ELSEVIER

International Journal of Antimicrobial Agents 15 (2000) 221–226

INTERNATIONAL JOURNAL OF
Antimicrobial
Agents

www.ischemo.org

Original article

Aminoglycoside-modifying enzymes in high-level streptomycin and gentamicin resistant *Enterococcus* spp. in Spain☆

R. del Campo [a], C. Tenorio [b], C. Rubio [a], J. Castillo [a], C. Torres [b,*], R. Gómez-Lus [a]

[a] *Departamento Microbiología, Facultad de Medicina, Universidad de Zaragoza, Zaragoza, Spain*
[b] *Area Bioquímica y Biología Molecular, Universidad de La Rioja, Avenida de la Paz 105, 26004 Logroño, Spain*

Received 2 November 1999; accepted 3 February 2000

Abstract

Aminoglycoside resistance was evaluated in 690 enterococcus strains isolated from different clinical sources originating from patients at the University Clinic Hospital of Zaragoza (Spain). The enterococci obtained from clinically significant samples (blood, urine, or exudates) showed more high-level resistance to gentamicin and streptomycin (65 and 42%, respectively) than those isolated from faecal samples (49 and 23%, respectively). Aminoglycoside-modifying enzymes (AME) from 119 of these high-level gentamicin and streptomycin resistant enterococcus strains were studied. The most frequent AMEs found were APH(3′) and AAC(6′)-APH(2″). More than one enzyme was detected in 71% of the strains (four different enzymes in 5% of the strains). Three *Enterococcus faecalis* strains had ANT(4′)(4″) enzymatic activity. Different enzymatic expressions of the bifunctional enzyme AAC(6′)-APH(2″) were demonstrated in strains in which the complete *aac(6′)-aph(2″)* gene was detected by PCR and hybridization: (i) AAC(6′) + APH(2″) activity; (ii) AAC(6′) only; (iii) APH(2″) only; and (iv) no activity of AAC(6′) or APH(2″).
© 2000 Elsevier Science B.V. and International Society of Chemotherapy. All rights reserved.

Keywords: Aminoglycoside-modifying-enzymes; *Enterococcus*; Gentamicin; Streptomycin

1. Introduction

Nosocomial infections caused by enterococci are becoming increasingly important. Enterococci have a natural low level resistance to aminoglycosides (Ag). In addition, enterococci can also show high level resistance to aminoglycosides (HLR-Ag) [1]. This is generally due to the acquisition of genes coding for aminoglycoside-modifying enzymes (AME) [2]. HLR-Ag in enterococci precludes any synergistic role of these antibiotics (generally gentamicin or streptomycin) with cell wall-active agents (such as penicillin or vancomycin). Such a combination may be important in the treatment of serious enterococcal infections [3].

Different AMEs have been detected in *Enterococcus* spp. [4–11]. High-level gentamicin resistance in enterococci is generally due to the bifunctional enzyme AAC(6′)-APH(2″) which also confers resistance to other aminoglycosides such as tobramycin, kanamycin, netilmicin and amikacin. The gene coding for this enzyme has been reported previously [9]. Other genes related to high or moderate levels of gentamicin resistance, *aph(2″)-Id* and *aph(2″)-Ic*, have been described in enterococci [5,11]. High-level resistance to streptomycin in enterococci can be mediated by the ANT(6) and ANT(3″)(9) enzymes [6,12]. Ribosomal mutation may also be involved in streptomycin resistance (MIC of > 32 000 mg/l) [13]. The ANT(4′)(4″) enzyme, that modifies kanamycin, tobramycin and amikacin, has been previously detected in *Enterococcus faecium* [4] as well as in strains of staphylococci [12].

The aim of this study was to determine the HLR-Ag frequency as well as the mechanism of resistance involved in enterococci isolated from clinically significant samples and from faecal samples.

☆ Part of this work was presented at the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA [Abstract No. C94].

* Corresponding author. Tel.: 34-941-299284; fax: 34-941-299274.
E-mail address: carmen.torres@daa.unirioja.es (C. Torres)

0924-8579/00/$20 © 2000 Elsevier Science B.V. and International Society of Chemotherapy. All rights reserved.
PII: S0924-8579(00)00169-2

2. Materials and methods

2.1. Bacterial strains and susceptibility testing

A total of 690 strains of various *Enterococcus* spp., isolated from different patients at the University Clinic Hospital of Zaragoza (Spain), between April and November 1995, were included in this study. The origins of the strains were as follows: urine samples (168 *E. faecalis*, seven *E. faecium* and four *E. durans*), blood/exudates (91 *E. faecalis*, six *E. faecium* and three *E. durans*) and faecal samples (29 *E. faecalis*, five *E. faecium* and 377 *Enterococcus* spp.). The enterococcal strains from the faecal samples were isolated on non-selective media (chocolate agar), and only one enterococcus strain per sample was selected for further studies. Strains from different origins were identified to the species level using the commercial PASCO system (Difco), the API 20 STREP system (BioMerieux) and various biochemical tests [14]. High-level gentamicin and streptomycin resistance were investigated using the agar diffusion method with high charge disks [15]. Lack of inhibition zones around disks of gentamicin (120 μg per disk) or streptomycin (300 μg per disk) were indicative of high-level aminoglycoside resistance. Inhibition zones of ≥ 10 mm suggested absence of high-level aminoglycoside resistance. When inhibition zones of 7–9 mm were detected, MICs were performed. High-level aminoglycoside resistance was taken to be an MIC > 2000 and > 500 mg/l for high level streptomycin and gentamicin resistance, respectively.

2.2. Testing for aminoglycoside-modifying enzymes

The AMEs were identified in sonic extracts of resistant enterococci obtained by ultrasonic disruption [16], using the phosphocellulose paper-binding assay as described previously [17]. The reaction was allowed to proceed for 30 min at 37°C. The bacterial strains to be analyzed were grown in brain heart infusion broth (Difco, Detroit, MI) supplemented with a subinhibitory gentamicin concentration (50 mg/l). Aminoglycosides used as substrates were: gentamicin C1a, gentamicin C1, tobramycin, netilmycin, amikacin, dactinomycin, streptomycin, spectinomycin, neomycin, butirosin, and lividomycin.

2.3. Mating experiments

Filter mating experiments were performed as previously described [18] using *E. faecalis* strain JH2-2 as the recipient (plasmid free, resistant to rifampicin and fusidic acid but without high-level resistance to aminoglycosides) [19], with a donor/recipient ratio of 1:10. Transconjugants were selected on brain heart infusion agar (Difco) supplemented with rifampicin (100 mg/l), fusidic acid (20 mg/l) and gentamicin (500 mg/l).

2.4. PCR amplification

Amplifications of the *aac(6′)-aph(2″)* and *aph(2″)-Id* genes used PCR, primers and conditions described previously [11,20]. *Escherichia coli* JM 109/SF 815A and *E. casseliflavus* UC73 (kindly supplied by P. Courvalin and J.W. Chow, respectively) were used for *aac(6′)-aph(2″)* and *aph(2″)-Id* amplifications, respectively; the *E. faecalis* strain JH2-2 was used as a negative control. The DNA for the PCR reactions was obtained using InstaGene™ Purification Matrix (BioRad) according to the manufacturer's instructions.

2.5. Hybridization

Probes were obtained as follows: *aac(6′)-Ie* as a 781 bp *Alu*I-*Sca*I fragment from the plasmid pSF 815AC, *aph(2″)-Ia* as a 1045 bp *Sca*I-*Alu*I fragment from the plasmid pSF 940AP and *aac(6′)-aph(2″)* as a 1500 bp *Alu*I fragment from the plasmid pSF 815A(9) (all these probes were kindly supplied by P. Courvalin).

For the dot blot hybridization, DNA was obtained by the alkaline method and transferred onto nylon membrane (Hybond, Amersham) as previously described [16]. For the Southern blot hybridizations, DNA was digested with *Alu*I according to the manufacturer's instructions and transferred onto nylon membrane using a vacuum system. The probes were labelled by random primer labelling (Rediprime, Amersham) with [^{32}P]dCTP (Redivue, Amersham). Prehybridizations and hybridizations were carried out in a rapid hybridization buffer (Amersham) at 65°C for 30 min and 18 h, respectively. The filters were washed twice at 65°C in $2 \times$ SSC ($1 \times$ SSC in 0.15 M NaCl and 0.015 M sodium citrate)-0.1% SDS, once with $1 \times$ SSC-0.1% SDS and once with $0.7 \times$ SSC-0.1% SDS. Autoradiography was carried out by exposing X-ray film (X-Omat, Eastman Kodak Co., Rochester, NY) to the filters for 72 h at -80°C.

3. Results and discussion

A total of 690 strains of *Enterococcus* spp. isolated from clinically significant samples ($n = 279$) and faecal samples ($n = 411$) were examined for the presence of high-level resistance to streptomycin and gentamicin by using an agar diffusion method with high charge disks and also by MIC determination. *Enterococcus* strains isolated from blood, exudate and urine samples showed higher levels of HLR to both streptomycin and gentamicin (60 and 70%, 60 and 36.6%, 68 and 43%, respectively) than those obtained from faecal samples (49.1 and 23.1%) (Table 1). Taking into account all the enterococcal strains investigated in this study, HLR to streptomycin was more frequently detected (55.6%)

Table 1
High level streptomycin and gentamicin resistance in 690 strains of *Enterococcus* spp. from different origins

Enterococci origin	No.	High level resistance[a] no. (%)		
		STR	GEN	STR+GEN
Urine	179	122 (68.2)	77 (43)	72 (49.7)
Exudate	90	54 (60)	33 (36.6)	31 (34.4)
Blood	10	6 (60)	7 (70)	4 (40)
Faeces	411	202 (49.1)	95 (23.1)	88 (21.4)
Total	690	384 (55.6)	212 (30.7)	195 (28.3)

[a] STR, streptomycin; GEN, gentamicin.

than HLR to gentamicin (30.7%). The occurrence of enterococci showing HLR-Ag varies substantially depending on the geographic location and the setting; frequencies of 7.5–65% have been reported for HLR to gentamicin in clinical enterococcal strains and frequencies of 21–51% for HLR to streptomycin [21–30].

A total of 119 strains of the 195 high-level gentamicin and streptomycin resistant enterococcal strains found in this study, were randomly selected for analysis of the detection of AMEs by the radioenzymatic method. The strains comprised: 50 *E. faecalis*, one *E. faecium* and one *E. durans* from urine samples, 18 *E. faecalis* and one *E. durans* from blood/exudates and 28 *E. faecalis*, five *E. faecium* and 14 *Enterococcus* spp. from faecal samples. The different AMEs detected in these 119 high-level gentamicin and streptomycin resistant enterococci are shown in Table 2. The most frequently detected AMEs were APH(3′) (67.1%) and AAC(6′)-APH(2″) (64.7%). These data suggest a wide dissemination of genetic determinants for kanamycin resistance, even though the strains investigated in this study were selected only for gentamicin and streptomycin resistance, these being aminoglycosides that are not substrates for APH(3′). In other studies, APH(3′)-III and AAC(6′)-APH(2″) were the most frequent enzymes detected in HLR-Ag enterococcal strains [31,32]. Other AMEs were also detected in this study: ANT(6) (30.3%), APH(3″) (13.4%), APH(2″) (9.2%), AAC(6′) (9.2%), ANT(3″)(9) (2.5%) and ANT(4)(4″) (2.5%). In 15 strains, no AMEs were detected by the radioenzymatic assay (12.6%).

In 87 of the 119 high-level gentamicin and streptomycin resistant strains analyzed, more than one AME was identified in the same strain (Table 2). The most common AME association detected was AAC(6′)-APH(2″) + APH(3′). It was frequently found in combi-

Table 2
Combinations of aminoglycoside-modifying enzymes in 119 enterococcal strains with HLR to both gentamicin and streptomycin

Aminoglycoside-modifying-enzymes	*E. faecalis*		Other enterococcal species	
	Clinical isolates ($n=68$)	Faecal isolates ($n=29$)	Clinical isolates ($n=3$)	Faecal isolates ($n=19$)
AAC(6′)-APH(2″)+APH(3′)	16	6		9
AAC(6′)-APH(2″)+APH(3′)+ANT(6)	12	8		3
AAC(6′)-APH(2″)	5	1		2
AAC(6′)-APH(2″)+APH(3′)+APH(3″)	5	2		1
APH(2″)+APH(3′)	3		1[a]	
AAC(6′)	3			
AAC(6′)+APH(3′)	3			
AAC(6′)-APH(2″)+APH(3″)	1	1		
AAC(6′)+APH(3′)+ANT(6)	1		1[a]	
AAC(6′)+ANT(6)	2			
APH(2″)+APH(3′)+ANT(6)+ANT(4)(4″)	1	1		
APH(2″)+APH(3′)+APH(3″)	1			
APH(2″)	1			
APH(3′)			1[b]	
AAC(6′)+APH(3′)+APH(3″)	1			
APH(2″)+APH(3″)	1			
APH(2″)+APH(3′)+ANT(3″)(9) +ANT(4)(4″)		1		
AAC(6′)-APH(2″)+ANT(6)	1	1		1
ANT(6)		3		1[b]
AAC(6′)-APH(2″)+APH(3′)+ANT(3″)(9)		2		
APH(2″)+APH(3′)+ANT(6)		1		
No aminoglycoside-modifying-enzymes	11	2		2

[a] *E. durans*.
[b] *E. faecium*.

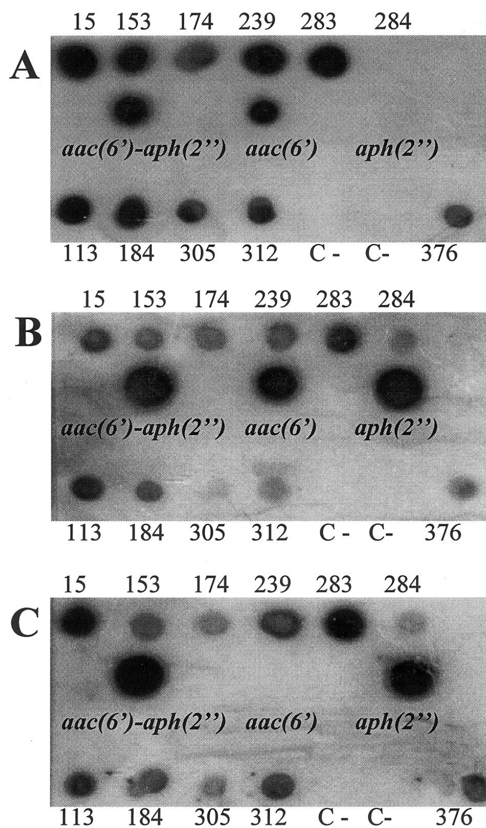

Fig. 1. Dot blot hybridization using genomic DNA of *Enterococcus* spp. with different expressions of the bifunctional enzyme: (1) AAC(6′)+APH(2″) (*E. faecalis* 305, 312, 376); (2) AAC(6′) (*E. faecalis* 15, 153, 174, 283 and *E. durans* 239); (3) APH(2″) (*E. faecalis* 113, 184). Probes: (a) *aac(6′)-Ie*; (b) *aph(2″)-Ia*; and (c) *aac(6′)-aph(2″)*.

nation with other modifying enzymes such as ANT(6), APH(3″) or ANT(3″)(9). In this way, the combination of AAC(6′)-APH(2″)+APH(3′), either alone or with other enzymes, was found in 48.5% of clinical *E. faecalis* strains, in 62% of the faecal enterococcal strains and in 63% of the non-*E. faecalis* strains obtained from faecal samples (Table 2). Lower detection rates of these two enzymes have been found by other authors (4.1%) (31). In 36 *E. faecalis* strains (37%), at least three different AMEs were observed (30.8% in *E. faecalis* strains of clinically significant samples and 51.7% in *E. faecalis* strains of faecal samples); in three of these strains, four different resistance mechanisms were detected. This high diversity of AME in one strain is also common in Gram negative rods [10]. The ANT(4′)(4″) enzyme was found in three *E. faecalis* strains (two isolated from faecal samples and one strain isolated from a urine sample), always associated with APH(3′) and APH(2″) without AAC(6′) activity. This ANT(4′)(4″) enzyme has been reported in both staphylococcal and *E. faecium* strains [12]; however, to our knowledge, this is the first time that this enzyme has been described in *E. faecalis* strains. These findings suggest the dissemination of the *ant(4′)-Ia* gene between different enterococcal species.

In 88 out of 119 enterococcal strains studied, a gentamicin resistance enzymatic mechanism was detected (77 with AAC(6′)-APH(2″) and 11 with APH(2″)). In 53 strains of the same group, an enzymatic mechanism for streptomycin resistance was found: ANT(6) (31 *E. faecalis*, three *E. faecium*, one *E. durans* and two *Enterococcus* spp.), ANT(3″)(9) (three *E. faecalis*) and APH(3″) (12 *E. faecalis* and one *Enterococcus* spp.). The detection of the *aadA* gene coding for a streptomycin/spectinomycin adenylyltransferase ANT(3″)(9) in *E. faecalis* has been recently described [6]. In our study, the ANT(6) enzyme was detected in 32% of the streptomycin resistant *E. faecalis* strains (25% in clinically significant strains and 48% in faecal strains). Ounissi et al. [12] found a positive hybridization with an *ant(6)-Ia* probe in 87% of streptomycin-resistant enterococci.

In 77 of the 119 high-level gentamicin and streptomycin resistant enterococcal strains, a typical expression of the bifunctional enzyme AAC(6′)-APH(2″) was detected. However, a dissociate expression was observed in 22 strains (20 *E. faecalis* and 2 *E. durans*). Eleven of these strains showed AAC(6′) without APH(2″) activity and 11 strains the opposite, APH(2″) without AAC(6′) activity. In the remaining 20 strains, no enzymatic activities compatible with AAC(6′) or APH(2″) were found. The dissociate expression (AAC(6′) without APH(2″) activity) could be confused with the activity of the enzyme codified by *aph(2″)-Id*, described recently in enterococci [11]. In all our 42 strains with dissociated or no enzymatic activity, a positive PCR amplification for the *aac(6′)-aph(2″)* gene was obtained with negative amplification for the *aph(2″)-Id* gene. In all these reactions, positive and negative results were obtained with positive and negative controls, respectively. Dot blot hybridization experiments using *aac(6′)-Ie*, *aph(2″)-Ia* and *aac(6′)-aph(2″)* probes gave positive results in 4/4 strains with AAC(6′) without APH(2″) activity (*E. faecalis* 15, 174, 283 and *E. durans* 239) and in 2/2 strains with APH(2″) without AAC(6′) (*E. faecalis* 113 and 184) (Fig. 1). Southern blot hybridization of genomic DNA digested with *Alu*I from *E. faecalis*, with different expressions of the bifunctional enzyme, was carried out using *aac(6′)-aph(2″)* as a probe; a positive hybridization was obtained on a 1.5 Kb band in 3/3 strains with AAC(6′)-APH(2″) activity and in 1/2 strains with only AAC(6′) activity. On the other hand, a positive hybridization on two bands of 0.9 and 1.5 Kb was detected in the *E. faecalis* strain 283 (with only AAC(6′) activity) (data not shown). Dissociate expression of the bifunctional enzyme has been previously reported in staphylococci [33]. The structures that carry the *aac(6′)-aph(2″)* gene in *E. faecalis* and other enterococcal strains are highly diverse [34–39].

The transfer of aminoglycoside resistance determinants was carried out by conjugation in six enterococcus strains with different expressions of the bifunctional enzyme, using *E. faecalis* strain JH22 as recipient. In all cases, the gentamicin resistance was transferred but no plasmids were observed in the donor and transconjugant strains. Complete AAC(6′)-APH(2″) activity was detected by the radioenzymatic assay and a positive *aac(6′)-aph(2″)* PCR amplification for all the transconjugant strains was obtained (data not shown). Transconjugants obtained with donors which were *enterococcus* strains with different expressions of the bifunctional enzyme, expressed both AAC(6′) and APH(2″) activities.

The results of this study show that HLR to streptomycin and gentamicin is frequently detected in enterococcal strains isolated from clinically significant samples and lower rates of resistance are detected in those strains isolated from faecal samples. Different AMEs are implicated in aminoglycoside resistance and three or more enzymes are usually involved.

Acknowledgements

We thank P. Courvalin for providing us with the *aac(6′)-aph(2″)*, *aac(6′) and aph(2″)* probes and J.W. Chow for the *E. casseliflavus* UC73 strain. We thank A. Portillo for helping us with bacterial identification. R. del Campo was supported by a grant from the Diputacion General de Aragon of Spain (P49/97).

References

[1] Murray BE. The live and times of the *Enterococcus*. Clin Microbiol Rev 1990;3:46–65.
[2] Carlier C, Courvalin P. Resistance of streptococci to aminoglycoside–aminocyclitol antibiotics. In: Schlessinger D Jr, editor. Microbiology–1982. Washington, DC: American Society for Microbiology, 1982:162–6.
[3] Leclercq R, Dutka-Malen S, Brisson-Noel A, et al. Resistance of enterococci to aminoglycosides and glycopeptides. Clin Infect Dis 1992;15:495–501.
[4] Carlier C, Courvalin P. Emergence of 4′-4″-aminoglycoside nucleotidyltransferase in enterococci. Antimicrob Agents Chemother 1990;34:1565–9.
[5] Chow JW, Zervos MJ, Lerner SA, et al. A novel gentamicin resistance gene in *Enterococcus*. Antimicrob Agents Chemother 1997;41:511–4.
[6] Clark NC, Olsvik O, Swenson JM, Spiegel CA, Tenover FC. Detection of streptomycin/spectinomycin adenyltransferase gene (*aadA*) in *Enterococcus faecalis*. Antimicrob Agents Chemother 1999;43:157–60.
[7] Courvalin P, Shaw WV, Jacob AE. Plasmid-mediated mechanisms of resistance to aminoglycoside-aminocyclitol antibiotics and to chloramphenicol in group D streptococci. Antimicrob Agents Chemother 1978;13:616–725.
[8] Courvalin P, Carlier C, Collatz E. Plasmid-mediated resistance to aminocyclitol antibiotics in group D streptococci. J Bacteriol 1980;143:541–51.
[9] Ferreti JJ, Gilmore KS, Courvalin P. Nucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase enzyme in *S. faecalis* and identification and cloning of gene regions specifying the two activities. J Bacteriol 1986;167:631–8.
[10] 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.
[11] Tsai SF, Zervos MJ, Clewell DB, Donabedian SM, Sahm DF, Chow JW. A new high-level gentamicin resistance gene, *aph(2″)-Id*, in *Enterococcus*. Antimicrob Agents Chemother 1998;42:1229–32.
[12] Ounissi H, Derlot E, Carlier C, Courvalin P. Gene homogeneity for aminoglycoside-modifying enzymes in gram-positive cocci. Antimicrob Agents Chemother 1990;34:2164–8.
[13] Eliopoulos GM, Farber BF, Murray BE, Wennersten C, Moellering RC Jr. Ribosomal resistance of clinical enterococcal isolates to streptomycin. Antimicrob Agents Chemother 1984;25:398–9.
[14] Facklam RR, Collins MD. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. J Clin Microbiol 1989;27:731–4.
[15] Sahm DF, Torres C. High-content aminoglycoside disks for determining aminoglycoside-penicillin synergy against *Enterococcus faecalis*. J Clin Microbiol 1988;26:257–60.
[16] Maniatis T, Fritsch EF, Sambrook J. Molecular Cloning, A Laboratory Manual. New York: Cold Spring Harbor, 1982.
[17] Haas MJ, Dowding JE. Aminoglycoside-modifying-enzymes. In: Methods in Enzymology. XLIII. Antibiotics. New York: Academic Press, 1975:611–40.
[18] Dunny GM, Craig RA, Carron R, Clewell DB. Plasmid transfer in *Streptococcus faecalis*. Production of multiple sex pheromones by recipients. Plasmid 1979;2:454–65.
[19] Jacob AE, Hobbs SJ. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J Bacteriol 1974;117:360–72.
[20] van de Klunder JAM, Vliegenthart JS. PCR detection of genes coding for aminoglycoside modifying enzymes. In: Persing DH, Smith TF, Tenover FC, White TJ, editors. Diagnostic Molecular Microbiology. Washington, DC: American Society for Microbiology, 1993:547–52.
[21] Bartoloni A, Stefani S, Orsi A, Nicoletti A, Difonzo AR, Paradisi F. High-level aminoglycoside resistance among enterococci isolated from blood cultures. J Antimicrob Chemother 1992;29:729–30.
[22] Caballero-Granado FJ, Cisneros JM, Luque R, et al. Comparative study of bacteremias caused by *Enterococcus* spp. with and without high-level resistance to gentamicin. J Clin Microbiol 1998;36:520–5.
[23] Coque TM, Arduino RC, Murray BE. High-level resistance to aminoglycosides: comparation of community and nosocomial fecal isolates of enterococci. Clin Infect Dis 1995;20:1048–51.
[24] Guiney M, Urwin G. Frequency and antimicrobial susceptibility of clinical isolates of enterococci. Eur J Clin Microbiol Infect Dis 1993;12:362–6.
[25] Ma X, Kudo M, Takahashi A, Tanimoto K, Ike Y. Evidence of nosocomial infection in Japan caused by high-level gentamicin-resistant *E. faecalis* and identification of the pheromone-responsive conjugative plasmid encoding gentamicin resistance. J Clin Microbiol 1988;36:2460–4.
[26] Murray BE, Tsao J, Panida J. Enterococci from Bangkok, Thailand, with high-level resistance to currently available aminoglycosides. Antimicrob Agents Chemother 1983;23:799–802.
[27] Smyth EG, Stevens PJ, Holliman RE. Prevalence and susceptibility of highly gentamicin resistant *E. faecalis* in a South London teaching hospital. J Antimicrob Chemother 1989;26:1287–91.

[28] Vandamme P, Vercauteren E, Lammens C, et al. Survey of enterococcal susceptibility patterns in Belgium. J Clin Microbiol 1996;34:2572–6.

[29] Watanakunakorn C. Rapid increase in the prevalence of high-level aminoglycoside resistance among enterococci isolated from blood cultures during 1989–1991. J Antimicrob Chemother 1992;30:289–93.

[30] Watanakunakorn C, Patel R. Comparison of patients with enterococcal bacteremia due to strains with and without high-level resistance to gentamicin. Clin Infect Dis 1993;17:74–8.

[31] 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.

[32] Weems JJ, Lowrance JH, Baddour LM, Simpson WA. Molecular epidemiology of nosocomial, multiply aminoglycoside resistant *E. faecalis*. J Antimicrob Chemother 1989;24:121–30.

[33] Goñi MP. Caracteristicas genéticas y bioquímicas de la enzima bifuncional AAC(6′)IV/APH(2″) y su posible expresión disociada. Ph.D. University of Zaragoza, Spain, 1991.

[34] Casetta A, Hoi AB, Cespedes GD, Horaud T. Diversity of structures carrying the high-level gentamicin resistance gene (*aac6-aph2*) in *Enterococcus faecalis* strains isolated in France. Antimicrob Agents Chemother 1998;42:2889–92.

[35] Hodel-Christian SL, Murray BE. Comparison of the gentamicin resistance transposon Tn*5281* with regions encoding gentamicin resistance in *Enterococcus faecalis* isolates from diverse geographic locations. Antimicrob Agents Chemother 1992;36:2259–64.

[36] Rice LB, Carrias LL, Marshall SH. Tn*5384*, a composite enterococcal element conferring resistance to erythromycin and gentamicin whose ends are directly repeat copies of IS*256*. Antimicrob Agents Chemother 1995;39:1147–53.

[37] Straut M, de Cespedes G, Horaud T. Plasmid-borne high-level resistance to gentamicin in *E. hirae*, *E. avium* and *E. raffinosus*. Antimicrob Agents Chemother 1996;40:1263–5.

[38] Straut M, de Cespedes G, Delbos F, Horaud T. Molecular typing of *E. faecalis* strains resistant to high levels of gentamicin isolated in Romania. J Antimicrob Chemother 1997;39:483–91.

[39] Thal LA, Chow JW, Clewell DB, Zervos MJ. Tn*924*, a chromosome-borne transposon encoding high level gentamicin resistance in *E. faecalis*. Antimicrob Agents Chemother 1994;38:1152–6.