Pathogenesis of nosocomial infections with Enterococcus faecalis
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Molecular Epidemiology of *Enterococcus faecalis* in Liver Transplant Patients at the University Hospital Groningen

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Abstract

We report the molecular epidemiology of *Enterococcus faecalis* in liver transplant patients transplanted at the University Hospital Groningen (The Netherlands) as determined by amplified fragment length polymorphism (AFLP) typing. A total of 133 *E. faecalis* isolates were cultured from the faeces and throat (95 isolates) or clinical sites (35 isolates) of 43 liver transplant patients. Among these 133 isolates, 15 different AFLP types could be identified with 90% AFLP similarity. Of these 15 groups, nine contained isolates from more than one patient, which may indicate transmission of *E. faecalis* isolates between patients. In five of these groups transmission could be explained by the fact that patients carrying identical strains were staying in the same ward at the same time. One of these epidemic isolates (AFLP type K) distinguished itself by colonizing 23 liver transplant patients during 15 months. Antimicrobial susceptibility testing did not reveal any multi-resistant isolates. This study showed that transmission of susceptible *E. faecalis* isolates occurs frequently on the liver transplant wards. Detection of this transmission and understanding of the mechanism is important, as it might also be an indicator of possible transmission of enterococci resistant to antibiotics.
Introduction

In recent years enterococci have become one of the leading causes of nosocomial infections and their incidence is increasing (131, 159). The seriousness of infections with enterococci has been underscored by different reports, which have shown that enterococcal bacteremia is associated with an increased mortality and prolonged duration of hospital stay (34, 137). Enterococcal infections are becoming more difficult to treat due to emerging antibiotic resistance (71). *Enterococcus faecalis* is an important cause of episodes of bacterial infection in patients receiving a liver transplant (LT) (93, 249, 264). The reason for the prominent role of this species in LT patients is not clearly understood. Selection and spread of more virulent and/or resistant strains during anti-microbial treatment may play a role as enterococci are not included in the spectrum of most prophylactic antibiotic regimens (6, 189).

Originally, enterococcal infections were thought to arise from a patient’s own endogenous flora or to be introduced into the abdomen during transplant surgery or its complications (6, 37). However, molecular epidemiological studies provided evidence for epidemic spread of enterococci in a hospital setting and nosocomial acquisition of enterococci (7, 131). Information about the nosocomial spread of enterococci is important to detect and prevent transmission of strains that might be virulent or resistant and cause infections in time. In an earlier study of the incidence of virulence factors in *E. faecalis* isolates from LT patients we reported that some *E. faecalis* surface proteins, like the enterococcal surface protein, might be associated with spread between LT patients (chapter 2).

Different molecular techniques are used to study the molecular epidemiology of bacteria. Pulsed field gel electrophoresis (PFGE) is often considered the standard reference method in the study of the nosocomial spread of strains in hospitals (134, 236). However, amplified fragment length polymorphism (AFLP) has similar power and has been more robust in studying hospital epidemics with vancomycin resistant *Enterococcus faecium* (VRE) (260, 261) and it has been suggested that AFLP could serve as “gold standard” for molecular epidemiology of VRE (4). Furthermore, AFLP typing has been used to study the molecular epidemiology of a wide range of bacterial species (52, 244).

In the present study, we report the screening of LT patients for *E. faecalis* combined with DNA typing using AFLP to investigate the molecular epidemiology of *E. faecalis* among these patients. Aim of this study was to gain insight in the epidemiology and the (high) infection rate with *E. faecalis* in LT patients.

Materials and Methods

Study design

Weekly routine surveillance cultures from stool and throat and other cultures from clinical sites with signs of infection were taken from consecutive patients who received a LT at University Hospital Groningen (The Netherlands) between March 1998 and March 2000. The liver transplant unit consisted of two wards (ward 1 and 2) and one intensive care unit (ICU). Ward 1 consisted of 31 beds for LT patients and patients with other gastro-intestinal
diseases. LT patients generally stayed on this ward during pre- and post-transplant evaluation. Ward 2 consisted of 30 beds for LT patients and patients undergoing hepatobiliary or vascular surgery. LT patients generally stayed on this ward for direct post-transplant treatment. The ICU contained 12 beds and patients demanding intensive care after surgery stayed on this unit. Nurses were generally dedicated to one ward, whereas medical doctors worked on the three different wards.

From every culture, one *E. faecalis* isolate was included in the study, presuming that enterococci from one culture with similar colony morphology and identification were identical isolates. The specimens were collected during the first month after the first LT. Patients with a re-transplant more than one month after the first transplantation and patients younger than 18 year were not included. To investigate the clonal relationship of *E. faecalis* isolates AFLP analysis was performed.

**Patients and bacteria**

The throat and stool specimens were cultured on blood agar plates CM55 (Oxoid) containing 5 % sheep blood and blood agar plates with 20 µg ml⁻¹ aztreonam added. Every putative enterococcus colony was subcultured on blood agar for further identification. The isolates were identified using a commercially available kit (API 20 Strep; bioMérieux).

**AFLP**

Chromosomal DNA of *E. faecalis* was isolated according to Boom et al. (21) with some minor modifications. The bacteria were grown overnight in brain-heart infusion broth (Oxoid) at 37 °C on a rotating incubator at 250 rpm. After centrifugation, the 1-ml culture pellet was resuspended in 50 µL water. The bacteria were lysed by incubation with mutanolysin (final concentration: 0.04 mg ml⁻¹; ICN Biomedicals) for 30 minutes at 37 °C. After lysis, 950 µl of a mixture of diatom suspension (Acros Organics) and L6 buffer (21) was added and mixed. After 10 min at room temperature, the tube was mixed again and centrifuged, and the supernatant was discarded. The pellet was subsequently washed twice with ethanol 70 % and once with acetone. After disposal of the acetone, the tube was dried at 37 °C for 30 min. The DNA was eluted by adding 200 µL water, and the vessel was mixed briefly and incubated for 10 min at 56 °C, mixed again, and centrifuged for 5 min. The AFLP analysis was performed as described by Willems et al. (261). In brief, the *E. faecalis* DNA was simultaneously digested with *Eco*RI and *Cfo*I and ligated to a single adapter that resulted in circularised DNA molecules. The molecules were amplified using two primers based on the adapter sequence, each with an extra selective base, and one primer was labelled with a fluorescent dye. The amplification products were separated on a 96 capillary ABI PRISM 3700 DNA analyser. AFLP patterns were analysed with BioNumerics software version 1.5 (Applied Maths). The Pearson product moment correlation was calculated and the unweighted pair group method with arithmetic averages was used for cluster analysis. In a previous study the similarity percentage of AFLP banding patterns of epidemiological related strains in a hospital outbreak was > 90 % (260, chapter 2). Therefore, in this study, strains were considered to be identical when AFLP banding patterns
were more than 90% similar. Isolates were considered epidemic when the same AFLP type was found in two or more patients, who could have been in contact, having stayed at the same ward during overlapping time periods.

**Antibiotic susceptibility testing**

From each patient, one *E. faecalis* isolate per AFLP group (total 56 isolates) was tested with agar disk diffusion for susceptibility to gentamicin (250 µg), erythromycin (78 µg), tetracycline (80 µg), ciprofloxacin (10 µg), amoxicillin (30 µg), and vancomycin (5 µg). The appropriate inoculum was derived from one or more colonies on blood-agar plates suspended in physiologic saline to a turbidity of 0.5 McFarland units, subsequently diluted 1:100 and applied to iso-sensitest agar plates (Oxoid). After the plates were dried for about 5 minutes at room temperature, the neosensitabs tablets (Rosco) were applied. After 24 h at 37°C the plates were checked for semiconfluent growth and the inhibition zones were determined, the zone of vancomycin was checked again after 48 h at 37°C. Inhibition zone diameters corresponding to minimal inhibitory concentration breakpoints and corresponding were read as determined for the University Hospital Groningen according to the Dutch national guidelines (51). The sensitivity and resistance breakpoints were respectively ≤ 1 and > 4 µg ml⁻¹ for gentamicin, ≤ 1 and > 2 µg ml⁻¹ for erythromycin, ≤ 1 and > 4 µg ml⁻¹ for tetracycline, ≤ 1 and > 2 µg ml⁻¹ for ciprofloxacin, ≤ 2 and > 16 µg ml⁻¹ for amoxicillin, and ≤ 4 and > 8 µg ml⁻¹ for vancomycin. Data from the antimicrobial susceptibility testing were analysed by the χ² method. Significance was defined as *P* ≤ 0.05.

**Results**

**Bacteria and patients**

During the study period 60 patients older than 18 years received LTs. We studied 133 *E. faecalis* isolates from 43 of these patients (chapter 2). Of these isolates, 35 were cultured from clinical sites: wound (10), bile (10), sputum (5), ascites (8), blood (1) and intravascular catheter (1). These clinical sites were cultured when there were signs of infection, however, it was not proven that the *E. faecalis* isolates from these sites caused infection. From 31 of the 43 LT patients in this study more than one isolate was collected (mean, 3.9 isolates/patient) and nine of these patients (patient number 2, 6, 7, 10-12, 18, 19, 22) carried isolates of more than one AFLP type. In 14 of these 31 patients (patient number 1, 2, 6, 12, 18, 22, 23, 25, 29, 30, 34, 40-42), *E. faecalis* with identical AFLP types (similarity > 90%) were isolated from both routine cultures and clinical sites. This indicates that in these patients the faeces or throat might provide reservoirs for enterococci that might cause infections. However, a common source (e.g., environment or hands) could also have led to colonisation of both faeces and/or throat and the clinical site.
Epidemiology of *E. faecalis*

Figure 1 shows the genetic relationship of the 133 *E. faecalis* isolates recovered from 43 LT patients. Among these isolates 15 different AFLP types (similarity > 90 %) were identified of which nine AFLP types (A, B, G, H, I, J, K, M and O) contained isolates from more than one patient, which is compatible with spread of *E. faecalis* isolates between patients. Transmission from patient to patient could be explained by geographic location and temporal relation between patients carrying isolates with AFLP type I, J, K, M and O. Of the five patients carrying AFLP type H isolates, transmission within two clusters of respectively two (patient 11 and 12) and three (patient 7, 9 and 10) patients could be explained by geographic location and temporal relation. However, transmission between the two clusters could not be explained. Transmission of the AFLP type G isolates between the two patients could be explained by survival of the *E. faecalis* on the hands of personnel or surfaces for one day, whereas transmission of AFLP type A and B isolates between the two patients could only be explained by survival of *E. faecalis* for long periods of two and one month respectively. Transmission from patient to patient of AFLP type A, B, I, J, G and H could have occurred on LT ward 1, transmission of AFLP type K and M on LT ward 1 or 2, whereas transmission of AFLP type O could only have occurred on the ICU.

Remarkable was the transmission of a genetically highly homologous strain (AFLP type K, similarity generally > 95 %) among 23 patients between September 1998 and December 1999. Nine other patients that also received a LT during this period and stayed at the same wards did not acquire this epidemic strain. Of these nine patients, six were transplanted during the first or last two months of the period that AFLP type K strain was predominant on the LT ward. Figure 2 shows the stay of the LT patients carrying AFLP type K *E. faecalis* on the different LT wards during the study period. All patients carrying the epidemic strain stayed on the same ward during the same period before or after the LT, which illustrates the possibility of transmission on the LT wards. Although cultures were only taken after the LT, transmission likely occurred during a previous stay in the hospital, e.g. for pretransplant monitoring, as in most cases the epidemic strain K was isolated immediately after the operation. The *E. faecalis* strain AFLP type K was not isolated during the study period before September 1998 or after December 1999 from LT patients nor from the faeces of healthy volunteers from the same geographical area or blood culture isolates from patients at the same hospital during the same period (data not shown) (chapter 2). These data demonstrate epidemic spread of one *E. faecalis* clone (AFLP type K) among LT patients.

The duration of hospital stay during one year before LT varied between one and 135 days, but the average duration did not differ significantly between the patients with and without the epidemic strain K. Three patients were not colonized with isolates from any of the epidemic groups, their average duration of stay before transplantation also did not differ significantly from the other patients.

**Characteristics of epidemic isolates**

The characteristics of the epidemic isolates (AFLP types A, B, G, H, I, J, K, M, and O; total 126 isolates), with respect to isolation site and antibiotic resistance, were compared with
Figure 1. Dendrogram of 133 *E. faecalis* isolates recovered from 43 liver transplant patients. Percentage on horizontal axis indicates percentage similarities (Pearson product-moment correlation coefficient). A-O, different AFLP-types with a similarity of > 90%. Patients are numbered 1 to 43; numbers behind the branches in the dendrogram indicate the patient from which the *E. faecalis* was isolated and the source (routine or clinical isolate).

*a*Routine surveillance isolates from faeces or throat, *b*includes wound (10), bile (10), ascites (8), sputum (5) blood (1) and intravascular catheter (1), *c*five patients had two different isolates and four patients had three different isolates.
non-epidemic isolates (AFLP type C, D, E, F, L, and N; total 7 isolates). Twenty-five percent of the epidemic isolates originated from clinical sites, in contrast to 43% of the non-epidemic isolates. In total, 26% of the isolates came from clinical sites. These data suggest that the epidemic isolates may spread well but mainly colonize the patients. Table 1 shows the susceptibility of 56 *E. faecalis* isolates to gentamicin (high level resistance), erythromycin, tetracycline, and ciprofloxacin as determined by agar disk diffusion. All isolates were susceptible to amoxicillin and vancomycin. The results of the $\chi^2$ test showed a significant difference in antibiotic resistance between epidemic and non-epidemic isolates for gentamicin, erythromycin, and ciprofloxacin; the epidemic strains were more resistant.

**Discussion**

We report the molecular epidemiology of *E. faecalis* in LT patients, a group prone to infections with this microorganism. We found that *E. faecalis* isolates susceptible to antibiotics, such as vancomycin or amoxicillin, had spread among LT patients. The finding of one particular clone that colonised 23 LT patients over a 15 months period was especially striking. During the remaining nine months of the study period this strain was not cultured again from a LT patient. This indicates that the strain might have disappeared from the transplantation wards. However, the end of transmission did not coincide with any change in antibiotic regimen, selective bowel decontamination or infection prevention on the LT wards. For five of the nine *E. faecalis* strains that were isolated from multiple patients, the epidemiology could be fully explained by the fact that patients carrying identical strains stayed in the same wards during overlapping periods of time. These findings suggest that there was transmission of *E. faecalis* strains on a LT ward, and this might have been caused by carriage on the hands of personnel, direct contact between the patients or adhesion and survival on abiotic surfaces e.g. medical equipment. Only one of the epidemic isolates described in this study was possibly transmitted on the ICU. This shows that both on ICUs and on other wards hygienic precautions should be carefully followed to prevent transmission of bacteria between patients.

<table>
<thead>
<tr>
<th>Antibiotic tested</th>
<th>Epidemic isolates N (%)</th>
<th>Non-epidemic isolates N (%)</th>
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<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Gentamicina</td>
<td>43 (86)</td>
<td>7 (14)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>43 (86)</td>
<td>7 (14)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>39 (78)</td>
<td>7 (14)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>41 (82)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>50 (100)</td>
<td></td>
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<tr>
<td>Amoxicillin</td>
<td>50 (100)</td>
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</table>

The epidemic isolates are AFLP types A, B, G, H, I, J, K, M, and O (50 isolates), the non-epidemic isolates are AFLP type C, D, E, F, L, and N (six isolates).

* high level resistance was tested
The genetic relatedness of *E. faecalis* strains was studied using AFLP. AFLP is a high throughput typing method, providing a high level of discrimination between isolates and is applicable without previous knowledge of genome sequences. AFLP has already proven its value in molecular epidemiological studies of various pathogens including *E. faecium* (28, 260, 261). In a previous study we applied AFLP to study the genetic relatedness of *E. faecalis* isolates recovered from faeces and clinical sites of liver transplant patients, other patients and volunteers. By AFLP we were able to discern different *E. faecalis* genogroups, where the strain within each group shared > 80 % of their restriction fragments. In this study AFLP was used to identify epidemic clones. The cut-off point of 90 % similarity to define identical isolates was chosen according to Willems et al. (260) who considered VRE strains to be epidemic if the AFLP pattern showed > 90 % similarity.

Reports of transmission of *E. faecalis* susceptible to clinical important antibiotics like amoxicillin and vancomycin are scarce as most reports focus on resistant enterococci. Sabria-Leal et al. (209) reported one *E. faecalis* strain, sensitive to clinically important
antibiotics, that was found in 11 out of 45 patient on an ICU as determined by PFGE. Information about the molecular epidemiology of *E. faecalis* still susceptible to clinically important antibiotics like amoxicillin and vancomycin, and not causing infections, is important to detect and prevent ‘silent’ transmission of isolates. As enterococci are able to acquire resistant determinants relatively easily, unrecognised transmission of susceptible isolates may finally result in outbreaks of multi-resistant strains. An example illustrating this was reported recently in which a VRE outbreak in a hospital in Poland originated from a vancomycin-susceptible strain that was endemic in the hospital, and that acquired the *vanB* transposon through horizontal transmission (129).

The incidence of colonization with *E. faecalis* in our study was 72 % (43 of 60 patients), which is relatively high compared to the incidence of 2.4 % found among all patients admitted to a University Hospital in eastern France (166). This difference might be explained by the fact that most of the risk factors for nosocomial acquisition of multi-resistant enterococci are present in the LT patients in our study. All patients have peri-operative antibiotic prophylaxis, previous surgery and most patients have longer hospitalisation and the need for advanced nursing care, factors frequently described as risk factors for acquisition of multi-resistant enterococci (131). Although the isolates we found were not multi-resistant, these risk factors might also apply to their acquisition. This is supported by the fact that the epidemic strain K was resistant to ciprofloxacin, one of the drugs used as prophylaxis during the LT. Conversely, data regarding colonization are often difficult to compare because culturing methods are very different. In this study we focused on the epidemiology of *E. faecalis*, therefore we cultured only weekly for four weeks. However to study the exact dynamics of colonization with *E. faecalis*, longer and more frequent culturing is necessary.

Epidemic isolates were cultured less frequently from clinical sites compared with non-epidemic isolates, which indicates that although these epidemic isolates might be capable of spreading, they are not more pathogenic. Previously, we reported that isolates of *E. faecalis* from LT patients, blood cultures and the faeces of healthy volunteers were genetically different and could be subdivided into different AFLP genogroups with > 80 % shared restriction fragments (chapter 2). The disclosure of different genogroups for isolates causing infections (e.g. blood culture isolates) and isolates from LT patients suggested the existence of genetically distinct pathogenic subpopulations. Our present finding that epidemic isolates from LT patients are not frequently isolated from clinical sites is in agreement with this previous study.

Adhesion of bacteria to biomaterial surfaces plays an important role in the initiation of infections and spread of bacteria (22, 45). Better adhesion to abiotic surfaces might explain the spread of *E. faecalis*, such as epidemic strain K in this study. We reported previously that *E. faecalis* strains expressing enterococcal surface protein or aggregation substance, surface proteins that are associated with infection and spread, adhere better to biomaterials compared to *E. faecalis* strains not expressing these surface proteins (chapter 5). Using polymerase chain reaction (PCR) we demonstrated the presence of the *esp* gene in the epidemic strain K. Furthermore, we determined that this strain was able to adhere to silicone rubber in comparable high numbers to other *esp* positive *E. faecalis* strains (data not shown). These data suggest that the observed high adherence to biomaterial surfaces
facilitated the epidemic spread of this strain. We have also reported that growth in bile significantly increased adhesion to biomaterial surfaces of esp positive strains (chapter 7). This underscores the possible role of esp of strain K in transmission as many LT patients have a bile drain for prolonged time after LT.

In summary, this study showed the epidemic spread of *E. faecalis* isolates susceptible to clinically important antibiotics at the LT wards of a university hospital. The spread of one strain among 23 patients during 15 months was especially interesting. The epidemic isolates were not more frequently isolated from clinical sites compared with the non-epidemic isolates. However, detection and prevention of epidemic spread of susceptible enterococcal strains is important, as it could be an indicator of and may precede transmission of, multi-resistant enterococcal strains.

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