Short Communication

Genotypic Analysis of Enterococci Isolated from Fecal-Polluted Water from Different Sources by Pulsed-Field Gel Electrophoresis (PFGE) for Application to Microbial Source Tracking

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(Received October 4, 2010—Accepted January 31, 2011—Published online February 26, 2011)

PFGE has potential applications in the source tracking of fecal pollution in aquatic environments. We tried to distinguish the genotypes of Enterococcus faecium collected from fecal-contaminated water using PFGE. Well-identified 115 strains of E. faecium were classified into 25 PFGE patterns, and characteristics distinctive to each genotype were recognized. Analysis of the characteristics of genotypes using PFGE can be used to track source of fecal pollution.

Key words: pulsed-field gel electrophoresis (PFGE), genotypic analysis, enterococci, fecal-polluted water, microbial source tracking

Recently, it has been reported that the risk of infection by pathogenic bacteria and viruses is not negligible in coastal areas, even in developed countries with advanced water conservation technologies and management (1, 3). To improve public health and to ensure safe living conditions in coastal urban areas, the development of a method of analysis for tracking sources of fecal contamination in aquatic environments is required (8, 9, 11, 15, 20–23). Several methods of biomolecular and genotypic analysis of target bacteria and viruses based on the polymerase chain reaction (PCR) have been developed and applied for bacteriological investigation (18). In particular, genotypic analysis has become an extremely valuable tool for tracking sources of pollution in aquatic environments. PCR is the most commonly used molecular method, and PCR-based approaches using repetitive DNA sequences (7), denaturing-gradient gel electrophoresis coupled with PCR (4), and host-specific 16S rRNA genetic markers (2) have also been developed. On the other hand, pulsed-field gel electrophoresis (PFGE), a method of molecular epidemiological genetic analysis, can be used to compare genotypic characteristics within the same species, and is an established standard tracking method for identifying the route of infection in epidemiological studies (12). This method has extremely high sensitivity, reproducibility, and discriminative ability compared with other microbial source tracking (MST) methods (17). In addition, pathogenic bacteria with resistance to antibiotics have been detected in river water and coastal water and characterized using PFGE (6). PFGE has also been utilized for the source tracking of Salmonella contamination in marine environments (16) and for comparing genotypes of Pseudomonas aeruginosa collected from the ocean and freshwater (13). However, there have been few cases of PFGE being applied to the MST of fecal bacteria in urban and catchment areas. Enterococci are Gram-positive bacteria that normally live in human and animal gastrointestinal tracts. Because of their long-term survival and low growth rate in the environment, they have been conventionally used as indicators of fecal pollution in aquatic environments (15, 19, 25). The objectives of this study were (i) to correctly identify Enterococcus faecium and Enterococcus faecalis as indicators of fecal pollution using genetic and biochemical methods among enterococcal strains isolated from fecal-contaminated water samples from different sources and (ii) to discriminate and compare the genotypes of collected indicator bacteria by applying PFGE analysis to aquatic environments.

Treated wastewater, referred to as community drainage, was collected from a community wastewater treatment plant, which treats the human waste from about 100 households without animals. Urban drainage was collected from a sewer that drains the treated wastewater from on-site wastewater treatment plants. Wastewater was collected from a municipal wastewater treatment plant. In addition, river water was sampled from the Yaegawa River in Miyazaki, Japan. The Yaegawa is a typical urban river derived from sewage treatment plants and on-site wastewater treatment plants. All water samples were collected from the surface, and stored in sterile 1-l polyethylene bottles. The sources of fecal pollution differed for each of the samples. KF Streptococcus agar (Difco, Becton Dickinson, Franklin Lakes, NJ, USA, KF agar) was used for the collection of enterococci. For the analysis of bacteria in the samples of community drainage and wastewater, a dilution series of up to 10−2 was prepared using each water sample together with a sterilized physiological saline solution. One hundred microliters of each diluted water sample was directly applied to a KF agar plate. Ten or 100 mL of each sample of urban drainage and river water was filtered through a 0.45-μm-pore membrane filter (47 mm diameter, sterile, mixed cellulose ester; Advantec, Tokyo, Japan), and incubated on a KF agar plate for 48 h at 37±1.0°C. From each sample, a single colony on the KF agar
plate was randomly streaked on a Todd Hewitt agar plate (Becton Dickinson, TH agar, added agar 1.5%), and incubated for 24 h at 37±1.0°C. To compare the genotypic characteristics of enterococcal strains by PFGE, it is very important to correctly identify the target strains of various enterococci. The identification of *E. faecium* and *E. faecalis* was carried out using a combination of PCR and the Api 20 Strept test.

Genomic DNA was extracted from a single colony on each TH agar plate that had been incubated for 24 h at 37±1.0°C using InstaGene Matrix (BioRad, Hercules, California, USA) in accordance with the manufacturer’s instructions. The extracted DNA was stored at −20°C until further use. The primers used in the PCR analysis for the identification of *E. faecium* (5) were Efm-forward (5'-TGGAGCCAGACCA GATTGACG-3') and Efm-reverse (5'-TATGACAGCGACT CCGATTCC-3'). Those used for *E. faecalis* (14) were Efs-forward (5'-GCGACTATTCTCTCGGACGC-3')and Efs-reverse (5'-GTCGCATCTTCGGAATAA-3'). PCR was carried out in 20 µL of a PCR mixture consisting of 2.0 µL of Takara 10× Ex Taq Buffer (Takara, Otsu, Japan), 1.6 µL of 2.5 mM dNTP mixture, 2.0 µL of 10 µM forward and reverse primers, 0.2 µL of 5 U µL⁻¹ Ex Taq, 4.2 µL of sterile distilled water, and 8.0 µL of template DNA extracted from an enterococcal strain. The amplification program used was as follows: initial denaturation at 94°C for 4 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 69°C (*E. faecium*) or 62°C (*E. faecalis*) for 30 s, and elongation at 72°C for 1 min. Both amplifications were followed by a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel using 1× Tris-borate-EDTA (TBE) buffer. All the enterococcal strains identified as *E. faecium* or *E. faecalis* by PCR were characterized using the Api 20 Strept test (bioMerieux, SA Lyon, France) in accordance with the manufacturer’s instruction. Enterococcal strains were identified as *E. faecium* and *E. faecalis* by both PCR and Api 20 Strept test. Then, their genotypic characteristics were analyzed by PFGE.

To clarify the genetic relatedness among the enterococcal strains, PFGE was carried out using the Genepath Group 1 reagent kit (BioRad) following the manufacturer’s instruction with a minor modification. The PFGE analysis was carried out according to previously published methods (10).

One hundred strains isolated from community drainage were identified by PCR, 76 and 4 strains of *E. faecium* and *E. faecalis* were retained, respectively (Table 1). Subsequently, all 80 strains identified as *E. faecium* or *E. faecalis* were confirmed using a biochemical test (Api 20 Strept test). Among the 76 *E. faecium* strains identified by PCR, 59 were also identified as *E. faecium* using the Api 20 Strept test (Table 1). The remaining 17 strains were *Enterococcus avium* (4 strains), *Aerococcus* spp. (4 strains), or *Leuconostoc* spp. (1 strain). Eight strains were not identified. The four *E. faecalis* strains identified by PCR were confirmed to be *E. faecalis*. Thus, among the 100 strains, 59 strains (59%) and 4 strains (4%) were identified as *E. faecium* and *E. faecalis*, respectively, by both PCR and the biochemical test. Furthermore, the 100 enterococcal strains collected from the other three water samples were identified using the same methods. *E. faecium* was identified more frequently than *E. faecalis* in the other three samples (data not shown).

Table 1. Identification of enterococcal strains using PCR and the Api 20 Strept test.

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR</th>
<th>Api 20 Strept</th>
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<tbody>
<tr>
<td><em>E. faecium</em></td>
<td>76</td>
<td>59</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Others</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>80</td>
</tr>
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</table>

From the results of the identification test, we concluded that *E. faecium* was a suitable species as an indicator of fecal pollution. *E. faecium* strains were isolated and identified from urban drainage, wastewater, and river water by the same methods; 25, 11, and 20 strains were collected from urban drainage, wastewater, and river water, respectively. These *E. faecium* strains, which were included in the 59 strains found in community drainage, were subjected to a fragment analysis by PFGE.

The 59 *E. faecium* strains isolated from the community drainage sample exhibited five PFGE types (CD-I–CD-V). *CD-II* (18 strains) and *CD-IV* (22 strains), were considered the major types. The 25 *E. faecium* strains isolated from the urban drainage sample also exhibited five PFGE types (UD-I–UD-V), with UD-I (20 strains) the major type. The 11 *E. faecium* strains isolated from the wastewater sample exhibited six PFGE types (WW-I–WW-VI). Although the smallest *E. faecium* strains were isolated from the wastewater, the number of PFGE types was larger than that for the community drainage or urban drainage. The 20 *E. faecium* strains isolated from the river water sample exhibited eight PFGE types (RW-I–RW-VIII). River water had the most diverse range of restriction band patterns among the four samples. The major PFGE type in river water was RW-VI (7 strains).

A dendrogram was prepared that included all the PFGE types obtained from each *E. faecium* strain and ATCC 19434 as a reference strain (Fig. 1). From the results of PFGE, Tenover *et al.* proposed that strains belonging to the same cluster with a 0.9 similarity level be considered related species (24). Based on the criteria, the 25 PFGE types were classified into 17 different clusters. No strain exhibited high similarity with the reference strain (ATCC 19434) at this level. Results indicated that the *E. faecium* strains isolated from the water samples exhibited different restriction band patterns from the reference strain. The clusters all consisted of PFGE types of strains isolated from the same sample, except for the cluster that consisted of CD-I and RW-VI. The characteristic genotype of *E. faecium* obtained using PFGE differed for each sample isolated from fecal-polluted water and was clearly distinguishable using the dendrogram. In the dendrogram-based analysis, the 25 PFGE types were classified into 17 clusters at a 0.9 similarity level. A 0.9 similarity level was also used for the restriction band patterns of strains isolated from the same sample to classify them into the same cluster, except the types CD-I and RW-VI. Strains from different samples with a greater than 0.9 similarity in restriction band patterns are highly likely to have an identical source. Therefore, by comparing the restriction band patterns of the strains of *E. faecium* isolated from potentially contaminated water with that of a sample suspected of being a
source of pollution, it is possible to track the source.

Over the last decade, various MST methods have been developed to distinguish different sources of human and/or nonhuman fecal pollution (22). Numerous review articles (8, 9, 15, 20, 21) and articles that included comparisons of molecular and biochemical methods for MST (11, 22, 23) have been published. The approach adopted in the present study; the identification of enterococci, a genotypic analysis with PFGE, and the use of a dendrogram, has the distinct advantage of distinguishing E. faecium, as an indicator of fecal pollution, at significantly high sensitivity, although the sampling plan employed did not account for the temporal variability and geographic heterogeneity of genotypes of E. faecium. Therefore, PFGE is a potential tool for MST that can be applied to areas of water used for recreation. An analysis of the restriction band patterns of enterococci strains isolated from recreational areas such as beaches for swimming and of samples of river and drainage water flowing into recreational areas will be carried out next to demonstrate the applicability of PFGE to MST.

References