

The prevalence and clonal expansion of high-level gentamicin-resistant enterococci isolated from blood cultures in a Dutch university hospital

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We studied the prevalence and clonality of high-level gentamicin-resistant enterococci (HLGRE) in a Dutch university hospital. Of 238 enterococcal strains isolated from blood cultures between 1991 and 1997, 57 were HLGRE. Genomic analysis of these strains revealed 19 different genotypes, two of which were encountered more frequently [type A (12/57), type B (23/57)]. The spread of these types largely explained the rise in HLGRE incidence from 14% in 1991 to 31% in 1997. However, the contribution of unique strains to the total HLGRE burden also increased from 4% to 16%. We conclude that both clonal expansion and the emergence of unique HLGRE have contributed significantly to the increasing incidence of HLGRE.

Introduction

Enterococci can cause serious infections, including bacteraemia and endocarditis.¹ Standard therapy with combinations of antibiotics generally results in a synergic, bactericidal effect.² A combination of a cell wall-active agent and an aminoglycoside is often used. In this and other regimens, susceptibility of enterococci to the aminoglycoside is critical to achieve the bactericidal effect. The aminoglycoside-modifying enzymes are encoded on plasmids or transposons that are transferable to other enterococcal species by conjugation.³ It is therefore not surprising that since the first high-level gentamicin-resistant enterococci (HLGRE) were reported in 1979 in France,⁴ the incidence of HLGRE in hospitals has increased worldwide.^{2,5} High-level gentamicin resistance arises in enterococci when they acquire or mutate genes for aminoglycoside-modifying enzymes, capable of inactivating the aminoglycoside agents. The gene *aacA/aphD*, which encodes the bi-functional enzyme aminoglycoside-6'-*N*-acetyltransferase/2-*O*-nucleotidyltransferase [AAC(6')/APH(2'')], mediates resistance to all aminoglycosides except streptomycin and neomycin. The *aphA3* gene codes for aminoglycoside-3'-*O*-phosphoryltransferase-III [APH(3'')III] and confers resistance to streptomycin, kanamycin and amikacin. The *aadC* gene codes for aminoglycoside-4,4'-*O*-nucleotidyltransferase [ANT(4'4)], mediating resistance to

amikacin, kanamycin and tobramycin.⁶ Consequently, the synergic effect with β -lactam agents and the bactericidal effect of the combination are lost and the treatment of serious infections is compromised.⁷ The recent emergence of glycopeptide resistance in enterococci has further limited the therapeutic alternatives and has increased mortality sometimes up to 60–70%⁸. Therefore, it is important to study the local epidemiology of resistant microorganisms within a hospital setting. The aim of this study was to determine the prevalence of HLGRE isolated from blood cultures in our hospital from 1991 to 1997 and to study the local spread and genetic relatedness of these strains.

Materials and methods

Bacterial isolates

Enterococcal strains ($n = 243$) were isolated from blood cultures taken from 243 patients hospitalized at the Erasmus University Medical Center in Rotterdam between June 1991 and June 1997 [1991–1992 ($n = 34$), 1992–1993 ($n = 28$), 1993–1994 ($n = 42$), 1994–1995 ($n = 39$), 1995–1996 ($n = 48$), 1996–1997 ($n = 52$)]. Five strains from the 1991–1992 collection were lost and were not available for further study.

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Identification

A presumptive identification of all HLGRE was made on the basis of colony morphology, Gram's stain, catalase and pyrase (Dryslide Pyrkite, Difco Laboratories, Detroit, MI, USA). Definitive identification was done by RAPID ID32 STREP (bioMérieux, Hertogenbosch, The Netherlands). The identification strips were read after 5 and 24 h of incubation at 37°C.

Antimicrobial susceptibility tests

All enterococcal strains isolated were tested for high-level gentamicin resistance on Mueller–Hinton agar (Difco Laboratories) with Etest strips (AB Biodisk, Solna, Sweden) following the manufacturer's instructions. All plates were incubated at 37°C and read after 18 h.

Pulsed field gel electrophoresis

PFGE was performed as described in our previous studies.⁹ The gel was stained with ethidium bromide and de-stained in distilled water for 1 h before photography. The gel pictures were inspected visually by two different investigators. The PFGE patterns were interpreted according to Tenover *et al.*¹⁰ Isolates that differed by one to three bands, consistent with a single differentiating genetic event, were assigned a numbered subtype. Four or more band differences between two strains defined different genotypes.

DNA isolation and PCR detection of aminoglycoside resistance genes

DNA was isolated according to Boom *et al.*¹¹ Diagnostic PCR assays targeting the various resistance genes (*aphA3*; *aacA/aphD*; *aadC*) were performed as described by Van Asselt *et al.*⁷ Three different primer pairs were used: *aphA3* 5'-GCCGATGTGGATTGCGAAAA and *aphA3* 3'-GCTTGATCCCCAGTAAGTCA (292 bp); *aacA/aphD* 5'-CCAAGAGCAATAAGGGCATA and *aacA/aphD* 3'-CCAAGAGCAATAAGGGCATA (220 bp); *aadC* 5'-GCAAGGACCGACAACATTTC and *aadC* 3'-TGGACAGATGGTCATAACC (165 bp). Per reaction 50 pmol of each primer was included. A PCR aiming for the 16S rRNA gene was used in each reaction as an internal positive control [*rrs* 5'-GGATTAGATACCCTGGTAGTCC; *rrs* 3'-TCGTTGCGGGACTTAACCCAAC (340 bp)]. Amplification of DNA was performed in a Biomed thermocycler (Model 60, Theres, Germany), using pre-denaturation at 94°C for 5 min. This was followed by 32 cycles of 30 s at 94°C, 45 s at 55°C and 2 min at 72°C. Amplicons were analysed by electrophoresis on 2% agarose gels (Hispanagar; Sphaero Q, Leiden, The Netherlands) in the presence of a 100 bp DNA ladder (Gibco/BRL Life Technologies, Breda, The Netherlands).

Results

Antimicrobial susceptibility tests

During the study period the following HLGRE incidences were found; 1991–1992: 4/29 (14%), 1992–1993: 5/28 (18%), 1993–1994: 12/42 (29%), 1994–1995: 9/39 (23%), 1995–1996: 11/48 (23%) and 1996–1997: 16/52 (31%). All enterococcal strains isolated had gentamicin MICs of >1024 mg/L. Two out of 57 HLGRE were identified as *Enterococcus faecium*, the rest appeared to be *Enterococcus faecalis*.

Pulsed field gel electrophoresis

A total of 57 HLGRE isolates were analysed by PFGE. Most of the PFGE banding patterns consisted of 15–20 bands. Nineteen different genotypes were identified. However, two genotypes [respectively 12/57 (Type A) and 23/57 (Type B)] predominated (see Table and Figure 1). The incidence of these two epidemic PFGE types increased during the study period from 10% to 15%. The prevalence of strains with a unique PFGE pattern increased from 4% to 16%.

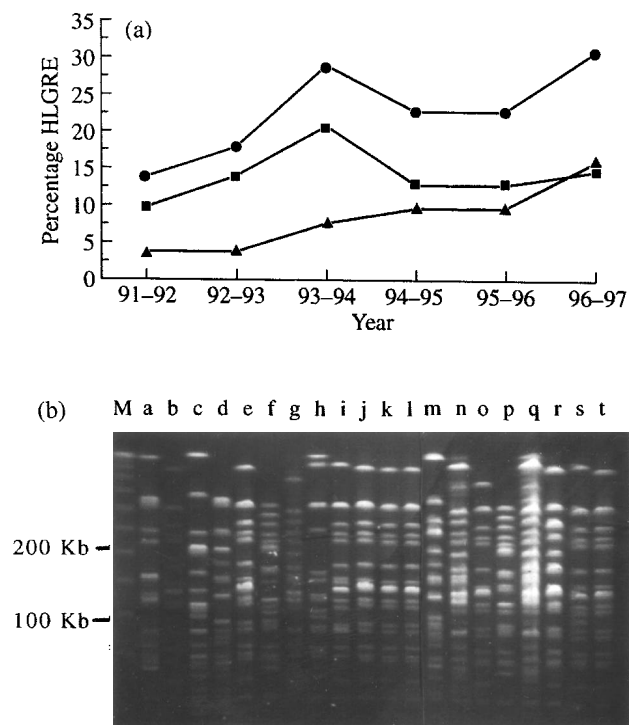


Figure. (a) The prevalence of clonal and unique HLGRE isolated from blood cultures in our hospital from 1991 to 1997. (b) Examples of PFGE patterns of HLGRE isolated from blood cultures. Strains b, e, i, j, k, l, o, s and t are clonal type A, strains n and q are type B. The lane marked M displays molecular length marker (50 kb ladder)

Table 1. Number and (percentages) of high-level gentamicin-resistant enterococci (HLGRE) isolated from blood cultures from June 1991 until June 1997

Period	Enterococcal isolates	HLGRE	PFGE type A	PFGE type B	<i>aacA/aphD</i>	<i>aacA/aphD</i> + <i>aphA3</i>	<i>aacA/aphD</i> + <i>aadC</i>
June 1991–1992	29	4 (14)	3 (10)	–	1	3	–
June 1992–1993	28	5 (18)	1 (4)	3 (11)	3	2	–
June 1993–1994	42	12 (29)	6 (14)	3 (7)	7	5	–
June 1994–1995	39	9 (23)	–	5 (13)	7	2	–
June 1995–1996	48	11 (23)	–	6 (13)	6	4	–
June 1996–1997	52	16 (31)	2 (4)	6 (12)	14	1	1

PCR

Genes that encode for aminoglycoside-modifying enzymes were found in all 57 HLGR enterococci. All 57 strains carried the gene *aacA/aphD*. Sixteen *E. faecalis* (28%) also had the *aphA3* gene and one *E. faecalis* (2%) contained the *aadC* gene (see Table). Nine of 12 clonal A-types carried both the *aacA/aphD* and *aphA3* genes. All clonal B-types identified during this study contained only the *aacA/aphD* gene.

Discussion

In recent years the enterococcus has emerged as a major nosocomial pathogen. Its propensity to acquire new resistance traits has led to the occurrence of multiply drug-resistant strains. Infections caused by these microorganisms are difficult to treat.¹² Aminoglycoside resistance was first reported in staphylococci in the 1970s, followed by reports of the acquisition of high-level gentamicin resistance in enterococci in the early 1980s. Although the clinical significance of HLGR in enterococci appears to be evident, no studies are available that describe its effect on morbidity and mortality.

In this study, we report a significant increase in the prevalence of HLGR in enterococci isolated from hospitalized patients with bloodstream infections, from 14% in 1991 to 31% in 1997. A similar rise in HLGRE detection was reported by others. Lavery *et al.*² documented an increase of HLGRE isolates from 17% to 60% in two Dublin hospitals during the period 1991–4. In South London a prevalence of 44% HLGRE was reported in the first quarter of 1991.¹³ A study in Oklahoma reported 16/27 (59%) enterococci isolated from blood that were highly resistant to gentamicin.¹⁴ Watanakunakorn¹⁵ reported an absence of HLGRE in blood cultures before 1985 in a teaching hospital in Ohio. However, in the periods 1985–1988 and 1989–1991 the prevalence of HLGRE increased from 9% to 36%. In contrast, a recent study by Barteloni *et al.*⁵ reported a significant decrease in the prevalence of *E. faecalis* highly resistant to gentamicin during the period 1993–1995, compared with the rate of HLGR in enterococci in 1990, 1991 and 1993. PFGE of the HLGRE found in our study revealed two possibly epidemic genotypes isolated in diverse years and different wards. These two epidemic enterococcal subtypes represent the majority of HLGR strains. However, this endemicity obscured another serious trend: unique types also increased in numbers during the years. Straut *et al.*¹⁶ reported a clonal spread of one HLGRE genotype. However, these strains were isolated from sources other than blood cultures. The emergence of new genotypes is a potential risk since these types may eventually colonize the hospital environment. Moreover, when selective pressure is applied (for example by antibiotics) these resistant strains may be selected.

With the use of the polymerase chain reaction we identified the genes that encode aminoglycoside-modifying enzymes in enterococci. All strains carried the gene *aacA/aphD*, 28% of all enterococci also carried the *aphA3* gene and 2% harboured the *aadC* gene. It remains to be assessed whether other genes involved in gentamicin resistance can be detected in the present collection of strains. Since all strains discussed are sensitive to vancomycin, the detection of the recently described, associated gene *aphA-Id* is not likely; also because this gene has only been documented in the absence of *aacA/aphD*.¹⁷

Nosocomial occurrence of HLGRE can follow either of two scenarios. First, HLGRE can be selected from the patients' gut flora, which presumes community-based carriage of HLGRE in otherwise healthy people. Since the prevalence of HLGRE in the community is unknown, surveillance studies involving non-hospitalized patients and healthy adults are warranted. Secondly, some strains of HLGRE may become endemic in the hospital and spread from one patient to another. Nosocomial spread of either resistant strains or resistance determinants may be reduced by prudent use of antimicrobial agents and by implementation of strict infection control measures.

The increasing and alarming problem of glycopeptide resistance in Gram-positive microorganisms worldwide has, somewhat diverted our attention from aminoglycoside resistance. However, against a background of increasing glycopeptide resistance, the increase in HLGR in enterococci reported in this study is a cause for additional alarm. More studies that clarify the epidemiology of aminoglycoside resistance in Gram-positive organisms in hospitals as well as in the community are required.

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