COMPARISON OF ARBITRARY PRIMER (AP) PCR AND PULSED-FIELD GEL ELECTROPHORESIS METHODS FOR GENOTYPIC DIFFERENTIATION OF ESCHERICHIA COLI O157 STRAINS

MARCIN WEINER AND JACEK OSEK

Department of Microbiology, National Veterinary Research Institute, 24-100 Pulawy, Poland
e-mail: josek@piwet.pulawy.pl

Received for publication July 03, 2003.

The genetic relatedness of 12 Escherichia coli O157 strains isolated from cattle and humans was examined by methods based on arbitrary primer amplification of DNA sequences (AP-PCR) and by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA digested with XbaI or NotI endonucleases. The usefulness of the methods for molecular differentiation of these isolates was evaluated using computer program analysis of the obtained DNA profiles with manually made corrections as well as with the Simpson’s index of diversity. The results indicated that PFGE with the XbaI restriction endonuclease, possessing the index of diversity $D = 1.00$, was the most powerful tool to reveal genetic relationship among E. coli O157 strains isolated from different sources.

Key words: E. coli O157, AP-PCR, PFGE, genotypic differentiation.

Escherichia coli O157 strains are an important group of food-borne pathogens causing severe human diseases, including bloody diarrhoea (haemorrhagic colitis - HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (1, 9, 16). E. coli O157 possess several factors implicated in the pathogenesis of the diseases, including Shiga toxins (Stx1 and Stx2) and the pathogenicity island called LEE, containing the eaeA gene encoding the intimin protein, involved in the intimate attachment of bacteria to enterocytes (14, 27). Moreover, the 60-MDa plasmid, carrying the ehlA gene encoding enterohaemolysin has been found in nearly all E. coli O157 strains and has been suspected to play a role in pathogenicity of infections (16, 27, 29).

Ruminants, especially cattle, are known to be the most important reservoir of E. coli O157 strains which are carried in their gastrointestinal tract and transmitted to humans through food contaminated with fecal material (1, 16). Moreover, transmission of E. coli O157 by person-to-person contact, drinking of raw milk, water or eating vegetables and fruits has also been described (1).

Because of the clonal nature of E. coli O157 strains, highly sensitive molecular biology-based subtyping methods are needed to differentiate and analyse strains recovered from the same or different sources (7, 34). Several assays have been used for genetic investigation of E. coli O157 isolates. These tests are based either on
restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) (2, 5, 13, 15) or amplification of DNA sequences with PCR (AP-PCR), i.e. Random Amplified Polymorphic DNA (RAPD-PCR), Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR), BOX-PCR and sequences located between the repetitive IS3 genomic element (IS-PCR) (4, 6, 11, 25, 32, 35).

The purpose of this study was to compare selected PCR-based methods with PFGE test for the determination of molecular relationship of E. coli O157 strains isolated from different sources.

**Material and Methods**

**Bacterial strains.** The E. coli strains used in this study are presented in Table 1.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Origin</th>
<th>Serotype</th>
<th>Virulence marker gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>stx1</td>
</tr>
<tr>
<td>344</td>
<td>C</td>
<td>O157:H7</td>
<td>+</td>
</tr>
<tr>
<td>408</td>
<td>C</td>
<td>O157:H7</td>
<td>+</td>
</tr>
<tr>
<td>409</td>
<td>C</td>
<td>O157:H7</td>
<td>-</td>
</tr>
<tr>
<td>414</td>
<td>C</td>
<td>O157:H7</td>
<td>-</td>
</tr>
<tr>
<td>420</td>
<td>C</td>
<td>O157:H7</td>
<td>-</td>
</tr>
<tr>
<td>422</td>
<td>H</td>
<td>O157:H7</td>
<td>+</td>
</tr>
<tr>
<td>424</td>
<td>H</td>
<td>O157:H7</td>
<td>-</td>
</tr>
<tr>
<td>441</td>
<td>H</td>
<td>O157:H7</td>
<td>-</td>
</tr>
<tr>
<td>442</td>
<td>H</td>
<td>O157:H7</td>
<td>-</td>
</tr>
<tr>
<td>443</td>
<td>H</td>
<td>O157:H7</td>
<td>+</td>
</tr>
<tr>
<td>451</td>
<td>H</td>
<td>O157:H7</td>
<td>+</td>
</tr>
<tr>
<td>454</td>
<td>H</td>
<td>O157:H7</td>
<td>+</td>
</tr>
</tbody>
</table>

*E. coli strains were isolated from cattle (C) or from humans (H)*

The strains were obtained from the E. coli Reference Laboratory in Dessau, Germany (numbers 344, 414, 441, 442, 443, 451), the National Institute of Hygiene in Warsaw, Poland (numbers 408, 422, 424), the Department of Food Hygiene, University of Warmia and Mazury, Olsztyn, Poland (number 420), and from the own strain collection (numbers 409, 454). The animal strains (one isolate from one animal) were recovered from 3-5-month-old cattle as described previously (22). No animal contacts between the Polish and German cattle farms were noted. Furthermore, the human strains were isolated from persons who had no contacts with the animals tested in the study. The E. coli isolates were previously analysed for the stx1, stx2, ehly, eaeA virulence factor genes, using single or multiplex PCR tests (18, 19).

**Preparation of genomic DNA for PCR tests.** Isolation and purification of genomic DNA was performed from E. coli bacteria grown in Luria Bertani (LB) broth using AquaPure Genomic DNA Isolation Kit (Bio-Rad, Hercules, USA). The purity and concentration of the DNA preparations were measured spectrophotometrically at 260 and 280 nm.

**AP-PCR amplification conditions.** All PCR primer sequences and concentrations used in the study are shown in Table 2.
<table>
<thead>
<tr>
<th>PCR technique</th>
<th>Primer code</th>
<th>Primer sequence</th>
<th>Primer concentration (μM)</th>
<th>Amplification conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD</td>
<td>1254</td>
<td>CCGCAGCCAA</td>
<td>0.5</td>
<td>94ºC 5 min, 35X (36ºC 1 min, 72ºC 2 min, 94ºC 1 min), 72ºC 10 min</td>
<td>24, 32</td>
</tr>
<tr>
<td>BOX</td>
<td>BOXA1R</td>
<td>CTACGGCAAGGCACGCTGACG</td>
<td>0.5</td>
<td>94ºC 5 min, 35X (40ºC 1 min, 72ºC 2 min, 94ºC 1 min), 72ºC 10 min</td>
<td>6</td>
</tr>
<tr>
<td>IS</td>
<td>IS3A</td>
<td>CACTTAGCCGCGTGTCC</td>
<td>0.5</td>
<td>94ºC 5 min, 35X (36ºC 1 min, 72ºC 2 min, 94ºC 1 min), 72ºC 10 min</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>IS3B</td>
<td>CGCTTAGGCTGCTGCTCCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERIC</td>
<td>ERIC1R</td>
<td>ATGTAAGCTCCTGGGATTCAC</td>
<td>0.2</td>
<td>94ºC 5 min, 35X (52ºC 1 min, 72ºC 2 min, 94ºC 1 min), 72ºC 10 min</td>
<td>11, 35</td>
</tr>
<tr>
<td></td>
<td>ERIC2</td>
<td>AAGTAAGTGACTGGGTTGAGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PCR reactions were performed in 50-μl reaction mixture consisting of *E. coli* genomic DNA (50 ng for RAPD-PCR, 10 ng for ERIC-, BOX- and IS-PCR, respectively), 1X Taq buffer (10 mM Tris-HCl [pH 8.8], 50 mM KCl, and 0.8% Nonidet P40), MgCl₂ in final concentration (mM) as follows: 2.0 (RAPD-PCR), 3.0 (BOX- and IS-PCR), and 5.0 (ERIC-PCR). 2 U of the Taq thermostable DNA polymerase (Fermentas, Vilnius, Lithuania), 200 μM of each dATP, dCTP, dGTP and dTTP (Fermentas), and DNase-, RNase-free, deionized water (ICN Biomedicals, Costa Mesa, USA). DNA amplification was carried out in a thermal cycler (PTC-100, MJ Research, Watertown, USA), using the program conditions as described in Table 2. The PCR products were visualized by standard gel electrophoresis in a 2% agarose gel (Type I, Low EEO, Sigma, St. Louis, USA) stained with ethidium bromide (5 μg/ml) for 1 min. The gels were photographed under UV light using the Gel Doc 2000 documentation system (Bio-Rad, Hercules, USA). All AP-PCR analyses were performed several times with slightly different amplification conditions until the band profiles were stable and highly reproducible. The fingerprints showed in this study based on at least three independent amplifications which provided the same AP-PCR pictures.

**Pulsed-field gel electrophoresis (PFGE).** Bacterial strains were grown in 10 ml of LB broth at 37ºC for 18 h with 150 rpm. After centrifugation at 10 000×g for 5 min at 4ºC, the bacterial pellet was washed with 10 mM Tris-HCl and resuspended to a final concentration of 2×10⁹ bacterial cells/ml. Equal volumes of the bacterial suspension and 2% agarose (low melting point; Sigma), pre-warmed at 55ºC, were mixed and immediately added into block formers (Bio-Rad). The cell lysis and DNA digestion were carried out essentially as described by Watanabe *et al.* (33). Two restriction endonucleases were used: *Xba*I (40 U) or *Not*I (50 U) (Fermentas), according to the manufacturer’s instruction. PFGE was performed with the CHEF DR II system (Bio-Rad) in 0.5X TBE at 14ºC for 23 h of a constant voltage of 200 V and the pulse time from 5 to 40 s. After electrophoresis, the gels were stained with ethidium bromide (5 μg/ml) for 25 min, rinsed in distilled water for 5 min and photographed using the GelDoc 2000 documentation system. A λPFGE DNA marker (Sigma) was included into each agarose run.

**Data analysis.** Gel images were scanned and analysed using the Molecular Analyst Fingerprinting Plus Software (Bio-Rad). Dendograms were created from a matrix of band matching using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) analysis. Some DNA bands, mistakenly identified as positive by the computer software were deleted. Moreover, some PCR amplicons not recognized automatically by the computer were manually added for the analysis. The isolates sharing less than 90% of the similarity were regarded as different (30). The discriminating ability of the tests used was determined by the Simpson’s index of diversity according to the following equation:

\[
D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j-1)
\]

where *N* is the total number of strains in the sample population, *s* is the total number of types being described, and *n_j* is the number of strains belonging to the *j*th type (12).
Results

Arbitrary Primer PCR analyses. RAPD-PCR amplification performed with the primer 1254 generated 5 to 8 weaker or stronger DNA bands with each of the *E. coli* isolates. The UPGMA analysis of the fingerprints revealed that 12 strains tested could be classified into 4 clonal groups (Fig. 1). The RAPD-PCR method possessed the discriminatory index $D = 0.79$.

![Agarose gel electrophoresis showing RAPD-PCR fingerprintings and the dendrogram outlining the clonal relationship of 12 *E. coli* O157 strains performed with UPGMA analysis and Dice’s coefficient.](image)

Fig. 1. Agarose gel electrophoresis showing RAPD-PCR fingerprintings and the dendrogram outlining the clonal relationship of 12 *E. coli* O157 strains performed with UPGMA analysis and Dice’s coefficient.
The genetic relationship among *E. coli* strains tested with BOX-PCR determined with the single BOXA1R primer resulted in 8 to 10 DNA amplicons. The strains were then classified into 7 clonal types with genetic similarity less than 90% (Fig. 2). The equation of Simpson’s index of diversity showed that BOX-PCR used in this study had the discrimination $D = 0.77$.

**Fig. 2.** Agarose gel electrophoresis showing BOX-PCR fingerprintings and the dendrogram outlining the clonal relationship of 12 *E. coli* O157 strains performed with UPGMA analysis and Dice’s coefficient.
The genetic analysis of the *E. coli* O157 isolates determined by IS-PCR resulted in 3 to 10 DNA amplicons that could be clustered into 9 different clonal groups (Fig. 3). The index of diversity was determined as D = 0.91.

**Fig. 3.** Agarose gel electrophoresis showing IS-PCR fingerprints and the dendrogram outlining the clonal relationship of 12 *E. coli* O157 strains performed with UPGMA analysis and Dice’s coefficient.
Further investigation of the genetic relationships among 12 *E. coli* O157 strains tested performed with ERIC-PCR resulted in 10 different fingerprints which had the degree of similarity less than 90% (Fig. 4). In this test the Simpson’s index was calculated as 0.97.

Fig. 4. Agarose gel electrophoresis showing ERIC-PCR fingerprints and the dendrogram outlining the clonal relationship of 12 *E. coli* O157 strains performed with UPGMA analysis and Dice’s coefficient.
**PFGE analysis.** Using the *XbaI* restriction endonuclease, 12 closely related PFGE profiles were observed among 12 *E. coli* O157 strains isolated from humans and cattle (Fig. 5).

![PFGE Patterns](image)

**Fig. 5.** PFGE patterns of *XbaI*-digested DNA of *E. coli* O157 strains and the dendrogram outlining the clonal relationship of these isolates generated using UPGMA analysis and Dice’s coefficient. Lane M - molecular size standard (50-kb λDNA).
UPGMA analysis with Dice coefficient and Simpson’s index of diversity showed that PFGE analysis performed with XbaI had the highest discrimination of \( D = 1.00 \). With the \textit{NotI} endonuclease, DNA of only 9 \textit{E. coli} O157 strains was digested whereas 3 isolates (i.e. 408, 443, 454) could not be differentiated with this enzyme. Moreover, digestion with \textit{NotI} generated only two DNA band types with a very low index of diversity \( D = 0.22 \) (data not shown).

**Discussion**

Several methods have been used for genetic differentiation of \textit{E. coli} O157 strains. Arbitrary amplification (AP-PCR) of repeated DNA sequences in \textit{E. coli} strains has been reported as a useful method for genetic characterization of these bacteria. There are several variants of this technique, such as RAPD-PCR, ERIC-PCR, BOX-PCR, and IS-PCR. It has been shown that all of them enable the generation of DNA fingerprints which discriminate \textit{E. coli} bacterial strains of the same serotype (4, 6, 11, 21, 23, 25, 32, 35).

In the present study four arbitrary amplification techniques for typing of \textit{E. coli} O157 isolates were compared. The results indicate that among them, ERIC-PCR test had the highest discriminatory capacity as calculated by Simpson’s index of diversity (0.97). On the other hand, Osek (20) previously showed that the DNA fingerprinting profiles received with BOX and ERIC primers were more effective for grouping of \textit{E. coli} strains isolated from pigs with diarrhoea than those obtained with RAPD and IS primers. In another study of Osek (21) performed with \textit{E. coli} O157:H7 isolates recovered from pigs, the genetic relatedness of the strains was evaluated with the BOX-PCR method. The results revealed that the isolates obtained from different farms and possessing the same virulence marker genes were closely related or identical as tested with the UPGMA analysis. However, the \textit{E. coli} O157 strains tested previously by Osek (21) were not the same as analysed in the present study.

One of the major advantages of the AP-PCR technique is that it is fast and can be used without previous knowledge of the nucleotide sequence of the bacterial target DNA. On the other hand, this method has been criticized due to lack of reproducibility (3, 10, 26). However, under the PCR conditions and with the oligonucleotide primers used in the present study, the stable fingerprints were obtained.

PFGE is widely used as a molecular subtyping method of \textit{E. coli} O157 strains, due to its high discriminatory power and good reproducibility. This method is based on the analysis of restriction endonuclease-digested genomic DNA fragments separated by pulsed-field gel electrophoresis (PFGE) (2, 5, 15, 17, 19, 28). The UPGMA analysis with Dice coefficient and Simpson’s index of diversity showed that PFGE with XbaI digestion possessed the index of discrimination \( D = 1.00 \) and therefore it was the most powerful tool to reveal genetic differences among \textit{E. coli} O157 strains analysed in the present study that were isolated from cattle and humans. Davis \textit{et al.} (5) found that in the absence of epidemiological data, similarity coefficients from a single-enzyme PFGE are poor measures of relatedness of \textit{E. coli} O157 isolates and even six or more restriction endonucleases are needed to provide a reasonable estimate using this discriminating method. Moreover, a poor correlation between similarities derived from the PFGE and randomly amplified DNA tests for \textit{E. coli} O157 was also observed (28). Other investigators have found that combination of results obtained with several typing methods is the best way to achieve the highest discriminatory power.
methods, including AP-PCR and PGFE, improve the discriminating power of the molecular differentiation tests used (8, 13).

In conclusion, the results obtained in the present study demonstrated that PFGE performed with the XbaI restriction endonuclease is a simple, powerful molecular tool to reveal genetic differences and clonal relationship among *E. coli* O157 strains of different origin and shows discriminating advantage over the four other AP-PCR methods used.

**Acknowledgments:** This paper was granted by the Open Society Institute ZUG Foundation, Project Code: 17013 (Log-in ID: 40004053).

**References**


