Phenotypic characteristics and population genetics of Enterococcus faecalis cultured from patients in Tehran during 2000–2001

Mohammad Mehdi Feizabadi, Atusa Aliahmadi, Fatemeh Mobasheri, Ahmad Asgharzadeh, Soroor Asadi, and Gelavizh Etemadi

Abstract: Conventional bacteriology techniques were used to identify enterococci isolates cultured from patients at different hospitals in Tehran during 2000–2001. The identification was confirmed using species-specific PCR targeting the α-alanyl-α-alanine ligase gene. A total of 59 isolates of Enterococcus faecalis were identified. The rates of resistance to different antibiotics were in the following order: penicillin 84%, ciprofloxacin 42%, high-level gentamicin 30%, nitrofurantoin 14%, imipenem 4%, and chloramphenicol 2%. Resistance to ampicillin was found to be rare among the Iranian isolates of E. faecalis. Multi-locus enzyme electrophoresis was then used to analyze the strains. Forty-five electrophoretic types were obtained when 10 enzyme loci were screened. Although the collection of bacterial isolates was limited in time and location, considerable heterogeneity was found. Analysis of strains for linkage disequilibrium demonstrated that the studied population is not clonal, since the index of association was not significantly different from zero (Ia = 0.0296). Enterococcus faecalis isolates recovered from patients in Tehran were genetically diverse and seemed to possess a high potential for genetic recombinations, though none were resistant to vancomycin.

Key words: Enterococcus faecalis, population genetics, MEE analysis, nosocomial infections.

Résumé : Nous avons employé en premier lieu des techniques conventionnelles de bactériologie afin d’identifier des isolats d’entérocoques cultivés provenant de patients de divers hôpitaux de Téhéran au cours de 2000–2001. Les identifications ont été confirmées à l’aide d’un PCR spécifique à l’espèce ciblant le gène de la α-alanyl-α-alanine ligase. Un total de 59 isolats de Enterococcus faecalis ont été identifiés. Le taux de résistance à différents antibiotiques fut dans l’ordre : pénicilline 84 %, ciprofloxacine 42 %, gentamicine de haut niveau 30 %, nitrofurantoin 14 %, imipenem 4 % et chloramphénicol 2 %. La résistance à l’ampicilline fut rarement signalée chez les isolats iraniens de E. faecalis. Une électrophorèse enzymatique multilocus fut ensuite employée pour l’analyse des souches. Quarante-cinq types électrophorétiques ont été obtenus lorsque 10 locus d’enzymes ont été criblés. Bien que la collection d’isolats bactériens était limitée dans le temps et l’emplacement, une hétérogénéité considérable fut constatée. L’analyse des souches pour un déséquilibre de liaison a démontré que la population étudiée n’était pas clonale puisque l’index d’association ne différéait pas significativement de zéro (Ia = 0,0296). Les isolats de E. faecalis recueillis de patients de Téhéran sont génétiquement diversifiés et semblent posséder un potentiel élevé de recombinations génétiques, bien qu’aucun ne soit résistant à la vancomycine.

Mots clés : Enterococcus faecalis, génétique de populations, analyse par EEM, infections nosocomiales.


M.M. Feizabadi,1 Department of Microbiology, Alzahra University, Vanak, Tehran, Iran, and Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran.
A. Aliahmadi. Department of Biological Science, Alzahra University, Vanak, Tehran, Iran.
F. Mobasheri. Department of Microbiology, Alzahra University, Vanak, Tehran, Iran.
A. Asgharzadeh. Soil and Water Research Institute, North Kargar Jalal Aleahmad Street, Tehran, Iran.
S. Asadi. Department of Infectious Disease, Labbafinehad Hospital, Pasdaran, Tehran, Iran.
G. Etemadi. Department of Infectious Disease, Chamran Hospital, Noboniad Sq, Tehran, Iran.

1Corresponding author (e-mail: feizabad@azzahra.ac.ir).
Nosocomial infections with enterococci are widespread and are responsible for 12% of all infections following hospitalization (NNIS System 1997). They are frequently cultured from urinary tract infections, but also from immunocompromised patients and from patients with endocarditis, sepsis, and intra-abdominal infections (Moellering 1995). Enterococcal infections can be endogenous and arise from the patient’s internal flora. Recent studies have demonstrated both intra- and inter-hospital spread of enterococci by different means, including contaminated hands of health care professionals (Huycke et al. 1998; Ma et al. 1998; Oana et al. 2001).

Infections with multi-drug resistant strains of enterococci are particularly important, since they can exchange antibiotic resistance genes with other bacterial genera; in some cases, these isolates are resistant to all standard therapies (Noble et al. 1992; Mundy et al. 2000). The most clinically important species of enterococci is *Enterococcus faecalis*, which accounts for about 90% of all cases of enterococcal infections at hospitals (Huycke et al. 1998). *Enterococcus faecalis* strains may have virulence factors, such as enterococcal surface protein, cytolysin, aggregation substance, and gelatinase (Eaton and Gasson 2001). These virulence factors act synergistically with the resistance of the organism to aminoglycosides and may seriously threaten the lives of infected patients.

The prevalence of infections with antibiotic-resistant strains in different geographical areas is not the same and partly depends on the usage of antibiotics in livestock and agriculture (Bates et al. 1993; Donnelly et al. 1996).

The epidemiology of infections with drug-resistant enterococci, including *E. faecalis*, has been studied in detail in different countries (Ma et al. 1998; Schouten et al. 2000; Oana et al. 2001). Multilocus enzyme electrophoresis (MEE) has been used to study the population genetics of different microorganisms and the molecular epidemiology of bacterial infections. The technique of MEE has allowed quantification of differences between populations of bacteria in exchanging genetic information during recombination events (Smith et al. 1993). It is also possible to estimate different combinations of alleles of each locus and compare the estimates with those obtained by MEE, using multilocus linkage analysis. Such analyses have not previously been done on populations of *E. faecalis*.

Infection with enterococci is endemic at Tehran hospitals, with 16.5% of isolates being resistant to at least three drugs (Feizabadi et al. 2002). Molecular techniques have not previously been applied to Iranian strains of *E. faecalis*, and consequently there is no information concerning the genetic relatedness of isolates within this species in the country.

To collect clinically important isolates of *E. faecalis* from Tehran hospitals for population genetic studies, we used conventional bacteriology to identify the strains to the species level (Facklam and Collins 1989; Manero and Blanch 1999). Consequently, 59 isolates were identified as *E. faecalis* in this study. Of these, 52 isolates were cultured from patients during October 2000 to October 2001 at different hospitals in Tehran. To compare the allelozyme patterns of the strains at different times, isolates recovered from patients in 1998 (n = 7) were also included in this study. The majority of isolates were cultured from urine (n = 49) and the remaining were from blood (n = 5), wounds (n = 3), ascites (n = 1), and drainage fluid (n = 1). The reference strains of *E. faecalis* ATCC 29212 and *Enterococcus faecium* TX0016 (kindly provided by B.E. Murray, University of Texas Medical School, Houston, Texas) were used as positive and negative controls, respectively, in all experiments.

Polymerase chain reaction (PCR) was then used to confirm the identity of bacterial isolates. DNA from each isolate was extracted by glass beads (Monstein et al. 1998) and assayed by PCR using primers for *ddl* *E. faecalis* (5'-ATCAAGTAC GTTATGCTT-3' and 5'-ACGATTCAAAGCTAACGT-3') under conditions described by Coque and co-workers (1998). Amplicons were analyzed by electrophoresis on 1.5% agarose gels (Roche diagnostics). The size of amplicons for all isolates was 941 bp. Later the identification of all strains to the species level were reconfirmed by PCR.

*Enterococcus faecalis* was the dominant species causing enterococcal infections at Tehran hospitals. The rate of infection with this species was less than 80%. Other enterococci, particularly *Enterococcus faecium*, were involved in such infections. Further studies in subsequent years showed that the ratio of *E. faecalis* to *E. faecium* isolates in the hospitals of Tehran was 4:1 when 250 isolates were screened (data not shown). This ratio is similar to those reported from Asia-Pacific and Europe, but much lower than ratios reported from Latin America, where it has been documented as 17:1 (Mutnick et al. 2003). The prevalence of enterococci other than *E. faecalis* and *E. faecium* in Tehran is also much lower than other countries (1%).

The isolates were screened for their susceptibility to vancomycin, gentamicin, and penicillin (Sigma, St. Louis, Mo.), using microbroth dilution. Isolates were categorized as susceptible or resistant using breakpoints as follows: penicillin, 8 mg/L; gentamicin (high-level), 500 mg/L; vancomycin, 4 mg/L (National Committee for Clinical Laboratory Standards 1993a). Disks containing antibiotics (BBL, BD Diagnostic Systems, Sparks, Md.) were used to assess the susceptibility of isolates to ampicillin, imipenem, chloramphenicol, ciprofloxacin, and nitrofurantoin, using standard methods (National Committee for Clinical Laboratory Standards 1993b). The rates of resistance to the antibiotics tested were as follows: penicillin 84%, ciprofloxacin 42%, nitrofurantoin 14%, imipenem 4%, and chloramphenicol 2%. Resistance to ampicillin was rare among the Iranian strains of *E. faecalis*. High-level gentamicin resistance was found in 30% of isolates. β-Lactamase production was checked by nitrocefin assay (BBL, BD Diagnostic Systems). All isolates were negative in this test, suggesting that other mechanism might be involved in the resistance of these strains to β-lactams antibiotics.

MEE analysis was then used to determine the genetic relationships among the isolates. Briefly, cells were cultured and incubated overnight at 37 °C in 200 mL of brain–heart infusion broth (Merck KGaA, Darmstadt, Germany). They were sedimented by centrifugation. The sediments were sonicated, and the lysates containing the enzymes were used for starch gel electrophoresis (Selander et al. 1986). The electrophoretic mobilities of 10 enzymes were determined by staining for specific enzyme activity. These enzymes include adenylyl kinase (ADK), esterase (EST), glucose-6-phosphate dehydrogenase (GPD), leucylglycine peptidase (LGG), nucleoside phosphorylase (NP), phosphoglucomutase isomerase (PGI), phos-
phoglucomutase (PGM), glutamate dehydrogenase (GDH), hexokinase (HEX), and 2,6-phosphogluconate dehydrogenase (PGD). Buffer A was used for enzyme electrophoresis (Selander et al. 1986).

Genetic diversity (h) for each enzyme locus examined in MEE was calculated from the formula 

\[ h = 1 - \sum \frac{P_i^2}{n(n - 1)} \]

where \( P_i \) is the frequency of the \( i \)th allele and \( n \) is the number of electrophoretic types (ETs) in the sample (Nei 1987). Genetic distances between ETs were calculated as the proportion of fixed loci at which dissimilar alleles occurred.

The 59 isolates of \( E. \ faecalis \) were divided into 45 ETs. All enzymes, except PGI and ADK, were polymorphic, with between two and five alleles (mean of 3.1 alleles). The mean genetic diversity for ten loci was 0.381. The locus GDH showed the most diversity, followed by PGM (0.685), GPD (0.609), EST (0.579), HEX (0.554), PGD (0.294), NSP (0.272), and LGG (0.095).

The genetic diversity for the loci, such as NP, PGM, PGD, and GPD, were significantly different from a previous study (Tomayko and Murray 1995), suggesting greater genetic diversity for these loci among the Iranian isolates.

The majority of isolates in this study were collected from Labbafinejad (\( n = 27 \)) and Shariati (\( n = 17 \)) hospitals. However, isolates from these hospitals were differentiated into 23 and 16 ETs, respectively. ET 4 consisted of three \( \beta \)-haemolytic isolates that had been recovered from outpatients at Labbafinejad hospital in May and June 2001 (Table 1). They also showed the same antibiotic resistant patterns and were \( \beta \)-haemolytic. Most of the isolates grouped in the ETs, such as ET 4, 9, 10, 19, 23, 29, and 41, showed similar drug-resistance patterns. Frequent isolation of strains with the same electrophoretic type was observed at one of the hospitals in 2 years. However, isolates with similar ETs were also found at different hospitals.

Eleven ETs contained more than one isolate (Table 1). Isolates with these ETs had been recovered from patients of different wards. There should be possibilities for dissemination of such strains to different hospital wards or sections, since isolates that grouped in ET 9, 17, 19, and 28 had been recovered from patients at different wards of the relevant hospitals (Table 1). For example, both isolates in ET 19 were \( \beta \)-haemolytic and produced the same drug-resistance pattern. Interestingly, they had been isolated from trachea and wounds of two different patients at orthopedic and intensive care wards. The dates of isolation for both isolates were just 2 days apart. Similarly, isolates in ET 4 had been cultured from outpatients. All three isolates in this ET were \( \beta \)-haemolytic and produced the same drug-resistance pattern.

It is not clear how such patients had been infected by identical isolates. Since the majority of patients in this study have a history of transplantation, they might have been infected shortly after operation, and the persistence of infection with these isolates may have lasted after they had left the hospital.

Data from MEE were analyzed using a statistical test designed to detect association between genes at different loci.

**Table 1.** Characteristics of strains that clustered in the same electrophoretic types (ETs).

<table>
<thead>
<tr>
<th>ET</th>
<th>Strainsa</th>
<th>Dates</th>
<th>Resistance patterns and virulence factors</th>
<th>Clinical specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>28Sh</td>
<td>11 April 2001</td>
<td>Cip, HLGR</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>31Sh</td>
<td>14 April 2001</td>
<td>Cip, HLGR</td>
<td>Urine</td>
</tr>
<tr>
<td>20</td>
<td>11Sh</td>
<td>15 November 2000</td>
<td>Cip</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>24Sh</td>
<td>30 January 2001</td>
<td>Cip, Van (intermediate)</td>
<td>Urine</td>
</tr>
<tr>
<td>17</td>
<td>51Ln</td>
<td>28 April 2001</td>
<td>Cip</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>91Ln</td>
<td>25 November 2001</td>
<td>–</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>68Cs</td>
<td>27 January 1998</td>
<td>–</td>
<td>Urine</td>
</tr>
<tr>
<td>4</td>
<td>54Ln</td>
<td>13 May 2001</td>
<td>Cip</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>55Ln</td>
<td>13 June 2001</td>
<td>Cip</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>57Ln</td>
<td>20 June 2001</td>
<td>Cip</td>
<td>Urine</td>
</tr>
<tr>
<td>10</td>
<td>33Ln</td>
<td>23 April 2001</td>
<td>Cip, HLGR</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>75Ln</td>
<td>26 October 2001</td>
<td>Cip, HLGR</td>
<td>Urine</td>
</tr>
<tr>
<td>23</td>
<td>35Ln</td>
<td>23 April 2001</td>
<td>Cip, HLGRb</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>47P</td>
<td>30 April 2001</td>
<td>Cip, HLGR</td>
<td>Blood</td>
</tr>
<tr>
<td>32</td>
<td>42Ct</td>
<td>18 December 1998</td>
<td>–</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>43Ct</td>
<td>18 December 1998</td>
<td>–</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>59Sh</td>
<td>30 April 2001</td>
<td>Cip, HLGR</td>
<td>Blood</td>
</tr>
<tr>
<td>29</td>
<td>17Sh</td>
<td>30 December 2000</td>
<td>–</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>37E</td>
<td>22 April 2001</td>
<td>–</td>
<td>Urine</td>
</tr>
<tr>
<td>19</td>
<td>39R</td>
<td>11 April 2001</td>
<td>HLGRb</td>
<td>Trachea</td>
</tr>
<tr>
<td></td>
<td>40R</td>
<td>13 April 2001</td>
<td>HLGRb</td>
<td>Wound drain</td>
</tr>
<tr>
<td>28</td>
<td>115Ln</td>
<td>6 January 2002</td>
<td>–</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>168K</td>
<td>18 January 2002</td>
<td>–</td>
<td>Blood catheter</td>
</tr>
<tr>
<td>41</td>
<td>111Sh</td>
<td>28 December 2001</td>
<td>–</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>154Ln</td>
<td>17 January 2002</td>
<td>–</td>
<td>Urine</td>
</tr>
</tbody>
</table>

Note: Cip, ciprofloxacin; Amp, ampicillin; HLGR, high-level gentamicin resistance. –, isolate not resistant.

a Followed by abbreviations for hospital names.

b \( \beta \)-hemolytic.

c Gelatinase producer.
To obtain the index of association ($I_p$), the expected variance of $K$ ($V_0$) and the observed variance of $K$ ($V_o$) were also calculated accordingly, where $K$ is the genetic distance between two individuals (Smith et al. 1993). $I_p$ was calculated as 0.0296. It showed that the population of $E. faecalis$ is not clonal. So, no dendrogram can be constructed for the population studied.

Numerous studies have been done for subspecific differentiation of $E. faecalis$ by genotyping techniques (Kuhn et al. 1995; Tomayko and Murray 1995; Barbir et al. 1996). However, little work has been done to structurally analyze the population of this microorganism by such techniques. The finding of 45 ETs for 59 isolates of $E. faecalis$ shows more heterogeneity in populations of this species in Tehran than in other parts of the world (Tomayko and Murray 1995). The results of MEE suggest that the population of $E. faecalis$ is not clonal, since $I_p$ was not significantly different from zero. It means that recombination probably occurs in the population. Evidence for such findings also has come from the ability of this species to transform large DNA particles mediated by pheromone-responsive plasmids (Wirth 2000). The Iranian strains of $E. faecalis$ also exchange such plasmids in vitro with high frequency (Feizabadi et al. 2003). Since the alleles in a panmictic population are randomly associated, no dendrogram can be constructed for determination of the genetic relatedness of the isolates in this study.

There are two outcomes for the current study: (i) absence of vancomycin-resistant isolates and (ii) high prevalence of aminoglycoside-resistant isolates. Both of these findings are slightly different from regional reports (Mutnick et al. 2003). Aminoglycosides, particularly gentamicin, have been in widespread use for at least three decades in Iran. They are still in use for treatment of very different infections in both hospitalized patients and outpatients. This may explain the high rate of high-level gentamicin resistance in Tehran. Conversely, vancomycin has not been used on a large scale, so it is still effective against $E. faecalis$ in Iran. However, the trend of resistance may change in the future, as our recent findings showed infections with vancomycin-resistant strains of $E. faecium$ have increased in Tehran (Feizabadi 2003). We suggest gentamicin to be used solely against susceptible strains in hospitalized patients. The rate of resistance to ciprofloxacin among Iranian strains was 42%. Frequent prescription of ciprofloxacin for urinary tract infections may be the cause of the high rate of resistance to these antibiotics in Tehran hospitals.

In our study, none of the strains produced $\beta$-lactamase. The results of the $\beta$-lactamase test were not changed when further strains were tested. It is assumed that, like in other countries, $\beta$-lactamase-producing strains of enterococci are also rare in Iran. $\beta$-Lactamase-producing strains of $E. faecalis$ were shown to belong to a certain clone by other investigators when they were analyzed by pulsed-field gel electrophoresis (Tomayko and Murray 1995; Murdoch et al. 2002).

We conclude that Iranian strains of $E. faecalis$ are genetically diverse. Despite this great heterogeneity, resistance to glycopeptides has not been observed yet. Resistance to other antibiotics, such as anti-cell-wall agents, aminoglycosides, and ciprofloxacin, are widespread among the strains. Such multidrug-resistant strains persist in the hospitals. Evidence for transmission of these strains to patients hospitalized in different wards was found by MEE.

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