Epidemiol. Infect. (2001), **126**, 197–204. Printed in the United Kingdom

Distribution of aminoglycoside resistance genes in recent clinical isolates of *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus avium*

N. KOBAYASHI[1]*, Md. MAHBUB ALAM[1], Y. NISHIMOTO[1], S. URASAWA[1], N. UEHARA[2] and N. WATANABE[2]

[1] *Department of Hygiene, Sapporo Medical University School of Medicine, S-1, W-17, Chuo-ku, Sapporo 060-8556, Japan*
[2] *Department of Laboratory Diagnosis, Sapporo Medical University School of Medicine, S-1, W-16, Chuo-ku, Sapporo 060-8543, Japan*

(*Accepted 21 November 2000*)

SUMMARY

Aminoglycoside modifying enzymes (AMEs) are major factors which confer aminoglycoside resistance on bacteria. Distribution of genes encoding seven AMEs was investigated by multiplex PCR for 279 recent clinical isolates of enterococci derived from a university hospital in Japan. The *aac*(6′)-*aph*(2′′), which is related to high level gentamicin resistance, was detected at higher frequency in *Enterococcus faecalis* (42·5%) than in *Enterococcus faecium* (4·3%). Almost half of *E. faecalis* and *E. faecium* isolates possessed *ant*(6)-*Ia* and *aph*(3′)-*IIIa*. The profile of AME gene(s) detected most frequently in individual strains of *E. faecalis* was *aac*(6′)-*aph*(2′′)+*ant*(6)-*Ia*+*aph*(3′)-*IIIa*, and isolates with this profile showed high level resistance to both gentamicin and streptomycin. In contrast, AME gene profiles of *aac*(6′)-*Ii*+*ant*(6)-*Ia*+*aph*(3′)-*IIIa*, followed by *aac*(6′)-*Ii* alone, were predominant in *E. faecium*. Only one AME gene profile of *ant*(6)-*Ia*+*aph*(3′)-*IIIa* was found in *Enterococcus avium*. The *ant*(4′)-*Ia* and *ant*(9)-*Ia*, which have been known to be distributed mostly among *Staphylococcus aureus* strains, were detected in a few enterococcal strains. An AME gene *aph*(2′′)-*Ic* was not detected in any isolates of the three enterococcal species. These findings indicated a variety of distribution profiles of AME genes among enterococci in our study site.

INTRODUCTION

Enterococci are becoming an important cause of nosocomial infections including bacteraemia, surgical wound infection, and urinary tract infection in various regions of the world [1]. Recently, enterococci has become increasingly resistant to broad ranges of antimicrobial agents [2], particularly, glycopeptides, beta-lactams, and aminoglycosides. Among these antibiotics, high level aminoglycoside resistance is reported worldwide, while glycopeptide or beta-lactam-resistant enterococci are prevalent mostly in the United States and European countries [3]. The presence of high level aminoglycoside resistance results in the loss of synergy between cell wall synthesis inhibiting antibiotics (penicillins and glycopeptides) and aminoglycosides, which makes the treatment of serious enterococcal infections difficult [3]. Hence, epidemiologic survey of aminoglycoside resistance in enterococci is of significance for control of enterococcal infections.

Although enterococci are intrinsically resistant to low levels of aminoglycosides, high level resistance to aminoglycosides (MIC $\geqslant$ 2000 μg/ml) is mostly due to acquisition of genes encoding aminoglycoside modifying enzymes (AMEs) [4, 5]. The high level resistance to gentamicin, a key therapeutic aminoglycoside, in enterococci is associated with the *aac*(6′)-*aph*(2′′) which encodes the bifunctional AME,

* Author for correspondence.

Table 1. *Primers and their sequences used in multiplex PCR to detect genes of aminoglycoside modifying enzymes*

Gene	Enzyme	Primer sequence (5′-3′)	Position*	Size of PCR product (bp)	Reaction number†	Reference of AME gene sequence
aac(6′)-Ii	AAC(6′)-I	tggccggaagaatatggaga	73–92	410	II	7
		gcatttggtaagacacctacg	462–482			
aac(6′)-aph(2″)	AAC(6′)-APH(2″)	ccaagagcaataagggcatacc	424–445	675	I	6
		accctcaaaaactgttgttgc	1078–1098			
ant(4′)-Ia	ANT(4′)-I	ggaagcagagttcagccatg	180–199	266	I	30
		tgcctgcatattcaaacagc	426–445			
ant(6)-Ia	ANT(6)-I	cgggagaatgggagactttg	83–102	563	II	31
		ctgtggctccacaatctgat	626–645			
ant(9)-Ia	ANT(9)-I	ggttcagcagtaaatggtggt	103–123	476	I	32
		tgccacattcgagctagggtt	557–578			
aph(2″)-Ic	APH(2″)-Ic	atacaatccgtcgagtcgct	61–80	837	II	8
		gttggccttatcctcttcca	878–897			
aph(3′)-IIIa	APH(3′)-III	ctgatcgaaaaataccgct	37–55	354	I	33
		acaatccgatatgtcgatggag	369–390			

* Position of primer sequence is expressed as nucleotide numbers from the first base of initiation codon of each AME gene.
† See text (Materials and Method).

AAC(6′)-APH(2″) [6]. Kanamycin and streptomycin resistances in enterococci are mediated by APH(3′) and ANT [6] enzymes encoded by *aph(3′)-IIIa* and *ant(6)-Ia*, respectively [4]. Moderate level resistance to gentamicin and other aminoglycosides in *E. faecium* is conferred by AAC(6′)-I and results in the marked decrease in penicillin-aminoglycoside synergism [7]. This AME gene is encoded by a chromosomal gene, *aac(6′)-Ii* in *E. faecium* [7]. Furthermore, aph(2″)-Ic and aph(2″)-Id which confer gentamicin resistance were recently identified in enterococci [8, 9]. The *ant(3″)-Ia* (*ant(3″)* (9) or *aadA1*) and *ant(4′)-Ia* gene, found in enterobacteria and *Staphylococcus aureus*, respectively, were also detected in a few enterococcal isolates [10, 11]. Although the prevalence of high level resistance to gentamicin or *aac(6′)-aph(2″)* gene in enterococci has been reported in many studies [11–14], data on distribution of other AME genes in each enterococcal species are limited. In this study, to estimate the present status of aminoglycoside resistance in enterococci, we investigated distribution of seven AME genes in recent clinical isolates by multiplex PCR.

MATERIALS AND METHOD

Bacterial strains, identification and characterization

A total of 279 strains comprising 226 *E. faecalis* strains, 46 *E. faecium* strains, and 7 *E. avium* strains were analysed. These bacterial strains were isolated from clinical specimens of 259 patients in Sapporo Medical University Hospital, Sapporo, Japan between January 1997 and December 1998. Approximately 50% of the isolates were derived from urine, followed by other samples such as pus, bile, sputum, vaginal secretion, and gastric juice, while isolates from blood sample were only six strains. Identification of bacterial species and antimicrobial susceptibility tests were performed by the use of MicroScan WalkAway™-96 (Baxter Diagnostics, Inc., West Sacrament, USA), and rapid ID32 STREP (bioMerieux) was also employed for several isolates. Identification of some *E. faecalis* and *E. faecium* strains were confirmed also by detecting with PCR enterococcal PBP5 genes, which are distinct between the two species [15, 16], using *E. faecalis* specific primers (5′-CAGGGATTC-AAGCAGAAGGA-3′ and 5′-TCACTGGTTCAGA-AGCGACTG-3′) and *E. faecium* specific primers (5′-GATGAATACCTCATTAGGTGA-3′ and 5′-TGG-TTGTTCAGGATTTTCTTC-3′).

During the study period, no vancomycin-resistant enterococcus was detected. MIC of the following aminoglycosides, gentamicin (GM), tobramycin (TOB), sisomicin (SISO), kanamycin (KM), streptomycin (SM) and spectinomycin (SPCM), were measured by broth microdilution assay as recommended by NCCLS [17].

E. faecalis strains were genetically typed by arbitrarily primed PCR (AP–PCR) using ERIC2 primer, as described previously [18].

Multiplex PCR for detection of AME genes

In this study, multiplex PCR was employed to detect seven AME genes which have been reported to be distributed among enterococci and staphylococci [4, 8, 11, 19]. AME genes examined were *aac*(6′)-*Ii*, *aac*(6′)-*aph*(2″), *ant*(4′)-*Ia*, *ant*(6)-*Ia*, *ant*(9)-*Ia*, *aph*(2″)-*Ic*, and *aph*(3′)-*IIIa*. Among these, the *ant*(9)-*Ia* has been detected only in *S. aureus* (4). The *ant*(4′)-*Ia* gene is also prevalent in *S. aureus* and has been reported only in a few enterococcal strains [11, 20]. AME genes such as *aac*(6′)-*aph*(2″), *aph*(3′)-*IIIa*, and *ant*(3″)(9) have been found in both *S. aureus* and enterococci [4, 10]. PCR primers specific for each AME gene are listed in Table 1. Multiplex PCR was carried out using two reaction tubes for a single bacterial isolate. In the reaction I, four pairs of primers specific for *aac*(6′)-*aph*(2″), *ant*(4′)-*Ia*, *ant*(9)-*Ia*, and *aph*(3′)-*IIIa* were contained and the reaction II tube contained three primer pairs for *aac*(6′)-*Ii*, *ant*(6)-*Ia*, and *aph*(2″)-*Ic*. These primers were designed to produce DNA fragments with distinct sizes from target AME genes in each reaction tube. Bacterial DNA was extracted using achromopeptidase as described previously [21]. Reaction mixture (100 μl) containing 1 μl template bacterial DNA, 2·5 unit of AmpliTaq DNA polymerase (Perkin–Elmer), 200 μM each of dNTP, 30 pM each of primer, 10 mM Tris–HCl (pH 8·3), 50 mM KCl, and 1·5 mM $MgCl_2$ were subjected to 30 PCR cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min), and primer extension (72 °C, 2 min) in Thermal Cycler. The PCR products were analysed by electrophoresis on 1% agarose gel stained with ethidium bromide.

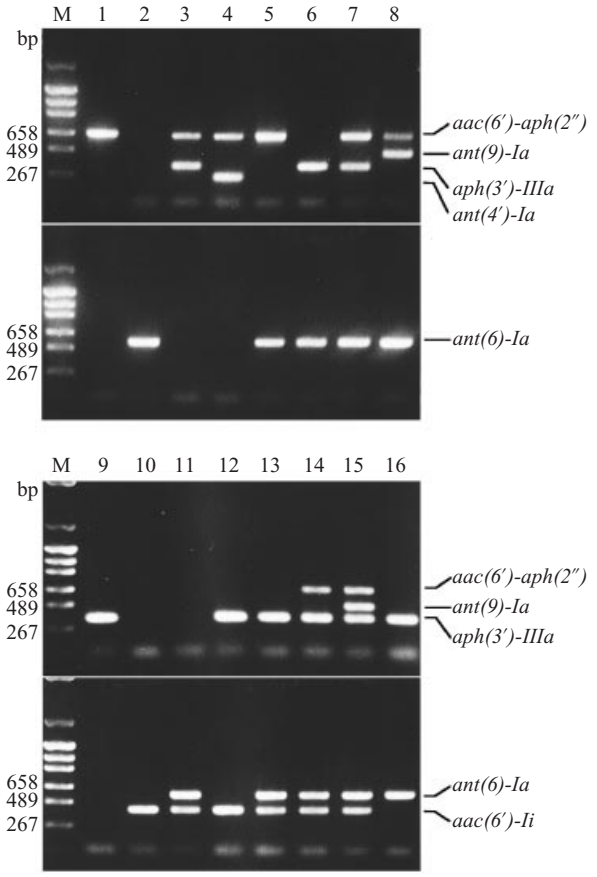

Fig. 1. Amplified products of AME genes generated by multiplex PCR, reactions I (upper portion) and II (lower portion), from enterococci with representative AME gene profiles. M, DNA size marker, lanes 1–9, *E. faecalis* strains (e69, e92, e282, e97, e6, e21, e308, e269, e216, respectively); lanes 10–15, *E. faecium* strains (e129, e100, e30, e137, e136, e185, respectively); lane 16, *E. avium* strain, e149.

RESULTS

Using multiplex PCR with 3 or 4 primer pairs designed in this study, individual AME gene-specific PCR products were generated and multiple PCR products in each reaction tube were clearly discriminated by their size, as shown in Figure 1. Detection rate of each AME gene were summarized in Table 2. The *aac*(6′)-*aph*(2″) gene was more frequently found in *E. faecalis* (42·5%) than in *E. faecium* (4·3%). In contrast, *aac*(6′)-*Ii* was detected exclusively and commonly in *E. faecium*. In both *E. faecalis* and *E. faecium*, almost half isolates possessed *ant*(6)-*Ia* and *aph*(3′)-*IIIa*. The *ant*(4′)-*Ia* was detected in four *E. faecalis* strains, and *ant*(9)-*Ia* was detected in a single strain each of *E. faecalis* and *E. faecium*. In *E. avium*, only two isolates possessed *ant*(6)-*Ia* and *aph*(3′)-*IIIa*, although the number of isolates was small. The *aph*(2″)-*Ic*, which was described recently as a novel GM resistance gene [8], was not detected in any enterococci.

Table 3 shows detection profiles of AME genes (AME gene pattern). In *E. faecalis*, 52·2% of isolates was found to have any AME gene. The most frequently observed AME gene profile was *aac*(6′)-*aph*(2″)+*ant*(6)-*Ia*+*aph*(3′)-*IIIa* found in 35·8% of *E. faecalis* isolates. This AME gene profile was found more frequently detected in *E. faecalis* strains derived from blood (75%, 3/4), bile (67%, 6/9) and pus (53%, 9/17) than in those from urine (36%, 43/119), gastric juice (21%, 5/24), vaginal secretion (17%, 2/12), sputum and pharyngeal swab (20%, 2/8) and all other specimens (31%, 11/35). When clonality of *E. faecalis* was analysed by AP–PCR, the enterococci with this major AME gene profile were differentiated

Table 2. *Incidence of aminoglycoside modifying enzyme (AME) genes in each enterococcal species*

AME gene	AME gene-positive isolates (%)		
	E. faecalis (226 isolates)	*E. faecium* (46 isolates)	*E. avium* (7 isolates)
aac(6′)-Ii	0 (0)	46 (100)	0 (0)
aac(6′)-aph(2″)	92 (42·5)	2 (4·3)	0 (0)
ant(4′)-Ia	4 (1·8)	0 (0)	0 (0)
ant(6)-Ia	105 (46·5)	27 (58·7)	2 (28·6)
ant(9)-Ia	1 (0·4)	1 (2·1)	0 (0)
aph(2″)-Ic	0 (0)	0 (0)	0 (0)
aph(3′)-IIIa	103 (45·6)	23 (50·0)	2 (28·6)

Table 3. *Profiles of aminoglycoside modifying enzyme (AME) genes and their incidence in each enterococcal species*

Enterococcal species	AME gene profile	No. of isolates (%)
E. faecalis (226 isolates)	AME gene-negative	108 (47·8)
	aac(6′)-aph(2″)	5 (2·2)
	ant(6)-Ia	2 (0·9)
	aph(3′)-IIIa	2 (0·9)
	aac(6′)-aph(2″) + *ant(4′)-Ia*	4 (1·8)
	aac(6′)-aph(2″) + *ant(6)-Ia*	3 (1·3)
	aac(6′)-aph(2″) + *aph(3′)-IIIa*	2 (0·9)
	ant(6)-Ia + *aph(3′)-IIIa*	18 (8·0)
	aac(6′)-aph(2″) + *ant(6)-Ia* + *ant(9)-Ia*	1 (0·4)
	aac(6′)-aph(2″) + *ant(6)-Ia* + *aph(3′)-IIIa*	81 (35·8)
E. faecium (46 isolates)	*aac(6′)-Ii*	18 (39·1)
	aac(6′)-Ii + *ant(6)-Ia*	5 (10·9)
	aac(6′)-Ii + *aph(3′)-IIIa*	1 (2·2)
	aac(6′)-Ii + *aph(6)-Ia* + *aph(3′)-IIIa*	20 (43·5)
	aac(6′)-Ii + *aac(6′)-aph(2″)* + *ant(6)-Ia* + *aph(3′)-IIIa*	1 (2·2)
	aac(6′)-Ii + *aac(6′)-aph(2″)* + *ant(6)-Ia* + *ant(9)-Ia* + *aph(3′)-IIIa*	1 (2·2)
E. avium (7 isolates)	AME gene-negative	5 (71·4)
	ant(6)-Ia + *aph(3′)-IIIa*	2 (28·6)

into 18 genetic types containing a dominant type that accounted for almost half of the isolates (data not shown). In *E. faecium*, 60·9% of isolates possessed one or more AME genes in addition to *aac(6′)-Ii*. The AME gene profile of *aac(6′)-Ii* + *ant(6)-Ia* + *aph(3′)-IIIa* was most frequently detected, followed by that of *aac(6′)-Ii*. Furthermore, it was of note that 4 or 5 AME genes including *aac(6′)-aph(2″)* were detected in the two *E. faecium* strains (e136, e185, Fig. 1).

MIC of aminoglycosides was measured for enterococcal strains with representative AME gene patterns in order to confirm consistency with their aminoglycoside resistance phenotype (Table 4). Most strains with *aac(6′)-aph(2″)* showed high level resistance (MIC $\geqslant$ 2000 μg/ml) to GM, as well as to SISO, KM, and TOB. Only one *E. faecium* strain (e136) with *aac(6′)-aph(2″)* exhibited moderate level resistance to GM, SISO, and TOB. *E. faecium* having *aac(6′)-Ii* was moderately resistant to SISO and TOB, and strains with *aph(3′)-IIIa* were highly resistant to KM. Strains with *ant(6)-Ia* and *ant(9)-Ia* were highly resistant to SM and SPCM, respectively. Thus, resistance to aminoglycosides of enterococcal strains were generally correlated with the presence of individual AME genes. However, the high level resistance to GM, SISO, KM, and TOB of *E. faecium* strain e129, SM resistance of *E. faecium* strain e30, and SPCM resistance of *E. faecalis* strain e6 could not be explained by the AME genes detected in this study. As for these three strains, an attempt was made by PCR to detect other AME

Table 4. *MIC of aminoglycosides against enterococcal isolates with different profiles of aminoglycoside modifying enzyme (AME) genes*

Enterococcal species	Isolate	AME gene profile	MIC (μg/ml)					
			GM	SISO	KM	TOB	SM	SPCM
E. faecalis	e66	AME gene-negative	8	4	16	4	64	32
	e69	*aac(6′)-aph(2″)*	> 2048	> 2048	> 2048	> 2048	128	32
	e92	*ant(6)-Ia*	8	4	16	32	> 2048	32
	e216	*aph(3′)-IIIa*	64	16	> 2048	32	128	128
	e97	*aac(6′)-aph(2″)* + *ant(4′)-Ia*	> 2048	> 2048	> 2048	> 2048	128	64
	e150	*aac(6′)-aph(2″)* + *ant(4′)-Ia*	> 2048	> 2048	> 2048	> 2048	128	64
	e6	*aac(6′)-aph(2″)* + *ant(6)-Ia*	> 2048	> 2048	> 2048	> 2048	> 2048	> 2048
	e139	*aac(6′)-aph(2″)* + *ant(6)-Ia*	> 2048	> 2048	> 2048	> 2048	2048	64
	e282	*aac(6′)-aph(2″)* + *aph(3′)-IIIa*	> 2048	> 2048	> 2048	> 2048	128	16
	e21	*ant(6)-Ia* + *aph(3′)-IIIa*	64	8	> 2048	32	> 2048	128
	e142	*ant(6)-Ia* + *aph(3′)-IIIa*	16	8	1024	16	> 2048	8
	e308	*aac(6′)-aph(2″)* + *ant(6)-Ia* + *aph(3′)-IIIa*	> 2048	> 2048	> 2048	> 2048	> 2048	16
	e68	*aac(6′)-aph(2″)* + *ant(6)-Ia* + *aph(3′)-IIIa*	> 2048	> 2048	> 2048	> 2048	2048	16
	e269	*aac(6′)-aph(2″)* + *ant(6)-Ia* + *ant(9)-Ia*	> 2048	> 2048	> 2048	> 2048	> 2048	2048
E. faecium	e146	*aac(6′)-Ii*	16	128	256	1024	64	64
	e129	*aac(6′)-Ii*	> 2048	> 2048	> 2048	> 2048	64	64
	e100	*aac(6′)-Ii* + *ant(6)-Ia*	8	128	128	256	> 2048	128
	e30	*aac(6′)-Ii* + *aph(3′)-IIIa*	64	256	> 2048	1024	> 2048	128
	e137	*aac(6′)-Ii* + *ant(6)-Ia* + *aph(3′)-IIIa*	32	256	> 2048	1024	> 2048	128
	e47	*aac(6′)-Ii* + *ant(6)-Ia* + *aph(3′)-IIIa*	32	256	> 2048	1024	> 2048	64
	e64	*aac(6′)-Ii* + *ant(6)-Ia* + *aph(3′)-IIIa*	32	512	> 2048	1024	> 2048	128
	e136	*aac(6′)-aph(2″)* + *aac(6′)-Ii* + *ant(6)-Ia* + *aph(3′)-IIIa*	256	512	> 2048	512	512	32
	e185	*aac(6′)-aph(2″)* + *ant(6)-Ia* + *aac(6′)-Ii* + *ant(9)-Ia* + *aph(3′)-IIIa*	2048	> 2048	> 2048	> 2048	> 2048	> 2048
E. avium	e132	AME gene-negative	4	4	16	4	32	64
	e149	*ant(6)-Ia* + *aph(3′)-IIIa*	4	32	> 2048	8	> 2048	8
Resistance break point *in vitro**			⩾ 128	⩾ 128	⩾ 512	⩾ 128	⩾ 256	⩾ 256

* Resistance break points were determined based on description or susceptibility data reported previously [11, 32].
In this study, MIC more than 2000 μg/ml of aminoglycosides was regarded as high level resistance, as described previously [3, 13, 19].

genes: *aph(2″)-Id*, *aac(3)-Ia*, *aac(3)-IIa*, *aac(3)-IIIa*, *aac(3)-IVa*, *aac(6′)-IIa*, *aac(2′)-Ia*, and *ant(2″)-Ia* for e129; and *aph(3″)-I*, *aph(6)-I*, *ant(3″)(9)(aadA1)*, *aadA2*, *aadA3*, *aadA4*, *aadA5*, *aadA6*, and *aadA7* for e6 and e30. However, none of these AME genes were detected in these strains.

DISCUSSION

Since its first emergence in late 1970s, enterococci with high level GM resistance have been disseminated in most countries and its incidence was remarkably increased in 1990s as shown in some studies [5, 20, 22, 23]. Since the high level of aminoglycoside resistance is recognized as a clinically serious problem, routine examination as well as surveillance of clinical isolates of enterococci are essential for the choice of appropriate therapy and infection control. Some genes encoding AMEs, major determinants of aminoglycoside resistance of bacteria, have been identified by PCR [10, 20]. Furthermore, in order to detect AME genes in a single strain, multiplex PCR using

several pairs of primers in one reaction mixture was developed [24, 25]. Using this method, prevalence of *aac*(6′)-*aph*(2″), *aph*(3′)-*IIIa*, and *ant*(4′)-*Ia* were examined for enterococci, group A streptococci, and methicillin resistant *S. aureus*. In the present study, we further developed a system of the multiplex PCR for enterococci to detect six AME genes, to obtain comprehensive knowledge on prevalence of AME genes. The multiplex PCR used in this study was demonstrated to be feasible for detection and differentiation of at least six AME genes without generating non-specific product, although isolates with *aph*(2″)-*Ic* was not detected.

High level GM resistance in enterococci is primarily associated with *aac*(6′)-*aph*(2″) gene encoding bifunctional enzyme AAC(6′)-APH(2″), which confer also resistance to clinically useful aminoglycosides (e.g. TOB, amikacin, netilmicin) [6]. The *aac*(6′)-*aph*(2″) gene is considered to have been conveyed to both enterococci and staphylococci via plasmid and transposons [5]. Incidence of recent enterococcal isolates showing high level GM resistance varies depending on countries as well as medical facilities. In a large scale survey involving 27 European countries, percentage of GM-highly resistant enterococcal isolates varied by country ranging from 1–49% (mean 22·6%), and by species (19·7%, *E. faecalis* and 13·6%, *E. faecium*) [14]. Another survey on isolates from European university hospitals reported the incidence of strains with high level GM resistance being 32% and 22% in *E. faecalis* and *E. faecium*, respectively [26]. The nationwide survey in the United States indicated the incidence of GM-highly resistant strains of 26% and 30·8% in *E. faecalis* and *E. faecium*, respectively [13]. In a study on *E. faecalis* isolates from a Japanese university hospital, 22·3% of isolates showed high level resistance to GM [27]. Since high level GM resistance is generally found at less than 30% of isolates as described above, the incidence (42·5%) of *aac*(6′)-*aph*(2″) in *E. faecalis* in our present study appears to be relatively high. Considering that *aac*(6′)-*aph*(2″) was found in genetically distinct 18 groups of *E. faecalis* in our study, this AME gene is suggested to be disseminated among various clones of *E. faecalis*, which then spread in the university hospital where our present study was done. In contrast, the incidence of *E. faecium* with this AME gene was 4·3%, which was markedly lower than not only that of *E. faecalis* in this study but also those of *E. faecium* with high level gentamicin resistance in other epidemiologic studies [13, 14]. Our results suggested that aminoglycoside resistant *E. faecium* may not be a serious nosocomial pathogen, compared to *E. faecalis* in this hospital.

The *ant*(6)-*Ia* and *aph*(3′)-*IIIa* responsible for resistance to SM and KM, respectively, have been commonly found in enterococci [11]. In our present study, it was of note that *ant*(6)-*Ia* was detected together with *aac*(6′)-*aph*(2″) in 37·6% of *E. faecalis*, and together with *aac*(6′)-*Ii* in 53·7% of *E. faecium* isolates. Since *E. faecalis* with *aac*(6′)-*aph*(2″) and *ant*(6)-*Ia* are highly resistant to both GM and SM, there will be no bactericidal regimen which is aimed at synergic activity between aminoglycosides and beta-lactams [3]. Similarly, *E. faecium* with *aac*(6′)-*Ii* and *ant*(6)-*Ia* may also restrict the choice of therapy, because AAC(6′)-I also reduce the synergy between beta-lactams and aminoglycosides with free 6′-amino groups [7]. Particularly, detection rate of *E. faecalis* with *aac*(6′)-*aph*(2″) and *ant*(6)-*Ia* in the present study was higher than those of *E. faecalis* that are resistant to both GM and SM reported in other studies [12, 24]. Therefore, in our hospital, precaution should be taken for such enterococci, although enterococci with vancomycin and beta-lactam resistance are still rare in Japan [27, 28]. In addition, it was noted in our present study that a few *E. faecalis* strains were resistant to ampicillin without producing beta-lactamase, although the mechanism of resistance is yet to be clarified (unpublished data). These strains mostly possessed *aac*(6′)-*aph*(2″) together with other AME gene(s) (*ant*(6)-*Ia*, *aph*(3′)-*IIIa* or *ant*(4′)-*Ia*). Hence it is also important to pay attention to beta-lactam resistance in *E. faecalis* viewed from the significance of synergic action with aminoglycosides.

In the present study, two AME genes, *ant*(4′)-*Ia* and *ant*(9)-*Ia* which are commonly found in *S. aureus* were detected in a few enterococcal isolates. The *ant*(4′)-*Ia* has been detected only in an *E. faecalis* isolate and an *E. faecium* isolate [11, 20], and the *ant*(9)-*Ia*, encoding ANT(9)-I conferring specific by resistance to spectinomycin has never been found in enterococci. It was also noted that *aad*(3″)(9)(*aadA*) gene which has been reported to exist in Gram-negative bacteria and *S. aureus* was recently detected in *E. faecalis* [10]. These findings indicated that all AME genes but *aph*(3″)-*I* distributed in staphylococci have been already found in enterococci. These facts may support a view that AME genes in enterococci might have been originated from staphylococci [5].

MIC of aminoglycosides measured for representative enterococci were almost consistent with the AME gene profile revealed by multiplex PCR in this

study. However, high level resistance to GM, SM and SPCM for three strains of e129, e30 and e6, respectively, was unexplainable by the presence of AME genes detected in this study. Furthermore, AME genes such as *aph*(2″)-*Id*, *aac*(3)-*Ia*, *aph*(3″)-*I* and *ant*(3″)(9) that have been previously described in enterococci or in other bacterial species were not detected by PCR. The presence of novel AME genes or genetic variation of the known AME genes may be possible. In addition, existence of resistance mechanism other than AME including ribosomal mutation reported for SM resistance [29] have been suggested. Thus, aminoglycoside resistance mechanisms of enterococci seem to be considerably variable, and surveillance of aminoglycoside resistance as well as further analysis on resistance mechanisms seem to be all the more important.

ACKNOWLEDGEMENT

This study was supported in part by a grant (no. 09770236) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Murray BE. The life and times of enterococcus. Clin Microbiol Rev 1990; **3**: 46–65.
2. Leclercq R. Enterococci acquire new kinds of resistance. Clin Infect Dis 1997; **24**(suppl 1): S80–4.
3. Murray BE. Vancomycin-resistant enterococcal infections. N Engl J Med 1999; **342**: 710–21.
4. Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev 1993; **57**: 138–63.
5. Simjee S, Gill MJ. Gene transfer, gentamicin resistance and enterococci. J Hosp Infect 1997; **36**: 249–59.
6. Ferretti JJ, Gilmore KS, Courvalin P. Nucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase 2″-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J Bacteriol 1986; **167**: 631–8.
7. Costa Y, Galimand M, Leclercq R, Duval J, Courvalin P. Characterization of the chromosomal *aac*(6′)-*Ii* gene specific for *Enterococcus faecium*. Antimicrob Ag Chemother 1993; **37**: 1896–903.
8. Chow JW, Zervos MJ, Lerner SA, et al. A novel gentamicin resistance gene in enterococcus. Antimicrob Ag Chemother 1997; **41**: 511–4.
9. Tsai SF, Zervos MJ, Clewell DB, Donabedian SM, Sahm DF, Chow JW. A new high-level gentamicin resistance gene, aph(2″)-Id, in *Enterococcus* spp. Antimicrob Ag Chemother 1998; **42**: 1229–32.
10. Clark NC, Olsvic O, Swenson JM, Spiegel CA, Tenover FC. Detection of a streptomycin/spectinomycin adenylyltransferase gene(*aadA*) in *Enterococcus faecalis*. Antimicrob Ag Chemother 1999; **43**: 157–60.
11. Ounissi H, Derlot E, Carlier C, Courvalin P. Gene homogeneity for aminoglycoside-modifying enzymes in gram-positive cocci. Antimicrob Ag Chemother 1990; **34**: 2164–8.
12. Gordon S, Swenson JM, Hill BC, et al. Antimicrobial susceptibility patterns of common and unusual species of enterococci causing infections in the United States. J. Clin Microbiol 1992; **30**: 2373–8.
13. Jones RN, Sader HS, Erwin ME, Anderson SC, Enterococcus Study Group. Emerging multiply resistant enterococci among clinical isolates. I. Prevalence data from 97 medical center surveillance study in the United States. Diagn Microbiol Infect Dis 1995; **21**: 85–93.
14. Schouten MA, Voss A, Hoogkamp-Korstanje JEE, the European VRE Study Group. Antimicrobial susceptibility patterns of enterococci causing infections in Europe. Antimicrob Ag Chemother 1999; **43**: 2542–6.
15. Signoretto C, Boaretti M, Canepari P. Cloning, sequencing and expression in *Escherichia coli* of the low-affinity penicillin binding protein of *Enterococcus faecalis*. FEMS Microbiol Lett 1994; **123**: 99–106.
16. Zorzi W, Zhou XY, Dardenne O, et al. Structure of the low-affinity penicillin-binding protein 5 PBP5fm in wild-type and highly penicillin-resistant strains of *Enterococcus faecium*. J Bacteriol 1996; **178**: 4948–57.
17. National Committee for Clinical Laboratory Standards. Approved standard M7-A3. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 3rd ed. National Committee for Clinical Laboratory Standards, Villanova, Pa., 1993.
18. van Belkum A, Bax R, Peerbooms P, Goessens WHF, van Leeuwen N, Quint WGV. Comparison of phage typing and DNA fingerprinting by polymerase chain reaction for discrimination of methicillin-resistant *staphylococcus aureus* strains. J Clin Microbiol 1993; **31**: 798–803.
19. Leclercq R, Dutka-Malen S, Brisson-Noel A, et al. Resistance of enterococci to aminoglycosides and glycopeptides. Clin Infect Dis 1992; **15**: 495–501.
20. van den Braak N, van Belkum A, Kreft D, te Witt R, Verbrugh HA, Endts HP. The prevalence and clonal expansion of high-level gentamicin-resistant enterococci isolated from blood cultures in a Dutch university hospital. J Antimicrob Chemother 1999; **44**: 795–8.
21. Kobayashi N, Wu H, Kojima K, et al. Detection of *mecA*, *femA*, and *femB* genes in clinical strains of staphylococci using polymerase chain reaction. Epidemiol Infect 1994; **113**: 259–66.
22. Lavery A, Rossney AS, Morrison D, Power A, Keane CT. Incidence and detection of multi-drug-resistant enterococci in Dublin hospitals. J Med Microbiol 1997; **46**: 150–6.
23. Marcus N, Peled N, Yagupsky P. Rapid increase in the prevalence of antimicrobial drug resistance among enterococcal blood isolates in southern Israel. Eur J Clin Microbiol Infect Dis 1997; **16**: 913–5.

24. van Asselt GJ, Vliegenthart JS, Petit PLC, van de Klundert JAM, Mouton RP. High-level aminoglycoside resistance among enterococci and group A streptococci. J Antimicrob Chemother 1992; **30**: 651–9.
25. Vanhoof R, Godard C, Content J, Nyssen HJ, Hannecart-Pokorni E, Belgian Study Group of Hospital Infections. Detection by polymerase chain reaction of genes encoding aminoglycoside-modifying enzymes in methicillin-resistant *Staphylococcus aureus* isolates of epidemic phage types. J Med Microbiol 1994; **41**: 282–90.
26. Schmitz FJ, Verhoef J, Fluit AC, SENTRY Participants Group. Prevalence of aminoglycoside resistance in 20 European university hospitals participating in the European SENTRY antimicrobial surveillance programme. Eur J Clin Microbiol Infect Dis 1999; **18**: 414–21.
27. Ma X, Kudo M, Takahashi A, Kanimoto K, Ike Y. Evidence of nosocomial infection in Japan caused by high-level gentamicin-resistant *Enterococcus faecalis* and identification of the pheromone-responsive conjugative plasmid encoding gentamicin resistance. J Clin Microbiol 1998; **36**: 2460–4.
28. Hirakata Y, Yamaguchi T, Izumikawa K, et al. In vitro susceptibility studies and detection of vancomycin resistance genes in clinical isolates of enterococci in Nagasaki, Japan. Epidemiol Infect 1997; **119**: 175–81.
29. Eliopoulos GM, Farber BF, Murray BE, Wennersten C, Moellering RC Jr. Ribosomal resistance of clinical enterococcal to streptomycin isolates. Antimicrob Ag Chemother 1984; **25**: 398–9.
30. Matsumura M, Katakura Y, Imanaka T, Aiba S. Enzymatic and nucleotide sequence studies of a kanamycin-inactivating enzyme encoded by a plasmid from thermophilic bacilli in comparison with that encoded by plasmid pUB110. J Bacteriol 1984; **160**: 413–20.
31. Ounissi H, Courvalin P. Appendix B. Nucleotide sequences of streptococcal genes. In: Ferretti JJ, Curtis R III, eds. Streptococcal genetics. Washington D.C: American Society for Microbiology, 1987: 275.
32. Murphy E. Nucleotide sequence of a spectinomycin adenyltransferase AAD(9) determinant from *Staphylococcus aureus* and its relationship to AAD(3″)(9). Mol Gen Genet 1985; **200**: 33–9.
33. Trieu-Cuot P, Courvalin P. Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3′5″-aminoglycoside phosphotransferase type III. Gene 1983; **23**: 331–41.