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Genetic relatedness among *Enterococcus faecalis* with transposon-mediated high-level gentamicin resistance in Swedish intensive care units

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We studied 45 isolates of *Enterococcus faecalis* with high-level gentamicin resistance (HLGR), all but one concomitantly resistant to ciprofloxacin, and 25 ciprofloxacin-resistant isolates without HLGR for genetic relatedness using pulsed-field gel electrophoresis (PFGE). *E. faecalis* were isolated from patients admitted to intensive care units at eight hospitals in southern Sweden from December 1996 through December 1998. Genomic analysis by PFGE resulted in three clusters of genetically related isolates (designated clusters I, II and III) and 23 unique clones. Cluster I was found predominantly in the eastern and central parts of southern Sweden and clusters II and III in south-western Sweden. Among the 45 isolates with HLGR, 69% belonged to cluster I, 20% to cluster II, and 11% had unique PFGE patterns, which suggests that the majority of isolates with HLGR are closely related. Among the 25 ciprofloxacin-resistant isolates without HLGR, 68% had unique PFGE patterns, 12% belonged to cluster I and 20% to cluster III, which suggests the ciprofloxacin-resistant isolates are not related. All isolates with HLGR contained the *aac(6′)Ie-aph(2″)Ia* gene, which was carried on a Tn*5281*-like transposon in all isolates except one. We conclude that HLGR in *E. faecalis* was mainly due to dissemination of genetically related clones during the time studied, and that HLGR in these isolates was due to the presence of the *aac(6′)Ie-aph(2″)Ia* gene.

Keywords: enterococcus, high-level gentamicin resistance, pulsed-field gel electrophoresis, transposon

Introduction

During recent decades, nosocomial infections caused by enterococci have become increasingly common in many countries.[1,2] Although enterococci have often been considered to be pathogens with low virulence, they are also known to cause serious infections such as bacteraemia and endocarditis.

A combination of a cell-wall active agent (i.e. ampicillin or vancomycin) and an aminoglycoside is recommended to achieve a synergic bactericidal effect in the treatment of serious enterococcal infections.[3] Unfortunately, enterococci can acquire high-level aminoglycoside resistance, which eliminates the synergic effect. In a 1997 European study including 27 countries, high-level gentamicin resistance (HLGR) in clinical isolates varied according to country from 1% to 49% (mean 23%), and concomitant resistance to ciprofloxacin was frequently encountered in these isolates.[4] In Sweden, the frequency of enterococci with HLGR has been reported to be low (2%), although there are local and regional differences.[5] In a previous study of enterococcal isolates collected between December 1996 and December 1998, based on clinical indications, from patients admitted to intensive care units (ICUs) at eight hospitals in southern Sweden, we have shown that 20% of *E. faecalis* isolates showed HLGR. No HLGR was found among *E. faecium*.[6]

Enterococci with HLGR have acquired genes that mediate production of aminoglycoside-modifying enzymes.[7,8] The most common and clinically important enzyme is the bifunctional enzyme Aac(6′)Ie-Aph(2″)Ia, with both adenyltransferase and phosphotransferase capacity. Enterococci that possess this enzyme are resistant to practically all commercially available aminoglycosides, i.e. gentamicin, amikacin, tobramycin, netilmicin and kanamycin, with the exception of streptomycin.[9] The corresponding gene, *aac(6′)Ie-Aph (2″)Ia*, has been found on plasmids, and has also been identified as being part of a

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transposon, located either on a plasmid or in the chromosomal DNA.[8] A conjugative transposon that carried the gene was characterized and designated Tn*5281* in 1991 by Hodel-Christian & Murray in an isolate from USA.[10] The aim of this study was to investigate the genetic relatedness of enterococci with HLGR and/or ciprofloxacin resistance, isolated from patients admitted to Swedish ICUs during a previous study conducted from 1996 through 1998,[6] and to detect and determine the location of the gene responsible for HLGR in these isolates.

Materials and methods

Bacterial isolates, susceptibility testing and breakpoints

Seventy *E. faecalis* isolates from a previous study that showed HLGR and/or ciprofloxacin resistance were chosen for the study of genetic relatedness.[6] Forty-five of these showed HLGR, and all except one were concomitantly resistant to ciprofloxacin. The remaining 25 *E. faecalis* isolates showed ciprofloxacin-resistance but not HLGR. Repeat isolates from the same patient were excluded. Identification of enterococci to the species level and susceptibility testing have been described previously.[6]

Three of the original 14 antimicrobial agents were re-tested to establish the antibiotype of each isolate: meropenem, ciprofloxacin and gentamicin. These agents were selected because they represent different groups of antimicrobial agents commonly used in ICUs. Resistant strains were defined according to the species-related MIC breakpoints of the Swedish Reference Group for Antibiotics (SRGA) (i.e. ciprofloxacin: S ≤ 0.12 mg/L, R >2 mg/L, meropenem: S ≤ 1 mg/L, R >8 mg/L, HLGR: ≥ 500 mg/L).[11]

Enterococcus faecalis ATCC 51299[12] and *E. faecalis* ATCC 29212 were used as positive and negative controls, respectively, in PCR detection of *aac(6′)Ie-aph(2″)Ia,* plasmid DNA preparations and detection of a Tn*5281*-like transposon by long-PCR and nested PCR.

Participating hospitals

The geographic locations of the participating hospitals are shown in Figure 1.

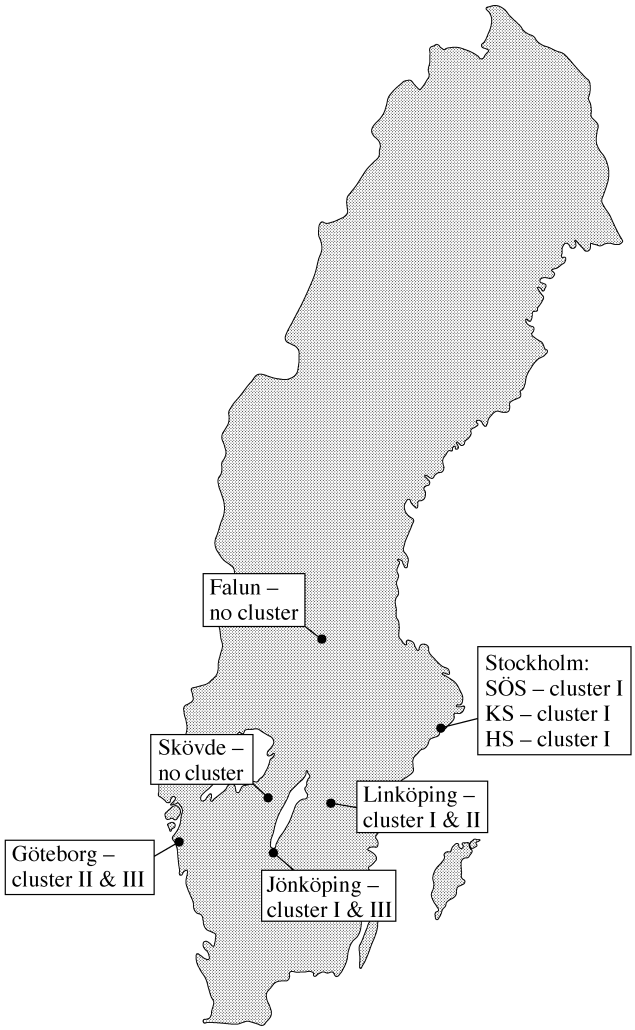

Figure 1. Map of Sweden demonstrating geographic location of hospitals and where genetically related isolates (cluster I, II and III) were found.

Pulsed-field gel electrophoresis

Genomic DNA extraction, endonuclease digestion and pulsed-field gel electrophoresis (PFGE) were carried out according to the GenePath Group 1 Reagent Instruction Manual Kit (Bio-Rad, Hercules, CA, USA), with some modifications of the electrophoretic conditions. Briefly, genomic DNA was prepared in agarose plugs and digested by *Sma*I restriction endonuclease (Bio-Rad). The electrophoresis was carried out using a CHEF DRII apparatus (Bio-Rad). The electrophoretic conditions were 14°C, 6 V/cm, 120° switch angle and a total run time of 24 h divided into two blocks; the first block had a run time of 20 h and a switch time ramp of 5–35 s, and the second block had a run time of 4 h and a switch time ramp of 5–10 s. This modification of the electrophoretic conditions gave a good separation of both small and large fragments.[13] The results of the PFGE were interpreted according to the criteria proposed by Tenover *et al.*, i.e. isolates with indistinguishable PFGE patterns were considered to be genetically related (a clone). Isolates differing from a clone by one to three bands, consistent with a single genetic event, were considered closely related to the clone and were assigned a certain subtype. Isolates that differed by four bands or more were considered to be genetically unrelated (unique clone).[14]

Plasmid preparation

The 45 isolates with HLGR, as well as the three isolates without HLGR belonging to cluster I (for details, see Results), were included for plasmid DNA preparation, PCR detection of the *aac(6′)Ie-aph(2″)Ia* gene and detection of a Tn*5281*-like transposon by long-PCR and nested PCR.

To extract plasmid DNA, a Qiagen Plasmid Mini Kit (Qiagen, GmbH, Hilden, Germany) was used, following a protocol for isolation of low-copy-number plasmids as recommended by the manufacturer. Minor modifications were added to the protocol. In brief, isolates were spread over a paper disc method (PDM) agar plate supplemented with defibrinated horse blood (AB Biodisk, Solna, Sweden), and grown overnight with a gentamicin disc (30 µg per disc) (AB Biodisk), to make sure that they had not lost the resistance gene. The bacteria were harvested directly from a single plate, resuspended in 2 mL of SET (200 mM sodium chloride, 25 mM EDTA, 20 mM Tris (pH 8.0)) and washed through centrifugation (2000 rpm, 4 min).[15] Before treatment with NaOH, bacteria were incubated with 10 mg/mL lysozyme as described elsewhere.[16] All plasmid DNA preparations were analysed by agarose gel electrophoresis followed by staining with ethidium bromide and visualized under UV light. Plasmid DNA preparations were analysed for presence or absence of contaminating chromosomal DNA by a PCR reaction with primers targeting the *parC* gene.[17]

PCR detection of aminoglycoside-modifying enzyme gene in bacterial DNA and plasmid preparations

For analysis of whole cell bacterial DNA, the specimens were grown on PDM plates supplemented with defibrinated horse blood with a gentamicin disc. Bacterial colonies close to the gentamicin disc were chosen for PCR amplification to make sure that they had not lost their resistance genes.

For analysis of plasmid DNA preparations, 2 µL of the plasmid DNA preparations was used as template for the PCR reaction. Primers were chosen as described earlier by Kobayashi *et al.*[18] to yield a PCR product of 675 bp. Amplifications were carried out with Ready-to-Go PCR lyophilized beads as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in a final volume of 25 µL, including 25 pmol of each primer, and supplemented with additional $MgCl_2$. The final reaction conditions were 2.5 mM $MgCl_2$, 1.5 units *Taq* DNA polymerase, 10 mM Tris–HCl pH 9.0, 50 mM KCl and stabilizers. After an initial denaturation procedure (94°C, 10 min), the reaction mixture was subjected to 30 PCR cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and primer extension (72°C, 2 min) in a programmable thermal controller (Mastercycler, Eppendorf, Hamburg, Germany). PCR products were analysed by agarose gel electrophoresis in the presence of a 100 bp DNA ladder. The gel was photographed in UV light and visually compared to the positive and negative controls.

Tn5281-like transposon detection by long-PCR and nested PCR.

To detect the presence of a Tn*5281*-like structure, a long-PCR protocol was used, including a Expand Long Template PCR system (Roche Diagnostics GmbH, Mannheim, Germany). Plasmid DNA preparations were used as templates and the IS256 forward primer (5′-CAGAACAGCTGGATCCTATGG-3′) as a single primer as described elsewhere.[19,20] The expected size of the amplicon is approximately 3470 bp. Amplifications were carried out as previously described[19] with some modification; extra $MgCl_2$ was added to each PCR reaction to a final concentration of 2.25 mM. After an initial denaturation procedure (94°C, 2 min) the reaction was subjected to 10 cycles of 94°C for 10 s, 57°C for 30 s and 68°C for 45 s, another 22 cycles of 94°C for 10 s, 57°C for 30 s and 68° C for 45 s + 20 s increase per cycle and a final elongation procedure of 68°C for 10 min in a programmable thermal cycler (PTC-100, MJ Research, SDS Falkenberg, Sweden) The long-PCR products were analysed by agarose gel electrophoresis as described in the previous section.

For nested PCR amplification, the long-PCR products were diluted 1:1000 with ultra pure water and 2 µL of the diluted long-PCR products were subjected to PCR amplification of *aac(6′)Ie-aph(2″)Ia* and analysed by agarose gel electrophoresis as described earlier.

Results

PFGE and antibiotypes

Seventy isolates were subjected to bacterial strain typing by PFGE. Most of the PFGE patterns consisted of 14–19 bands, resulting in three main banding patterns, designated clusters I, II and III, and 23 unique patterns (Figure 2). Thirty-four isolates belonged to cluster I and were found in five hospitals located in the eastern and central parts of southern Sweden, i.e. Stockholm (18 isolates), Linköping (14 isolates) and Jönköping (two isolates) (Table 1, Figure 1). Nine isolates belonged to cluster II, and with the exception of one isolate found in Linköping, all were found in Göteborg. Four isolates belonged to cluster III, three of which were found in Göteborg, and one of which was found in Jönköping (Table 1, Figure 1). Each cluster contained a clone with several subtypes. Cluster I had seven subtypes (IA–IG), cluster II had five subtypes (IIA–IIE), and cluster III had four subtypes (IIIA–IIID).

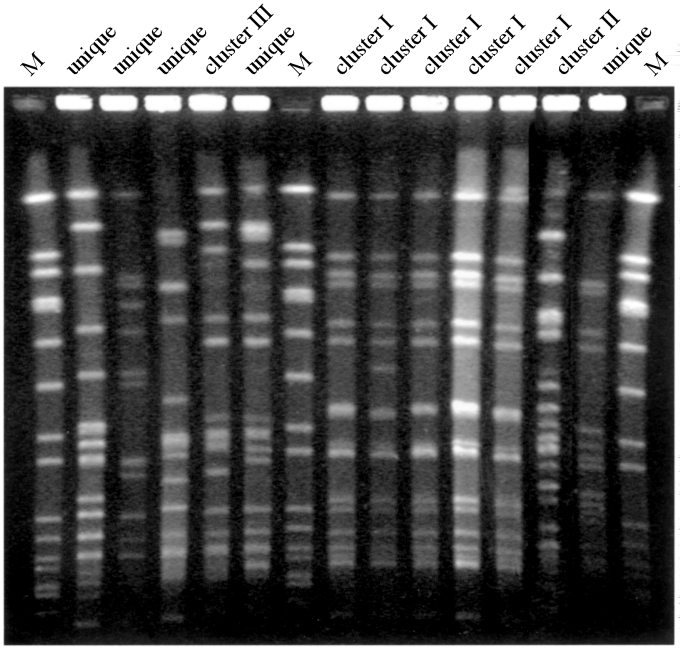

Figure 2. PFGE patterns.

Isolates with HLGR were found in six of eight hospitals, whereas isolates with ciprofloxacin resistance and no HLGR were found in all hospitals. Among the 45 isolates with HLGR, five isolates (11%) had unique PFGE patterns, 31 (69%) belonged to cluster I, and another nine isolates (20%) with HLGR belonged to cluster II (Table 1, Figure 3).

Among the 25 ciprofloxacin-resistant isolates with no HLGR, 18 (72%) had unique PFGE patterns, three (12%) belonged to cluster I, and four isolates (16%) belonged to cluster III (Table 1, Figure 3). Multiresistance, i.e. concomitant HLGR and resistance to ciprofloxacin and meropenem, was found in two of 34 isolates (6%) in cluster I, in all isolates (100%) in cluster II, in no isolates in cluster III, and in one of 23 isolates (4%) with unique PFGE patterns (Table 1).

PCR

The *aac(6′)Ie-aph(2″)Ia* gene was found in all isolates with HLGR as well as in the positive control *E. faecalis* ATCC 51299, both in bacterial DNA from whole cells as well as in plasmid DNA preparations. It was not present in any of the isolates without HLGR belonging to cluster I, nor in the negative control *E. faecalis* ATCC 29212.

Plasmid preparation

No visible bands were found after electrophoresis and ethidium bromide staining of plasmid preparations, except for four isolates (three with unique PFGE patterns and one belonging to cluster I, data not shown) where weak bands of approximately 20 kb were found. Nevertheless, the *aac(6′)Ie-aph(2″)Ia* gene was found in all 45 preparations from isolates with HLGR as well as in the positive control, *Enterococcus faecalis* ATCC 51299. However contaminating chromosomal DNA (*parC*) was found to be present in plasmid preparations.

Table 1. Location of isolates within clusters (same PFGE pattern, differing in ≤3 bands) or unique PFGE patterns with different antibiotypes

Cluster	Antibiotype			No. of isolates	Location of isolates
	gentamicin	ciprofloxacin	meropenem		
I	no HLGR	R	R	3	KS, HS, Linköping
I	HLGR	R	S+I	29	KS, HS, SÖS, Linköping, Jönköping
I	HLGR	R	R	2	Linköping
II	HLGR	R	R	9	Göteborg, Linköping
III	no HLGR	R	S+I	4	Göteborg, Jönköping
Unique	no HLGR	R	S+I	14	KS, SÖS, Linköping, Skövde, Falun
Unique	no HLGR	R	R	4	KS, HS, Göteborg,
Unique	HLGR	S+I	S+I	1	HS
Unique	HLGR	R	S+I	2	KS, HS
Unique	HLGR	R	R	2	KS, SÖS

S, susceptible; I, indeterminate; R, resistant. KS, Karolinska Sjukhuset; HS, Huddinge Sjukhus; SÖS, Södersjukhuset, all located in Stockholm.

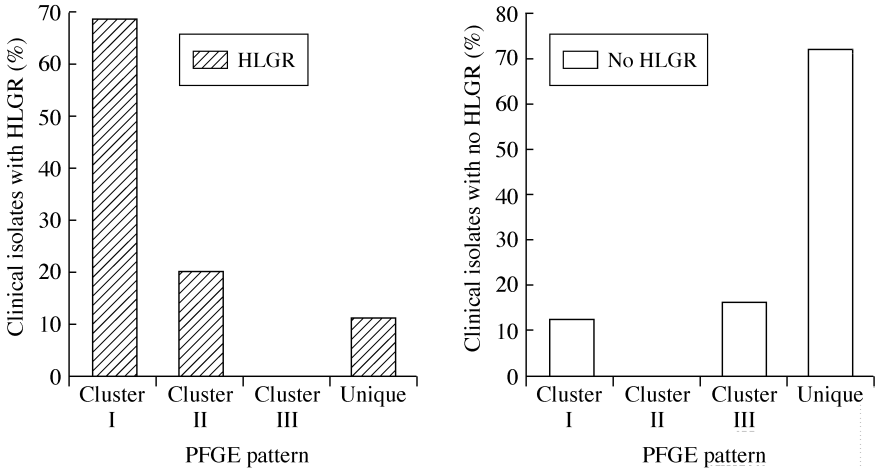

Figure 3. Clinical isolates within different clusters with HLGR ($n = 45$) or no HLGR ($n = 25$).

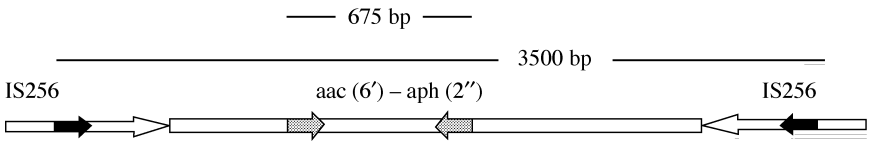

Figure 4. Schematic picture of Tn*5281*. The resistance gene *aac(6′)Ie-aph(2″)Ia* is flanked by IS256 in inverse orientations. The primers used in the nested PCR are shown as patterned arrows. Filled arrow = IS256 forward primer. Striped arrow = aac(6′)-aph(2″) forward and reverse primers.

Detection of a Tn5281-like transposon

For long-PCR, only one primer was used, making use of the fact that in Tn*5281*, *aac(6′)Ie-aph(2″)Ia* is flanked by IS256 in inverse directions so that a forward primer would anneal to the inverted IS256 and act as a 'reverse primer' (Figure 4). A PCR product of approximately 3500 bp was present in all plasmid DNA preparations of isolates with HLGR except one (KS 20, with unique PFGE pattern). Weak bands of approximately 2500 bp were also found in several plasmid DNA preparations suggesting the presence of another IS256 flanked structure in these isolates. Nested PCR, i.e. detection of the *aac(6′)Ie-aph(2″)Ia* gene on long-PCR products, were positive in all isolates with HLGR including KS 20, but was not found in the three isolates without HLGR belonging to cluster I (Table 1).

Discussion

Our results indicate dissemination of genetically related *E. faecalis* isolates, with HLGR among patients in Swedish ICUs. This could be one explanation for the higher frequency of *E. faecalis* isolates with HLGR that was seen in our study compared with earlier Swedish reports.[5,6]

With respect to these results, one can speculate regarding two possible scenarios. First, a clone of enterococci with HLGR could be common and still remain undetected among healthy carriers in the community. If such a carrier was admitted to hospital and received antimicrobial chemotherapy, enterococci with HLGR could be selected from the patient's gut flora and might then be the cause of

an infection. Few studies have been conducted on the prevalence of enterococci with HLGR in healthy individuals. Silverman *et al.* examined stool samples from 200 patients within 48 h after admission to hospital for carriage of resistant bacteria as well as risk factors for carriage. They found 78 *E. faecalis* isolates, of which 11% showed HLGR. Prior hospitalization was significantly more common in patients carrying enterococci with HLGR than in controls.[21] Others have detected enterococci with HLGR in 0–1% of healthy carriers.[22–24]

In our study we focused on patients admitted to the ICU, and the prevalence of carriage in healthy individuals was not assessed.

In the second scenario, clones of resistant enterococci might have become established in the ICU and spread from one patient to another. Nosocomial transmission of resistant bacteria between patients, as well as the impact of infection control measures, have been thoroughly studied and well documented.[25–27] Concerning enterococci, focus has been on vancomycin-resistant enterococci (VRE).[28,29] Establishment of endemicity of VRE was recently reviewed. It seems that if VRE are not controlled, sporadic cases may evolve into a monoclonal outbreak, which may then evolve into polyclonal endemicity.[30] The same pattern of establishment seems likely for other resistant bacteria with transferable antimicrobial resistance genes, such as the isolates with HLGR found in this study.

Considering this, nosocomial spread of hospital isolates seems a more likely cause of the high prevalence of genetically related enterococcal isolates with HLGR in this study. The fact that genetically related isolates were more prevalent in certain geographic areas might support this assumption. Nevertheless, more studies to assess the prevalence of carriage of enterococci with HLGR, in the community as well as in hospitalized patients, would be of value, in Sweden as well as in other countries.

In contrast to the isolates with HLGR and concomitant resistance to ciprofloxacin, most isolates with ciprofloxacin resistance but without HLGR were not genetically related. Ciprofloxacin resistance is due to mutational changes in the chromosomal genes encoding DNA gyrase (mainly *gyrA*) or topoisomerase IV (mainly *parC*).[17,31] This kind of resistance is not transmissible, and consequently dissemination of resistant clones could have explained the relatively high frequency of ciprofloxacin-resistant isolates in our study, but this was not found. The majority of the isolates (18 of 25 isolates) had unique PFGE patterns, and only four isolates belonged to cluster III and three isolates to cluster I (Table 1). Therefore, fluoroquinolone resistance mutations seem to have occurred in a diverse population of enterococci.

The gene encoding the most common and clinically important aminoglycoside-modifying enzyme in enterococci, *aac(6′)Ie-aph(2″)Ia*, conveys resistance to virtually all commercially available aminoglycosides except streptomycin.[9,32] It has been associated with both broad and narrow range plasmids of heterogeneous sizes and restriction patterns.[8,19] It has also been found to be a part of a conjugative transposon, situated either in the chromosomal DNA or on a plasmid.[10,19,33] The transposon was first identified by Hodel-Christian & Murray and was designated Tn*5281*. It was found to be similar, if not identical, to the transposon Tn*4001* found in *Staphylococcus aureus,* consisting of the *aac(6′)Ie-aph(2″)Ia* gene flanked on both sides by the insertion element IS256 in inverse orientations (Figure 4).[10] Tn*5281*-truncated elements have also been described, lacking the entire flanking IS256 or parts of it, on either side.[19,33]

In this study, the *aac(6′)Ie-aph(2″)Ia* gene was found in all isolates expressing HLGR. Despite the fact that we used a plasmid DNA isolation procedure previously described in the literature, we could not show unequivocally that a plasmid carrying the resistance gene was present in the isolates analysed.[15,16] This could be due to several factors on which one can only speculate, for example: a very large plasmid might be difficult to recruit from the bacteria, or a plasmid with very low copy numbers might not be detectable upon agarose analysis. The resistance gene might be incorporated in the chromosomal DNA and not plasmid-mediated because chromosomal DNA contamination was found in the plasmid preparations. However, in all isolates with HLGR except one, we found that the *aac(6′)Ie-aph(2″)Ia* gene was flanked by IS256 on both sides, and that this element was of the same size as Tn*5281*, i.e. a transposon similar to Tn*5281*, if not identical. This Tn*5281*-like element was not found in the three isolates without HLGR, which belonged to cluster I (all three belonging to different subtypes). This indicates that loss or acquisition of a Tn*5281*-like element does not necessarily alter the PFGE pattern by more than two bands. Transfer of the Tn*5281*-like element to an aminoglycoside-susceptible strain was not assessed, however, others have found transferability of the *aac(6′)Ie-aph(2″)Ia* gene without observing a plasmid in the donor or transconjugant strains.[34]

The majority (89%) of all isolates with HLGR in our study belonged to one of two genetically related clusters, which might suggest that the spread of enterococci with HLGR in Swedish hospitals is a rather recent event, and endemicity and polyclonal establishment have not yet occurred. The latter is supported by the fact that the frequency of HLGR in enterococci in Sweden has previously been reported to be low (2%).[5] In our study, HLGR was only detected in *E. faecalis* isolates. This is in accordance with findings in other countries. Although HLGR was first detected in *E. faecalis* in 1979, HLGR in *E. faecium* was not discovered until 1987.[35,36] If efficient infection control measures are not taken, establishment of endemicity may occur, thereby increasing the risk of transmission of the *aac(6′)Ie-aph(2″)Ia* gene to other clones of *E. faecalis* as well as to *E. faecium* isolates.

We conclude that HLGR in *E. faecalis* was mainly due to dissemination of genetically related clones during the time studied, and that HLGR in these isolates was due to the presence of the *aac(6′)Ie-aph(2″)Ia* gene which was situated on a Tn*5281*-like transposon. Infection control is of major importance in avoiding or diminishing establishment of endemicity of multiresistant enterococci.

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