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In vitro susceptibility studies and detection of vancomycin resistance genes in clinical isolates of enterococci in Nagasaki, Japan

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SUMMARY

Glycopeptide resistance in enterococci is now a cause of clinical concern in the United States and Europe. However, details of vancomycin resistance in enterococci in Japan have been unknown. We measured minimum inhibitory concentrations (MICs) of various antimicrobial agents for a total of 218 clinical strains of enterococci isolated in our hospital in 1995–6 in addition to 15 strains with known genotypic markers of resistance. We also screened vancomycin resistance genes using a single step multiplex-PCR.

In clinical isolates, only two strains of *Enterococcus gallinarum* were of intermediate resistance to vancomycin (MIC, 8 μg/ml), while the others were all susceptible. Glycopeptides (vancomycin and teicoplanin) and streptogramins (RP 58500 and RPR 106972) showed potent antimicrobial effects for the isolates. In addition, ampicillin was also potent for *Enterococcus faecalis*, while ampicillin, minocycline and gentamicin were potent for *Enterococcus avium*. No *vanA* or *vanB* genes were detected, while $vanC_1$ and $vanC_{23}$ genes were detected from two and four strains, respectively. Our results suggest that incidence of VRE in Japan may be estimated as still very low at this time.

INTRODUCTION

Enterococci are increasingly responsible for nosocomial infections such as endocarditis, bacteremia, urinary tract infections, and neonatal sepsis [1, 2]. Glycopeptide resistance in enterococci is now a cause of clinical concern. Three glycopeptide resistance phenotypes can be distinguished on the basis of the level and inducibility of resistance to vancomycin and teicoplanin [3]. The VanA type is characterized by acquired inducible resistance to both vancomycin and teicoplanin [4]. Strains of the VanB type have acquired inducible resistance to various levels of vancomycin but not to teicoplanin [5]. Constitutive low-level resistance to vancomycin (VanC type) is an intrinsic property of motile enterococci [6, 7]. The *vanA* [3] and *vanB* [5] genes are associated with VanA and VanB types of glycopeptide resistance, respectively. Glycopeptide resistance genotypes of VanC type of enterococci were divided in to three species-specific types as $vanC_1$, $vanC_2$ and $vanC_3$ for *Enterococcus gallinarum* [6], *Enterococcus casseliflavus* [7] and *Enterococcus flavescens* [7], respectively.

At present vancomycin resistant enterococci (VRE) are frequently reported from the United States and occasionally in Europe. VRE occupied 14% of enterococci isolated in the United States [8]. However, details of VRE in Japan have been unknown.

In this paper, we measured antimicrobial susceptibility of clinical isolates of enterococci isolated in

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our hospital for various antimicrobial agents including glycopeptides (vancomycin and teicoplanin), gentamicin and streptogramins (RP 59500 and RPR 106972). We also screened vancomycin resistance genes using a single step multiplex-polymerase chain reaction (PCR) assay, which allows identification of four glycopeptide resistance genotypes described for enterococci (*vanA*, *vanB*, $vanC_1$ and $vanC_{23}$) by boiling colonies for DNA preparation [9] and using mixture of four sets of primers [1].

MATERIALS AND METHODS

Bacterial strains

Clinical strains of enterococci isolated in Nagasaki University School of Medicine Hospital and Clinics, a 829-bed hospital in Nagasaki, Japan in 1995–6 were used. The clinical strains consisted of 49 *Enterococcus faecalis*, 80 *Enterococcus faecium*, 30 *Enterococcus avium*, and 59 other enterococci. The kinds and numbers of clinical specimens, from which the enterococci were isolated, are listed in Table 1. Enterococci were identified by Gram staining; absence of catalase; inability to produce gas; presence of Lancefield antigen group D; and growth on 40% bile, in 6·5% sodium chloride, in 0·1% methylene blue and at pH 9·6. Species were identified by Vitek Gram-Positive Identification cards (bioMerieux-Vitek, Inc., MO). Enterococci other than *E. faecalis*, *E. faecium* and *E. avium* were also identified by api 20 strep (bioMerieux, Marcy I'Etoile, France). Only three and one isolates were identified by api 20 strep as *E. casseliflavus* and *Enterococcus durans*, respectively, while species of the others could not be identified.

As positive controls for the PCR study, well-characterized glycopeptide-resistant enterococci *E. faecalis* A256 (*vanA* genotype) [10], *E. faecalis* ATCC51299 (*vanB* genotype) [11], *E. gallinarum* BM4174 ($vanC_1$ genotype) [12], and *E. casseliflavus* ATCC25789 ($vanC_2$ genotype) [13] were used. For antimicrobial susceptibility testing, *E. faecium* BM4147 [4], *E. faecium* 228 [14], *E. faecalis* CA21, CA22, CA23, and CA24 (*vanA* genotype), *E. faecalis* V583 [15], *E. faecium* D366 [16] (*vanB* genotype), *E. gallinarum* VR42 [9] ($vanC_1$ genotype), *E. casseliflavus* ATCC25788 [13] ($vanC_2$ genotype), and *E. flavescens* CCM429 [13] ($vanC_3$ genotype) were also added. These strains were kindly supplied by Dr FC Tenover, Centers for Diseases Control and Prevention, Atlanta, Georgia, USA (*E. faecalis* A256, ATCC51299, V583, *E. faecium* 228, D366 and *E. gallinarum* VR42), Dr P. Courvalin, Institute Pasteur, Paris Cedex, France (*E. faecium* BM4147, *E. gallinarum* BM4174) and Dr R. Pompei, University of Cagliari, Cagliari, Italy (*E. faecalis* CA21, CA22, CA23 and CA24, *E. casseliflavus* ATCC25788 and ATCC25789, and *E. flavescens* CCM429). *E. faecalis* ATCC29212 was used for negative control for the PCR study and as a quality control for antimicrobial susceptibility tests.

Antibiotics and antimicrobial susceptibility tests

Antimicrobial susceptibility tests for enterococci were performed by the microdilution method with cation-adjusted Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, MD) according to National Committee for Clinical Laboratory Standards (NCCLS) recommendations [17]. MICs of potent antibiotics were measured for ampicillin (ABPC; Meiji Seika Kaisha, Ltd., Tokyo, Japan), erythromycin (EM; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), clarithromycin (CAM; Dainabot Co., Osaka, Japan), clindamycin (CLDM; Sigma Chemical Co., St Louis, MO), minocycline (MINO; Nihon Lederle Pharmaceutical Co., Ltd., Tokyo, Japan), gentamicin (GM; Schering-Plough KK., Osaka, Japan), vancomycin (VCM; Shionogi and Co., Ltd., Osaka, Japan), teicoplanin (TEIC; Nippon Hoechst Marion Roussel Ltd., Tokyo, Japan), and RP 59500 and RPR 106972 (Rhone-Poulenc Rorer Japan, Inc., Tokyo, Japan). The titres of drugs were calibrated according to their potencies. MICs were judged after 20 h incubation [17]. In some study, MICs were also measured after 48 h incubation.

Preparation of samples and DNA amplification

Preparation of template DNA for PCR was performed by heat-lysis of bacteria in PCR mixture as described by NC Clark and colleagues [9]. Briefly, one or two bacterial colonies from a plate incubated overnight were emulsified in 100 μl PCR mixture containing 2·5 U of recombinant *Taq* DNA polymerase (Takara Shuzo Co. Ltd., Shiga, Japan), 10 mM tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 0·2 mM deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 0·5 M each primer, and 20 μl of diluted bacterial suspension. The oligonucleotide primers were selected from the published sequences as follows; *vanA* (product size 732 bp), sense, 5′-GGG AAA ACG ACA ATT GC-3′; antisense, 5′-GTA CAA TGC GGC CGT TA-3′ [1], *vanB* (product size 433 bp), sense, 5′-GTG ACA AAC CGG AGG CGA GGA-3′;

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Table 1. *Kinds and numbers of clinical specimens, from which the enterococci were isolated*

Specimens	Numbers of enterococci isolated			Other enterococci
	E. faecalis	*E. faecium*	*E. avium*	
Respiratory tract	12	21	4	13
Urinary tract	21	12	4	8
Pus, Wound	2	17	11	23
Blood	1	8	1	1
Bile	0	6	1	3
Ascitis	12	9	4	8
Others	1	7	5	3

antisense, 5′-CCG CCA TCC TCC TGC AAA AAA-3′ (18), *vanC*$_1$ (product size 822 bp), sense, 5′-GGT ATC AAG GAA ACC TC-3′; antisense, 5′-CTT CCG CCA TCA TAG CT-3′ [1], *vanC*$_{23}$ (product size 439 bp), sense, 5′-CTC CTA CGA TTC TCT TG-3′; antisense, 5′-CGA GCA AGA CCT TTA AG-3′ [1]. For multiplex PCR study, four sets of primers were inoculated in each PCR mixture. A Perkin–Elmer Cetus model 9600 DNA thermocycler was programmed as follows: lysis and denaturation step for 10 min at 95 °C; 35 cycles with a 30-s denaturation step at 94 °C, a 30-s annealing step at 58 °C, and a 30-s extension step at 72 °C; and a holding step at 4 °C until analysis. PCR products were resolved by electrophoresis on a 1·5% agarose-Tris-borate-EDTA gel. The gels were stained with 10 μM ethidium bromide, and the PCR products bands were visualized and photographed by using u.v. transillumination. As a molecular size maker, 100 base-pair ladder (Pharmacia Biotech, Milwaukee, USA) was used.

RESULTS

Antimicrobial susceptibility tests

The MICs of antimicrobial agents tested against VRE with known genotypic markers of resistance are summarized in Table 2. Since some VRE associated with *vanB* gene were judged as vancomycin-susceptible 20 h after inoculation, the results at 48 h after incubation were also included. MICs of VCM for the strains with *vanB* gene and those of TEIC for some strains with *vanA* gene significantly shifted (more than fourfold) to resistant between 20 and 48 h after inoculation. ABPC was the most potent for VRE with *vanA* gene. RP 59500 and RPR 106972 also showed strong antimicrobial effects for some strains. For strains with *vanB* gene, TEIC and ABPC were potent. Most strains with *vanC* genes were susceptible to various antimicrobial agents.

The ranges of MICs, MICs for 50% strains, and MICs for 90% strains of antimicrobial agents for clinical isolates measured after 20 h incubation period were listed in Table 3. MICs of VCM for clinical isolates did not significantly shift to resistance in contrast to genetically known VRE described above if measured after 48 h incubation period (data not shown). Only two enterococci other than *E. faecium*, *E. faecalis* and *E. avium* were intermediate resistant to VCM (MIC, 8 μg/ml), while the others were all susceptible (MIC, $\leqslant$ 4 μg/ml). Most *E. faecalis* strains were highly resistant (MIC $>$ 512) to GM. Glycopeptides and ABPC showed potent antimicrobial effects for clinical isolates of *E. faecalis* followed by streptogramins. *E. faecium* were susceptible to glycopeptides and streptogramins but not to ABPC. For *E. avium*, ABPC, MINO and GM were also potent in addition to glycopeptides and streptogramins.

Detection of vancomycin resistance genes by PCR

Using control strains carrying vancomycin resistance genes, the responsible genes were detected by the combination of heat-lysis DNA preparation and multiplex-PCR (Fig. 1). We applied the method for detection of the genes from all of clinical isolates. No *vanA* or *vanB* genes were detected, while *vanC* genes were detected from six clinical isolates of enterococci other than *E. faecalis*, *E. faecium* or *E. avium* as follows; *vanC*$_1$ from two enterococci which showed MICs of VCM as 8 μg/ml, *vanC*$_{23}$ from the other four strains associated with MIC of 4 μg/ml (Fig. 2). By species-specificity of resistant genes, the former two strains were identified as *E. gallinarum* and the others were identified as *E. casseliflavus* or *E.*

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Table 2. *Antimicrobial susceptibility of VRE for various antimicrobial agents judged 20 and 48 h after inoculation*

Genotype strain	Time (h)	MICs (μg/ml) for*									
		VCM	TEIC	ABPC	EM	CAM	CLDM	MINO	GM	RP	RPR
vanA											
E. faecalis A256	20	> 64	16	0·5	> 64	> 64	> 64	32	2	8	2
	48	> 64	32	0·5	> 64	> 64	> 64	32	4	32	4
E. faecalis CA21	20	> 64	32	1	> 64	> 64	> 64	16	> 512	8	4
	48	> 64	> 64	1	> 64	> 64	> 64	32	> 512	32	32
E. faecalis CA22	20	> 64	32	1	> 64	> 64	> 64	16	> 512	16	4
	48	> 64	> 64	1	> 64	> 64	> 64	32	> 512	64	16
E. faecalis CA23	20	> 64	32	1	> 64	> 64	> 64	16	> 512	16	4
	48	> 64	> 64	2	> 64	> 64	> 64	32	> 512	64	16
E. faecalis CA24	20	> 64	8	0·5	> 64	> 64	> 64	32	2	8	2
	48	> 64	> 64	0·5	> 64	> 64	> 64	32	4	32	8
E. faecium 228	20	> 64	32	2	> 64	> 64	> 64	32	8	1	0·5
	48	> 64	64	4	> 64	> 64	> 64	32	16	2	1
E. faecium BM4147	20	> 64	> 64	8	2	1	8	32	8	1	0·5
	48	> 64	> 64	8	4	2	16	32	16	2	0·5
vanB											
E. faecalis ATCC52199	20	4	0·12	0·5	> 64	> 64	> 64	0·12	> 512	16	4
	48	16	0·25	0·5	> 64	> 64	> 64	0·12	> 512	32	16
E. faecalis V583	20	8	0·12	0·5	> 64	> 64	> 64	0·12	> 512	8	4
	48	> 64	0·25	0·5	> 64	> 64	> 64	0·12	> 512	32	16
E. faecalis D366	20	32	0·12	4	> 64	> 64	64	32	8	2	0·5
	48	> 64	0·25	4	> 64	> 64	> 64	64	8	8	1
vanC$_1$											
E. gallinarum VR42	20	8	0·25	0·25	0·5	0·5	16	0·06	4	2	0·5
	48	8	0·5	0·25	1	0·5	32	0·06	4	4	0·5
E. gallinarum BM4174	20	8	0·5	0·12	0·12	0·06	16	2	4	2	0·5
	48	8	0·5	0·12	0·25	0·12	16	4	4	4	0·5
vanC$_2$											
E. casseliflavus 25788	20	0·25	0·06	0·25	0·12	0·06	8	0·12	4	2	0·5
	48	0·5	0·06	0·25	0·12	0·06	16	0·12	4	2	0·5
E. casselifavus 25789	20	4	0·25	0·12	4	4	8	0·06	2	1	0·25
	48	8	0·5	0·25	4	4	16	0·12	2	1	0·5
vanC$_3$											
E. flavescens CCM429	20	0·25	0·25	0·25	1	0·25	16	0·12	4	1	0·25
	48	0·25	0·25	0·5	1	0·5	32	0·12	8	1	0·5

* VCM, vancomycin; TEIC, teicoplanin; ABPC, ampicillin; EM, erythromycin; CAM, clarithromycin; CLDM, clindamycin; MINO, minocycline; GM, gentamicin; RP; RP 59500; RPR; RPR 106972.

Table 3. *Antimicrobial susceptibility of clinical isolates of enterococci for various antimicrobial agents*

Organism (no. of strains)	Antimicrobial agents*	Range of MIC (μg/ml)	50% MIC (μg/ml)	90% MIC (μg/ml)
E. faecalis (49)	VCM	0·5–4	1	2
	TEIC	< 0·03–0·25	0·12	0·25
	ABPC	0·25–1	0·5	1
	EM	0·25– > 64	> 64	> 64
	CAM	0·12– > 64	16	> 64
	CLDM	2– > 64	> 64	> 64
	MINO	0·12–32	16	32
	GM	1– > 512	> 512	> 512
	RP 59500	2–8	4	8
	RPR 106972	1–4	2	2
E. faecium (80)	VCM	0·12–4	0·5	1
	TEIC	< 0·03–1	0·5	0·5
	ABPC	0·06– > 64	32	> 64
	EM	< 0·03– > 64	> 64	> 64
	CAM	< 0·03– > 64	> 64	> 64
	CLDM	0·06– > 64	> 64	> 64
	MINO	0·06–64	8	32
	GM	0·25– > 512	8	16
	RP 59500	0·25–4	0·5	2
	ROR 106972	0·12–1	0·25	0·5
E. avium (30)	VCM	0·06–1	1	1
	TEIC	0·12–2	1	1
	ABPC	0·12–32	8	16
	EM	0·06– > 64	> 64	> 64
	CAM	< 0·03– > 64	> 64	> 64
	CLDM	0·06– > 64	> 64	> 64
	MINO	0·06–16	8	16
	GM	1–4	2	4
	RP 59500	0·25–4	4	4
	RPR 106972	0·12–2	1	1
Other enterococci (59)	VCM	0·06–8	1	4
	TEIC	< 0·03–1	0·5	0·5
	ABPC	< 0·03– > 64	32	> 64
	EM	< 0·03– > 64	> 64	> 64
	CAM	< 0·03– > 64	> 64	> 64
	CLDM	< 0·03– > 64	> 64	> 64
	MINO	< 0·03–32	16	32
	GM	0·25–16	4	8
	RP 59500	< 0·03–8	0·5	4
	RPR 106972	0·12–2	0·25	1

MICs were judged 20 h after inoculation.
* See Table 2.

flavescens. Both *E. gallinarum* isolates identified by PCR were reported as *E. casseliflavus* by conventional method.

Although one strain of *E. faecalis* and one strain of *E. faecalis* showed 4 μg/ml of MIC of VCM, no vancomycin-resistance genes (*vanA*, *vanB*, $vanC_1$, $vanC_{23}$) were detected from both of them.

DISCUSSION

In spite of the prevalence of vancomycin resistance among enterococci in the United States and in some parts of Europe, no strains with inducible type vancomycin resistance associated with *vanA* or *vanB* could be found in this study. To our knowledge, the only clinical strain of *E. gallinarum* carrying both

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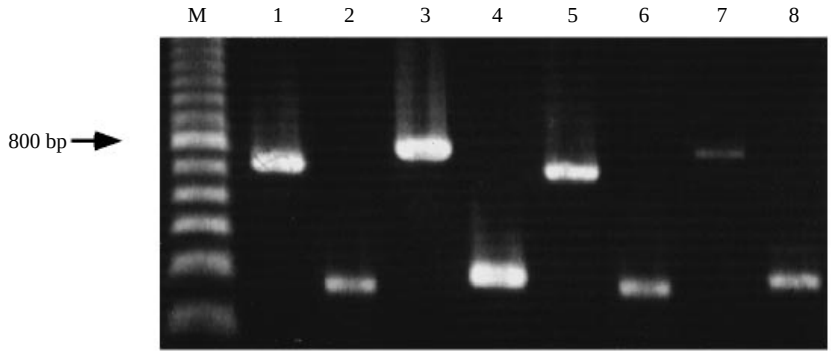

Fig. 1. Detection of vancomycin resistance genes by multiplex-PCR following heat-lysis DNA preparation from positive control strains. M, molecular size marker (100 base-pair ladder, Pharmacia Biotech); Lanes 1–4, PCR products by a single pair of primers (*vanA*, *vanB*, *vanC*$_1$ and *vanC*$_{23}$ for Lanes 1, 2, 3 and 4, respectively); Lane 5–8, PCR products by multiplex-PCR; Lane 1 and 5, *E. faecalis* A256 (*vanA*, 732 bp); Lane 2 and 6, *E. faecalis* ATCC51299 (*vanB*, 433 bp); Lane 3 and 7, *E. gallinarum* BM4174 (*vanC*$_1$, 822 bp); Lane 4 and 8, *E. casseliflavus* ATCC25789 (*vanC*$_{23}$ 439 bp).

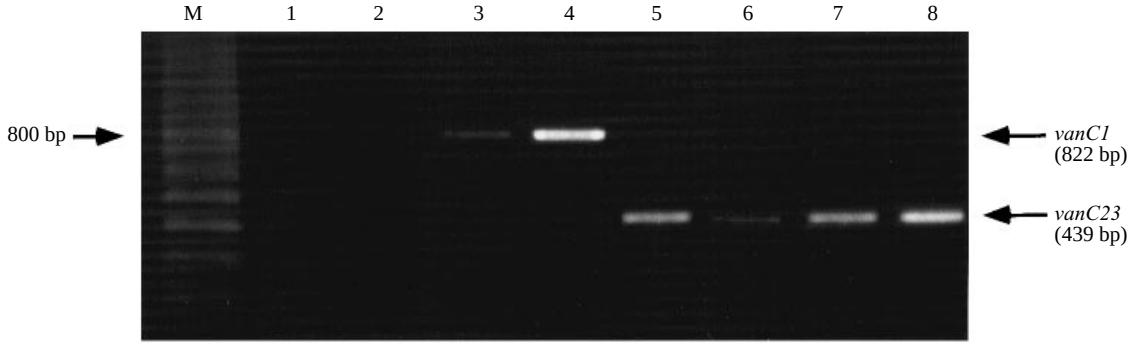

Fig. 2. Detection of vancomycin resistance genes by multiplex-PCR following heat-lysis DNA preparation from clinical isolates. M, molecular size marker (100 base-pair ladder, Pharmacia Biotech); Lane 1, *E. faecalis* (MIC of VCM; 4 μg/ml) negative for all vancomycin resistance genes; Lane 2, *E. faecium* (MIC of VCM; 4 μg/ml) negative for all vancomycin resistance genes; Lane 3 and 4, *E. gallinarum* (MIC of VCM; 8 μg/ml) positive for *vanC*$_1$ gene; Lane 5 and 8, *E. casseliflavus*/*E. flavescens* (MIC of VCM; 4 μg/ml) positive for *vanC*$_{23}$ gene.

vanB and *vanC* genes has been recently reported in Japan [19]. We first speculated that the very low rate of VRE in Japan is because susceptibility tests of vancomycin for enterococci were not determined routinely in clinical laboratories. Our results, however, suggest that incidence of VRE in Japan may be very low currently. The limited usage of VCM for infection due to methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* in Japan may contribute to the low incidence of VRE in our country.

In our study, some strains carrying *vanC* genes have been clinically isolated. The identification of enterococci other than *E. faecalis*, *E. faecium* and *E. avium* is frequently difficult in the clinical laboratory. In fact, there were discrepancies between the results by conventional method and those by PCR for species-specific genes in this study. *E. gallinarum* strains carrying *vanC*$_1$ gene were identified as *E. casseliflavus*, while *E. casseliflavus*/*E. flavescens* strains carrying *vanC*$_{23}$ could not be identified by conventional method.

In antimicrobial susceptibility tests using VRE obtained from Europe and the United States, ABPC and streptogramins showed relatively higher antimicrobial potency. In some VRE strains MICs of various antimicrobial agents drastically shifted to resistance dependent on inoculation time. Since such strains may be judged as susceptible for antimicrobial agents including VCM, 48 h incubation is thought to be needed.

Although no vancomycin-resistant clinical isolate of *S. aureus* has been reported to date, experimental evidence for the transfer of VCM resistance from enterococci to *S. aureus* [20] suggests that there is concern that MRSA resistant to VCM will emerge [21]. Therefore, alternative therapies to VCM are necessary [22]. Since prevalence of methicillin resistance in *S. aureus* is high in Japan, early detection and prevention of spreading of VRE is necessary.

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Although VRE with *vanA* have not yet been isolated in Japan, we should be aware of the emergence of VRE, which is expected to occur in the near future. Routine measurement of MICs of VCM, detection of vancomycin-resistant genes for clinical isolates, or use of selective media for detection of VRE [23] is desirable.

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