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SHORT REPORT
Molecular epidemiology of vancomycin-resistant enterococci isolated from non-tertiary-care and tertiary-care hospitals in Korea

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SUMMARY

This study compared the molecular characteristics of vancomycin-resistant *Enterococcus faecium* (VREF) isolates recovered from 20 non-tertiary-care hospitals (36 isolates) and three tertiary-care hospitals (26 isolates) in diverse geographical areas of Korea from October 2010 to April 2011. All isolates carried the *vanA* gene only, but 42% and 73% of non-tertiary and tertiary-care isolates expressed the VanB phenotype (teicoplanin minimum inhibitory concentration $\leqslant 16\,\mu$g/ml). All isolates harboured insertion sequences, IS*1542* and IS*1216V*, within Tn*1546*. The isolates from tertiary-care hospitals tended to have reduced Tn*1546* lengths by deletion of sequences adjacent to IS elements. Multilocus sequence typing revealed eight sequence types within clonal complex 17 (CC17), but DNA fingerprinting by rep-PCR did not show clonal relatedness between the intra- and inter-hospital isolates. These results suggest that *vanA*, which has prevailed in tertiary-care hospitals of Korea since the 1990s, had been transferred horizontally to non-tertiary-care hospitals while the genetic rearrangement driven by evolutionary adaptation to adverse environments may have occurred in tertiary-care hospitals.

Key words: Clonal complex 17, insertion sequence, tertiary-care hospital, Tn*1546*, *vanA*, vancomycin-resistant *Enterococcus faecium*.

Since their first report in Korea in 1992, vancomycin-resistant enterococci (VRE) have become endemic as an important nosocomial pathogen [1]. In earlier periods, most VRE infections in Korea occurred in large tertiary-care or university hospitals [1] and since then, most clinical research on VRE colonization and infection in Korea has focused on isolates from tertiary-care hospitals, but there is a lack of molecular epidemiological studies on VRE isolates from primary- or secondary-care hospitals.

VRE is an important concern because infections with these organisms are difficult to treat not only in clinical practice, but they can also spread within and between hospitals and further to different regions or countries. Molecular epidemiological studies are necessary to clarify the genetic relatedness and molecular evolution of such clones and thereby inform patterns of spread and consequently measures for infection control. This study aims to examine the evolutionary relationships of VRE isolates from non-tertiary and tertiary-care hospitals in Korea by a comparative analysis of the antimicrobial and molecular characteristics of isolates.

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We studied a total of 62 clinical vancomycin-resistant *Enterococcus faecium* (VREF) isolates from diverse geographical areas, including an island, around Korea from October 2010 and April 2011. Thirty-six isolates were collected from 20 primary- or secondary-care hospitals including small medical institutions, medium-sized general hospitals, and geriatric care hospitals (0–544 beds). For comparison, 26 isolates were collected from three different university hospitals (1087–2093 beds). The latter are teaching tertiary-care hospitals with organ transplantation services, cancer centres, emergency services, intensive care units, and various medical specialities in Seoul and Suwon, Korea. VREF were identified by conventional biochemical reactions with the Vitek identification system (bioMérieux Inc., USA) and the API Strep kit system (bioMérieux Inc.). We analysed the minimum inhibitory concentrations (MICs) of vancomycin and teicoplanin, resistance genotype, transposon (Tn) *1546* structures, multilocus sequence typing (MLST), and a dendrogram of DNA fingerprinting created by automated repetitive extragenic palindromic PCR (rep-PCR) in all VREF isolates. VREF strain BM4147 was used as a control. Antimicrobial susceptibility testing of vancomycin and teicoplanin for VREF was performed by the E-test according to Clinical and Laboratory Standards Institute (CLSI) recommendations [2]. DNA of VREF was extracted from agar grown cultures with the Qiagen DNeasy kit (Qiagen GmbH, Germany) according to the manufacturer's instructions, and multiplex PCR was performed to investigate the presence of *vanA, vanB, vanC1*, and *vanC2*/*C3* genes as published previously [3]. For structural analysis of Tn*1546*, PCR amplification of overlapping internal regions of Tn*1546* was performed as described previously [4, 5]. The purified PCR products were directly sequenced on an ABI Prism 3100 machine (Applied Biosystems, USA) and were analysed using DNASIS for Windows v. 2.6 (Hitachi Solutions America Ltd, USA). MLST was performed on VREF isolates according to Homan *et al.* [6]. The allele number for each gene was assigned according to the *E. faecium* MLST database (http://www.efaecium.mlst.net). Automated rep-PCR analysis was performed for bacterial strain typing and determination of genetic relatedness of the isolates as previously described [7]. DNA for rep-PCR analysis was extracted with the UltraClean™ Microbial DNA Isolation kit (MoBio Laboratories Inc., USA) according to the manufacturer's instructions. Data were analysed with the web-based DiversiLab® Analysis software version 3.4 (bioMérieux, USA), the Pearson correlation coefficient was calculated to determine distance matrices, and the unweighted pair-group method with arithmetic mean (UPGMA) was used to create dendrograms.

As with most VREF isolates found in Korea and worldwide [1], all of the isolates studied here harboured the *vanA* resistance gene and additionally were negative for *vanB, vanC1*, and *vanC2*/*C3* genes. The *vanA* gene typically corresponds to the VanA phenotype which is characterized by high-level resistance to both vancomycin and teicoplanin, whereas the *vanB* gene is responsible for the VanB phenotype (resistance to vancomycin but not to teicoplanin) [3, 8]. However, the *van* genotype in this study was not completely consistent with the phenotype as shown in earlier reports [4, 8]. The MICs of vancomycin and teicoplanin for VREF isolates from the non-tertiary-care hospitals ranged from 48 to >256 μg/ml, and from 2 to >256 μg/ml, respectively. All tertiary-care isolates showed vancomycin MICs >256 μg/ml and teicoplanin MICs from 4 to 96 μg/ml. Fifteen (41·7%) of 36 isolates from non-tertiary-care hospitals and 19/26 (73·1%) from tertiary-care centres exhibited the VanB phenotype: vancomycin MICs of 48 to >256 μg/ml and teicoplanin MICs of 2–16 μg/ml (Table 1). We used the breakpoint of teicoplanin for defining the VanB phenotype according to Lauderdale *et al.* [8]. The *vanA* genotype-VanB phenotype VREF isolates were more frequently identified in tertiary-care than non-tertiary-care hospitals ($P<0{\cdot}05$, χ^2 test). The molecular basis of the *vanA* genotype-VanB phenotype discrepancy has yet to be identified, but it has been suggested that impairment of accessory proteins VanY and VanZ, genetic rearrangement including deletion of both *vanY* and *vanZ* genes following insertion of IS*1216V*, or mutations in the *vanS* regulatory gene may be responsible for the loss of teicoplanin resistance [4, 5, 8].

In this study, Tn*1546* structure analysis showed that none of the isolates was identical to the prototype (*E. faecium* BM4147) and no mutations were identified in the central *vanS* regulatory gene regions. Insertion sequence (IS) *1542* or IS*1216V*-IS*1542* was inserted at nucleotide position 3932 in the *orf2*-*vanR* intergenic region, and IS*1216V* was located within or downstream of *vanX* in all isolates, and complete or partial deletion of nucleotides adjacent to the IS sites was evident in all. The isolates were classified into three main types according to Tn*1546* structural differences from the prototype (Fig. 1). Type I isolates

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Table 1. *Characteristics of 62 vancomycin-resistant* E. faecium *isolates from non-tertiary-care and tertiary-care hospitals in Korea*

		MICs (μg/ml)				
No. of isolates	MLST type	VM	TEI	Location (city)	Tn*1546* type	Source
Non-tertiary-care hospitals (*n* = 20)						
1	ST17	48	4	GG	II	Urine
1		96	3	GW	III	Urine
1		128	6	SE	III	Urine
3		>256	16	GB, GG, SE	II	Bile juice, stool, urine
6		>256	32	GG, SE	I, II	Rectal swab, stool, urine
1		>256	64	SE	II	Stool
1		>256	>256	GG	II	Wound
1	ST192	>256	2	GG	II	Urine
3		>256	16	GB, SE	II	Rectal swab, stool, urine
4		>256	32	GG, JJ, SE	II	Rectal swab, stool, wound
1		>256	48	JJ	II	Stool
1		>256	96	JJ	III	Stool
1	ST78	>256	6	JB	III	Urine
1		>256	32	GG	II	Stool
1		>256	48	SE	II	Rectal swab
1		>256	128	GG	II	Stool
1		>256	>256	GG	II	Urine
1	ST262	>256	8	GN	III	Stool
2		>256	16	GG, SE	I, II	?, Stool
1		>256	48	SE	II	Rectal swab
1	ST18	>256	16	GG	II	Stool
1		>256	32	GB	II	Urine
1		>256	48	GB	II	Urine
Tertiary-care hospitals (*n* = 3)						
3	ST78	>256	8	GG, SE	II, III	Urine, throat
2		>256	16	GG	III	Peritoneal fluid, tissue
1		>256	32	SE	III	Sputum
1	ST18	>256	4	GG	III	Urine
1		>256	6	GG	III	Urine
1		>256	16	GG	II	Wound
1		>256	32	GG	I	Abscess exudates
1		>256	48	GG	II	Urine
1	ST192	>256	16	GG	II	Sputum
1		>256	32	GG	III	Sputum
1		>256	48	GG	I	Urine
1		>256	96	GG	II	Urine
1	ST202	>256	8	GG	II	Sputum
2		>256	16	GG	II, III	Bronchial aspirate, peritoneal fluid
2	ST203	>256	16	SE	II, III	Blood, urine
1		>256	48	SE	I	Sputum
1	ST205	>256	8	SE	II	Urine
2		>256	16	SE	III	Blood, urine
2	ST17	>256	16	GG	II, III	Blood, urine

MLST, Multilocus sequence typing; MIC, minimum inhibitory concentration; VM, Vancomycin; TEI, teicoplanin; ST, sequence type; GB, Gyeongbuk; GG, Gyeonggi; GN, Gyeongnam; GW, Gangwon; JB, Jeonbuk; JJ, Jeju island; SE, Seoul.

(*n* = 5, 8·1%) were characterized by an IS*1542* insertion in the *orf2-vanR* intergenic region and IS*1216V* insertion in the *vanX-vanY* intergenic region. Type II isolates (*n* = 39, 62·9%) had IS*1542* and IS*1216V* inserted at the left end of the Tn*1546* with a deletion of *orf1* and/or *orf2* regions as well as IS*1216V* insertion in the *vanX-vanY* intergenic region, and in type III isolates (*n* = 18, 29%) the left and right ends of

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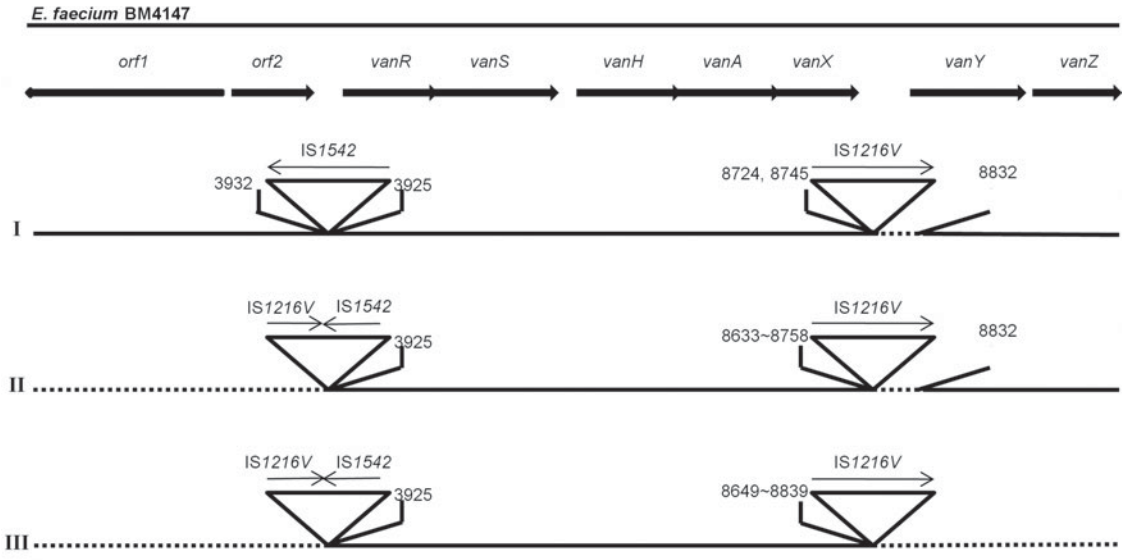

Type	Non-tertiary-care hospitals (*n*=20)	vs. Tertiary-care hospitals (*n*=3)
I	2/36 (5·5%)	3/26 (11·5%)
II	29/36 (80·6%)	10/26 (38·5%)
III	5/36 (13·9%)	13/26 (50·0%)

Fig. 1. Genetic maps of Tn*1546* types of 62 vancomycin-resistant *E. faecium* isolates from Korean hospitals. The positions of genes, open reading frames (*orf1* and *orf2*) and the direction of transcription are marked by arrows at the top. The inverted triangles with vertical lines represent insertion sequence (IS) elements. Deletions are indicated by dotted lines. The positions of the first nucleotide upstream and the first nucleotide downstream from the IS insertion sites are depicted.

Tn*1546* were totally deleted and accompanied with IS insertion. Fifteen (83·3%) of the type III group representing the deletion of *vanY* and *vanZ*, showed the VanB phenotype, while 15 isolates (44·1%) of the VanB phenotype were Tn*1546* type III. The complete deletion of both *vanY* and *vanZ* genes followed by an insertion of IS*1216V* may be one of factors responsible for the impaired resistance to teicoplanin.

Tn*1546* structural analyses, in addition to traditional methods like pulsed-field gel electrophoresis typing, have been used to investigate dissemination mechanisms of VRE [5]. The genetic rearrangements of Tn*1546* reported so far include point mutations, insertion of IS elements, and deletions of the left (*orf1* side) ends and/or the right (*vanZ* side) ends of the transposon [4, 8, 9]. We found that regardless of the hospital size, Tn*1546* exhibited IS*1542* and IS*1216V* in the genomes of all 62 isolates from diverse geographical areas of Korea (Fig. 1). Either IS*1542* or IS*1216V*-IS*1542* was inserted into the *orf2*-*vanR* intergenic region at nucleotide position 3932 in all isolates which is consistent with other studies [4, 9]. However, different integration sites of IS*1216V* in the *vanX*-*vanY* intergenic region indicated that the strains did not originate from clonal spread. This suggests that horizontal transfer of Tn*1546* with IS*1542* and IS*1216V* may have already occurred throughout Korea. IS*1542* appears to be restricted to clinical and poultry VRE isolates from Europe, China, and Korea, while IS*1216V* of *vanA* elements is ubiquitous [1, 9–11].

Internal size variations due to the presence of IS elements in Tn*1546*-like elements have been described previously [11]. In this study, IS insertions were accompanied by complete or partial deletion of nucleotides adjacent to the insertion site. Park *et al.* reported that the rearrangement of Tn*1546* such as integration of IS elements and deletions of nucleotides was associated with the evolution of the *vanA* gene cluster, and these elements may modify its transferability [11]. Tn*1546* sequences tended to be shortened as time passed, especially at the left or the right end of the transposon, and their transferability increased which suggests that the truncated sequences may play a role in the rapid dissemination of VRE [1, 11]. There was no significant difference in the incidence of Tn*1546* type I (5·5%, 11·5%) between the non-tertiary and tertiary-care hospitals [$P > 0{\cdot}05$, comparison of two rates by MedCalc version 12.7 (MedCalc Software, Belgium)]. The incidence of

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type II (80·6%, 38·5%) was significantly higher in the non-tertiary hospitals, whereas type III (13·9%, 50·0%) was more frequent in the tertiary-care hospitals ($P<0·05$). As Tn*1546* type increased, the number of VREF from the tertiary-care hospitals increased [$P<0·05$, linear by linear association by SPSS software v. 17·0 (SPSS Inc., USA)]. Tn*1546* type III was defined as the shortest type reducing their lengths by deleting sequences adjacent to IS elements. In tertiary-care hospitals, VRE have been exposed to adverse conditions such as intensive use of antibiotics or a variety of newly developed antibiotics over long periods, and we assumed that such genetic rearrangement most likely occurred due to an evolutionary adaptation of VRE to the hospital environment.

MLST is generally acknowledged to be an appropriate technique to establish an unambiguous international database of *E. faecium* genetic lineages in different laboratories or countries [6] and has been increasingly used to investigate the molecular evolution of VRE in regional and long-term global epidemiological studies [1, 6]. We identified eight different sequence types (STs) of clonal complex 17 (CC17) in the 62 VREF isolates investigated (Table 1). ST17 (25·8%), the predicted founder of CC17, was predominant followed by ST192 (22·6%), ST78 (17·7%), ST18 (12·9%), ST262 (6·5%), ST202 (4·8%), ST203 (4·8%) and ST205 (4·8%). The regional distribution of these sequence types are shown in Supplementary Figure S1. CC17 represents the globally dispersed nosocomially adapted clonal lineage [1] and organisms in this complex are typically ampicillin- and quinolone-resistant and are enriched for putative virulence factors that may assist with hospital adaptation and spread [1]. CC17 is therefore an example of evolutionary processes that have improved the relative fitness of bacteria in the hospital environment. It has shown rapid spread and subsequent acquisition of *vanA* or *vanB* genes [1]. ST17 (2/26, 7·7%) was the least common of the isolates from tertiary-care hospitals, but was the most common type (14/36, 38·9%) from the non-tertiary-care hospitals (Table 1). This most likely reflects a shift in the predominant sequence type in tertiary-care hospitals. ST192, ST78, ST18, ST202, ST203 and ST205 are single or double locus variants of ST17 while ST262 is a single locus variant of ST18. ST262, which has been reported in VREF isolates from Europe and Russia [12, 13], was detected only in isolates from the non-tertiary-care hospitals in diverse geographical areas (Table 1). There was no association between Tn*1546* type and MLST type. The dendrogram created with the automated rep-PCR results showed no clonal relatedness between the intra- and inter-hospital VREF isolates (data not shown). Taken together, these findings strongly suggest that *vanA* with IS*1542* and IS*1216V* have spread horizontally throughout Korea from tertiary-care hospitals to non-tertiary-care hospitals.

A limitation of this study is that the numbers of isolates investigated may be insufficient to chart unequivocally the evolutionary relationships between VREF from non-tertiary and tertiary-care hospitals and further investigations are needed to elucidate the mechanisms of transmission and molecular evolution of these organisms in tertiary-care hospitals.

In summary, all VREF isolates harboured *vanA* with IS*1542* and IS*1216V* integrated into Tn*1546*. IS*1542* was inserted at the same position in the *orf2-vanR* intergenic region of all the isolates. Although all isolates belonged to hospital-adapted CC17, a shift in the dominant sequence type in tertiary-care hospitals may have occurred. VREF from the latter hospitals tended to exhibit a VanB phenotype and shortened Tn*1546* lengths by the deletion of sequences adjacent to IS elements. We assume that the genetic rearrangements of VREF isolates from tertiary-care hospitals occurred during the continuing process of evolutionary adaptation.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268813003543.

DECLARATION OF INTEREST

None.

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