Eur J Clin Microbiol Infect Dis (2010) 29:25–30
DOI 10.1007/s10096-009-0810-9

ARTICLE

Clonal spread of CC17 vancomycin-resistant *Enterococcus faecium* with multilocus sequence type 78 (ST78) and a novel ST444 in Taiwan

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Received: 24 February 2009 / Accepted: 13 August 2009 / Published online: 15 September 2009

Abstract From May 2007 to January 2008, 30 isolates of vancomycin-resistant enterococci (VRE), including 29 *Enterococcus faecium* (96.7%) and 1 *E. faecalis* (3.3%) were obtained from various clinical specimens of 30 patients treated at a university hospital in Taiwan. Among these patients, 27 had VRE infections, including urinary tract infection (n=16), bacteremia (n=5), wound infection (n=5), and central nervous system infection (n=1). Three patients had VRE colonization. All of these isolates belonged to the *vanA* genotype with vancomycin minimum inhibitory concentrations of 64≥128 μg/ml. The isolate of *E. faecalis* had VanB phenotype-*vanA* genotype. All these isolates were susceptible to linezolid and were inhibited by tigecycline at 0.25 μg/ml. Multilocus sequence typing (MLST) analysis of the *E. faecium* isolates showed that 82.8% were ST78, which belongs to lineage C1. Transposon typing classified the 30 isolates of VRE into three types and most of the Tn*1546*-like elements contained an IS*1251*-like insertion sequence. Mating experiments showed that the *vanA* gene clusters were transferable at a frequency of about 10^{-6} to 10^{-7}. Our findings indicate that nosocomial spread of VRE resulted from dissemination of lineage C1 *E. faecium* clones, including a novel *E. faecium* MLST type (ST444), and the horizontal transfer of Tn*1546* elements among enterococci.

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Introduction

Enterococci, being part of the normal intestinal flora in human, are generally thought to be harmless pathogens, but they can cause opportunistic infection in immunocompromised patients. Along with the increase in clinical use of glycopeptide antibiotics against multiresistant Gram-positive bacteria, the isolation of vancomycin-resistant enterococcus (VRE) was first reported in 1988 in Britain and France [1, 2]. Until now, VRE have been reported widely and caused nosocomial infection in the United States [3], Europe [4], and Eastern Asia [5, 6]. The increased incidence of VRE infection has had a major impact both on the mortality of hospital patients and on the aspect of medical cost [7]. Molecular typing of VRE found that either clonal dissemination or horizontal transfer of a high-level glycopeptide resistance element resulted in the emergence of VRE [8–10]. In the USA and Europe, *vanA* is the predominant resistance mechanism among VRE. Gene encoding the VanA-phenotype resistance is located on a small mobile genetic element designated transposon Tn*1546*, which is transferable by conjugation [11]. Genetic variation, including the presence of insertion sequences or deletions in nonessential genes and intergenic regions, can be detected in the transposable elements [12].

In Taiwan, VRE was first isolated in 1996 [13]. Since then, VRE have become endemic and hard to eradicate in several tertiary hospitals [5, 14–16]. In recent years, nosocomial infection caused by VRE was noted to be increasing due to the high selective pressure of antibiotics [5, 14].

The aim of this study was to investigate the genotype and VanA Tn*1546* transposon diversity in VRE isolates recovered from Taipei Medical University-Wanfang Hospital, where VRE was seldom seen, but which has experienced an outbreak of VRE since June 2007. Since then, VRE has become endemic in Taipei Medical University-Wanfang Hospital.

Materials and methods

Hospital setting and bacteria

Taipei Medical University-WanFang Hospital is a 750-bed tertiary-care teaching hospital located in northern Taiwan. From May 2007 to January 2008, a total of 30 isolates of VRE were recovered. The most common specimen sources were urine (17 isolates) followed by blood (5 isolates), wound pus (5 isolates), catheter tips (2 isolates), and cerebrospinal fluid (1 isolate). Duplicate strains from the same patient were not included in the study.

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) for enterococci to vancomycin, ampicillin, erythromycin, tetracycline, ciprofloxacin, daptomycin, linezolid, tigecycline, and gentamicin were determined by the agar dilution methods, which were performed and interpreted according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) [17]. Susceptibility to teicoplanin was tested using the Etest (AB Biodisk, Solna, Sweden). *Staphylococcus aureus* ATCC 29213, and *E. faecalis* ATCC 29212 were used as control strains.

Detection of *vanA*, *vanB* genes, and *esp* genes

Total DNA of the isolates was purified by using the DNase Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The PCR conditions and primers used to detect *vanA*, *vanB*, and *esp* genes have been described previously [16, 18].

Multilocus sequencing type

Multilocus sequencing type (MLST) was performed according to the scheme described previously [19]. PCR products were purified by using a QIAquick PCR purification kit (Qiagen) prior to their sequence determination with an Applied Biosystems 3700 capillary sequencer. The obtained sequences were submitted to the *E. faecium* MLST database (http://www.mlst.net) for assignment of alleles at each locus and a sequence type. Cluster analysis of the data was performed using the MLST database and the e-BURST algorithm.

Characterization of Tn*1546* elements

Ten primer pairs were used as described previously to amplify 10 overlapping fragments of the Tn*1546*, an element responsible for VanA glycopeptides resistance [20]. *E. faecium* BM4147 was used as a positive control strain.

Conjugation experiments

Filter matings were performed as previously described with *E. faecium* GE-1 as the recipient strain [21]. Transconjugants were selected on plates containing vancomycin (6 mg/l), fusidic acid (25 mg/l), and rifampin (100 mg/l). Conjugation frequencies were calculated with reference to the donor isolate. Transconjugants were verified by detection of Tn*1546* elements by PCR.

Results

MLST genotyping

The new allele and ST identified in this study were deposited in the MLST database. Twenty-nine isolates of *E. faecium* were subjected to MLST genotyping. Twenty-four out of 29 isolates (82.8%) belonged to ST78 (Table 1). One isolate belonged to ST359, 1 to ST343, and 1 to ST18. Two isolates had a new allele 23 to the *adk* sequence, and ST444 was assigned to the new allelic profiles 47-1-1-1-1-1-23 (Table 1). eBURST analysis showed that ST359 was the founder of a clonal complex with ST18 and ST78 (Fig. 1). All isolates of *E. faecium* except for one (ST343) belonged to lineage C1.

Antimicrobial susceptibilities

All isolates were resistant to vancomycin, and susceptible to daptomycin and linezolid (Table 1). All isolates of ST78, except for one isolate (isolate 24), expressed high-level glycopeptide resistance (MIC of vancomycin, >128 mg/l; MIC of teicoplanin, 32–128 mg/l), and were resistant to ampicillin, erythromycin, tetracycline, and ciprofloxacin. Isolates of ST359, ST343, and ST18 also expressed high-

Table 1 Characteristics of 30 vancomycin-resistant enterococci isolates in this study

Patient (isolate)	Date (year/mo./day)	MIC (mg/l) for antibiotics										Sequence type (allelic profile)	*esp*	Transposon type	Conjugation frequency
		VAN	TEI	AMP	E	TE	CIP	DAP	LIN	TIG	GM				
1	20070519	>128	64	128	>128	32	>128	0.25	1	0.06	>128	78 (15-1-1-1-1-1-1)	+	B	4×10^{-7}
2	2007/06/04	64	64	64	>129	0.5	128	1	1	0.03	>128	359 (7-1-1-1-1-7-1)	+	B	
3	2007/06/08	>128	64	128	>128	32	>128	0.25	1	0.06	>128	78	+	B	
4	2007/06/30	>128	32	64	>128	64	>128	0.25	1	0.06	>128	78	+	B	
5	2007/07/03	>128	64	128	>128	32	>128	0.5	1	0.06	>128	78	+	B	
6	2007/07/27	>128	64	128	>128	32	>128	0.5	1	0.06	>128	78	+	B	
7	2007/07/27	>128	64	128	>128	32	32	0.25	1	0.03	>128	78	+	B	
8	2007/08/13	>128	32	128	>128	32	>128	0.25	1	0.06	>128	78	+	B	
9	2007/09/03	>128	64	128	>128	32	>128	0.25	1	0.06	>128	78	+	B	
10	2007/09/17	>128	32	128	>128	32	>128	0.25	1	0.06	>128	78	+	B	
11	2007/09/20	>128	32	128	>128	16	>128	2	1	0.06	>128	78	+	B	
12	2007/09/20	>128	32	128	>128	32	>128	0.25	1	0.06	>128	78	+	B	
13	2007/09/23	>128	32	128	>128	32	>128	0.5	1	0.06	>128	78	+	B	
14	2007/09/24	>128	8	1	>128	64	64	1	2	0.12	>128	*Enterococcus faecalis*	−	A	
15	2007/09/23	>128	64	128	>128	32	>128	0.5	1	0.06	>128	78	+	B	
16	2007/10/04	>128	32	128	>128	32	32	0.25	1	0.06	>128	78	+	C	10^{-6}
17	2007/10/04	>128	64	128	>128	32	>128	1	1	0.06	>128	78	+	C	
18	2007/10/23	>128	32	128	>128	4	>128	2	0.5	0.12	>128	343(15-1-1-39-1-20-1)	+	C	
19	2007/11/26	>128	64	128	>128	32	>128	1	1	0.06	>128	78	+	C	
20	2007/11/26	>128	64	128	>128	32	>128	0.25	1	0.06	>128	78	+	C	
21	2007/12/07	>128	64	128	>128	32	16	0.5	1	0.06	>128	444 (47-1-1-1-1-1-23)	+	A	7×10^{-7}
22	2007/12/08	>128	64	128	>128	64	>128	0.5	1	0.25	2	444	+	A	
23	2007/12/09	>128	128	128	>128	16	>128	2	1	0.06	>128	78	+	C	
24	2007/12/20	8	32	0.5	0.12	0.25	0.5	2	1	0.03	2	78	+	C	
25	2007/12/19	>128	64	128	>128	32	>128	0.25	1	0.06	4	78	+	C	
26	2007/12/21	>128	64	>128	>128	32	>128	0.25	1	0.06	>128	78	+	C	
27	2007/12/23	>128	64	128	>128	32	>128	1	1	0.03	>128	78	+	C	
28	2007/12/29	>128	64	32	>128	0.5	>128	2	1	0.06	64	18 (7-1-1-1-5-1-1)	+	B	
29	2008/01/14	>128	64	>128	>128	32	>128	0.5	1	0.06	>128	78	+	B	
30	2008/01/14	>128	64	128	>128	32	>128	0.25	1	0.06	>128	78	+	C	

VAN: vancomycin; TEI: teicoplanin; AMP: ampicillin; E: erythromycin; TE: tetracycline; CIP: ciprofloxacin; DAP: daptomycin; LIN: linezolid; TIG: tigecycline; GM: gentamicin

Fig. 1 An eBURST diagram: ST359 was the predicted founder of a group of 3 sequencing types

level glycopeptide resistance, and were resistant to ampicillin, erythromycin, and ciprofloxacin, but susceptible to tetracycline. Two isolates, belonging to ST444, expressed high-level glycopeptide resistance, and were resistant to ampicillin, erythromycin, tetracycline, and ciprofloxacin. One isolate of *E. faecalis* was susceptible to teicoplanin and ampicillin, but resistant to erythromycin, tetracycline, ciprofloxacin, and high-level gentamicin. The *E. faecalis* isolate presented with VanB phenotype. All isolates were inhibited by tigecycline at ≦0.25 mg/l, and 27 out of 30 (90%) isolates were inhibited by tigecycline at ≦0.06 mg/l.

Detection of *vanA*, *vanB*, and *esp* genes

The *vanA* gene was detected in all VRE isolates. Sequencing of the *vanS* gene of the *E. faecalis* isolate with VanB phenotype found three point mutations in the *vanS* gene at positions 148 (T-≥G), 160 (G-≥C), and 207 (A-≥T). Using PCR screening, all VRE isolates but *E. faecalis* carried the *esp* gene.

Characterization of Tn*1546* elements

By using 10 pairs of primers to study the diversity of Tn*1546*-like elements among 30 VRE isolates, there were three different Tn*1546* types (Table 2). Three isolates exhibited patterns the same as those of *E. faecium* BM4147, which was designated type A. Sixteen isolates exhibited B type, which had amplicons larger than those of *E. faecium* BM4147 by using the 6th primers. Sequencing the larger amplicon in B type found that there was an insertion of IS*1251*-like within the intergenic regions between the *vanS* and *vanH* genes. The sequences of the IS*1251*-like element were identical to those of accession number AF148130. Eleven isolates exhibited C types, which lack the PCR product, by using the first pair of primers, and had larger amplicons compared with those of *E. faecium* BM4147, by using the sixth primers. Sequencing the larger amplicon in C type also revealed that there was an insertion of IS*1251*-like within the intergenic regions between the *vanS* and *vanH* genes.

Conjugation results

Isolates 1, 16, and 21 representing each transposon type were selected to study the conjugation frequency (Table 1). Vancomycin-resistant transconjugants were obtained at a frequency of 10^{-6} to 10^{-7}.

Discussion

In the USA, VRE has been an important nosocomial pathogen since the 1990s [22]. Lineage C1, which was renamed clonal complex (CC) 17 and was characterized by the allele *purK1*, resistance to ampicillin and quinolone, and carrying mobile elements and a putative pathogenicity island including the *esp* gene, was responsible for the

Table 2 Polymerase chain reaction (PCR) patterns of *vanA* elements in 30 isolates by using 10 primer pairs

Type	PCR products by 10 primers										Number of isolates
	1	2	3	4	5	6	7	8	9	10	
A (*E. faecium* BM4147)	+	+	+	+	+	+	+	+	+	+	3
B	+	+	+	+	+	++	+	+	+	+	16
C	−	+	+	+	+	++	+	+	+	+	11

+: amplicon size was the same as those from *E. faecium* BM4147; −: no amplicon produced compared with those from *E. faecium* BM4147; ++: amplicon size was larger than those from *E. faecium* BM4147

majority of hospital-related VRE isolates in the USA [10, 23]. It was assumed that acquisition of insertion sequence (IS) elements helped CC17 to increase its genome plasticity to facilitate adaptation in a hospital environment [24]. In the study, we found that the spread of an *E. faecium* clone caused an outbreak of VRE in a teaching hospital in Taiwan. MLST analysis revealed that the epidemic strain was ST78. The spread of ST78 has been reported to cause an increase in VRE bloodstream infections in Italy and was common in Korea [25, 26]. ST78 was a single locus variant of ST17, which was the predicted founder of CC17. ST359, ST444, and ST18 were double locus variants of ST17. ST78, along with ST18, ST359, and ST444 disclosed in the study, belonged to CC17 (lineage C1). Consistent with previous study, all of the isolates of CC17 but one in the study were resistant to ampicillin, erythromycin, and ciprofloxacin [23]. Of note, isolate 24, harboring the *vanA* gene and belonging to ST78, presented low-level resistance to vancomycin and was susceptible to ampicillin and ciprofloxacin. The glycopeptides resistance mechanism in isolate 24 is worthy of further study.

Except for the *E. faecalis* isolate, all isolates of *E. faecium* harbored the *esp* gene. The result was also coherent with previous findings that *E. faecium* isolates with the presence of Esp, a surface-exposed protein involved in virulence and biofilm formation, was strongly associated with epidemicity [27].

Tn*1546* typing disclosed that there were two types different from the prototype. One type displayed an IS*1251*-like insertion sequence within the intergenic regions of the *vanS* and *vanH* genes (B type); the other displayed an IS*1251*-like insertion sequence and left-end deletions © type). VRE isolates with an IS*1251*-like insertion sequence within the intergenic regions of the *vanS* and *vanH* genes have also been reported in the USA, Norway, Ireland, and Poland [28, 29]. In the study, the same Tn*1546* type was found in different species of enterococci, and different Tn*1546* types were found in isolates belonging to the same genotype. These findings suggested that horizontal gene transfer might have played a role in the spread of glycopeptide resistance.

In general, the *vanA* gene cluster confers the VanA phenotype, which has high-level resistance to vancomycin and teicoplanin; the *vanB* gene cluster confers the VanB phenotype, which has resistance to vancomycin, but not to teicoplanin. In Japan and Taiwan, VRE strains with VanB phenotype-*vanA* genotype were reported because of point mutations located in the putative sensor domain of *vanS* [30]. In the study, we also found an isolate of *E. faecalis* with VanB phenotype-*vanA* genotype incongruence. Sequencing of the *vanS* gene of the *E. faecalis* isolate found identical point mutations in the *vanS* gene at the same position, as previous reports described in Japan and Taiwan [30, 31].

In conclusion, our study identified that an epidemic clone ST78 with positive *esp* belonging to a specific genetic lineage, CC17, caused a nosocomial VRE outbreak in the hospital. Given the heterogeneous antimicrobial resistance pattern within the same sequence type, the appearance of new allele and ST, and the existence of the gene transfer of glycopeptide resistance between enterococci during the study period, we believed that VRE was in a rapid evolutionary process in the hospital. More rigorous infection control policy and strict antibiotic restriction are needed to control the emergence of VRE.

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