 4 μg/ml, and their prevalence was shown to be 0.09%. In France, the France Teaching Hospital showed a VISA prevalence of 0.07% [29], which is similar to the result of this study, and in the case of the U.S.A., VISA prevalence in hospitals in the Detroit metropolitan area has been 0.3–2.3% for 22 years [30]. In Asia, VISA prevalence's in Thailand (0.8%, 3/361 MRSAs) and Japan (0.24%, 6/2,446 MRSAs) were slightly higher that the result of this study [13, 22].

In this study, about 70% of all *S. aureus* isolated from participating hospitals in Korea were methicillin-resistant. Because vancomycin is one of the frequently prescribed antimicrobial agents for treatment of these MRSA infections, the appearance of VISAs is expected to increase. In addition, to reduce the rate of failure in treating MRSA patients along with VISA patients, hVISA should also be investigated. In this study, the PAP–AUC method was used



Isolates	PFGE	SE	SCC*mec*-ST	Yr
V40*	B1	*gi*	IV-72	01
V231	B1	*gi*	IV-72	02
V236*	B1	*gi*	IV-72	02
V21*	B1	*gi*	IV-72	01
V411*	B2	*gi*	IV-72	04
V18	A2	*a*	III-239	01
V91	A2	*a*	III-239	01
V19	A1	*a*	III-239	01
V331	A1	*a*	III-239	03
V333	A1	*a*	III-239	03
V517	A1	*a*	III-239	05
V520	A1	*a*	III-239	05
V522	A1	*a*	III-239	05
V519	A3	*a*	III-239	05
V518*	A3	*a*	III-239	05
V521	A4	*a*	III-239	05
V605	A5	*a*	III-new	06
V23	D	*a*	IIIa-239	01
V24*	E	*h*	IV-1	01
V229*	C3	*cgi*	II-5	02
V303*	C3	*cgi*	II-5	03
V88*	C4	*cgi*	II-5	01
V28*	C1	*cgi*	II-5	01
V61*	C1	*cgi*	II-5	01
V259*	C1	*cgi*	II-5	02
V253*	C2	*cgi*	II-5	02
V335*	C5	*cgi*	II-5	03
V202*	C6	*cgi*	II-5	02
V26*	C6	*cgi*	II-5	01
V222	C6	*cgi*	II-5	02
V27*	C6	*cgi*	II-5	01
V89	C7	*cgi*	II-5	01
V95*	C7	*cgi*	II-5	01

Fig. 1. Characteristics of 33 VISA isolates.
SE, staphylococcal enterotoxin genes; *a(sea), c(sec), g (seg), h(seh), i(sei)*. SCC*mec*, staphylococcal cassette chromosome *mec* (SCC*mec*) types were determined by Oliveira *et al.*[26] ST, sequence type by MLST. new, ST identified in this study. Yr, year. *, hVISA was identified by PAP–AUC ratio (Wootton *et al.*[38]).

to screen VISA and hVISA from specimens; the PAP is being suggested as a method to identify hVISA and VISA in spite of controversies over the clinical importance of hVISA strains and hVISA detection methods [9, 12]. In this study, 18 out of 33 VISA strains were identified as hVISA by PAP–AUC ratio. In the U.S.A., 112 hVISA (7.5%) strains were identified out of 1,499 *S. aureus* strains, and, of these, 84.6% showed a vancomycin MIC of 4 μg/ml [30]. In the case of Japan, hVISA prevalence was shown to be 3% (34/1,149 MRSAs) in one study, but no hVISA was found in any other study conducted in the same year [14, 17]. In Italy, Germany, France, and The Netherlands, the prevalence of hVISA in MRSA was 1.1%, 0.21%, 0.6%, and 6%, respectively [3, 23, 28, 36]. Thus, hVISA prevalence appears to occur with slight differences among countries, although this is partially due to detection methods. Nevertheless, some actual differences among countries do occur.

Currently, the CDC in the U.S.A. uses BHI agar containing 6 μg/ml of vancomycin as a vancomcyin-screening medium, and CLSI criteria for VISA were changed, which means there is a possibility of missing VISA. When 33 VISA strains confirmed in our study were investigated for growth on BHI supplemented with 6 μg/ml vancomycin, only 14 strains grew on screening plate of 6 μg/ml vancomycin. Therefore, we propose a routine use of screening plate containing 4 μg/ml vancomycin to reinforce the screening of reduced susceptibility to vancomycin.

Thirty-three VISA isolates were tested for susceptibility to daptomycin, and, based on the results, 15 isolates were nonsusceptible. This reduced susceptibility of VISA strains to daptomycin was reported in 2006 by Cui *et al.* [8], and

the report was shown to be consistent with the result that the reduced susceptibilities of VISAs to vancomycin and daptomycin are correlated with each other.

The domestic epidemiology of VISAs was identified through PFGE and MLST, and, as a result, a lineage with three patterns of independent genetic traits could be identified. These isolates are PFGE A with SCC*mec* III and ST239, PFGE B with SCC*mec* IV and ST72, and PFGE C with SCC*mec* II and ST5. Given that genetic similarities were shown among the restriction patterns of PFGE, even though the strains were isolated from different hospitals and from different specimens, it is possible that the prevalent clones in hospitals in Korea, such as ST5, ST239, and ST72, obtained resistance to vancomycin or that clonal transmissions occurred among VISA isolates in the hospital. Moreover, it is assumed that these VISAs had reduced susceptibility to vancomycin as a result of clinical administrations of vancomycin. Kim *et al.* [18] reported that out of 12 vancomycin (4 μg/ml) patients, 11 patients had been exposed to glycopeptide antibiotics for long periods (56 days on average).

In conclusion, 33 VISA strains with vancomycin MIC of 4 μg/ml were reported in Korea, but no VRSA was detected. Since most VISA strains showed the characteristics of epidemic strains found in hospitals in Korea, we assume that VISAs were exposed to vancomycin, and this subsequently reduced their susceptibility to vancomycin, enabling them to spread in some hospitals. Therefore, the prudent use of antibiotics is very important in order to suppress the appearance of VISAs/VRSAs. Continued surveillance and infection control are essential in preventing the occurrence and spreading of VISAs/VRSAs.

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