MOLECULAR ANALYSIS OF ENTEROTOXIGENIC, SHIGATOXIGENIC AND ENTEROAGGREGATIVE ESCHERICHIA COLI STRAINS ISOLATED FROM SUCKLING PIGLETS WITH DIARRHOEA BY THE USE OF PULSED-FIELD ELECTROPHORESIS

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Abstract

Genetic relationship between Escherichia coli strains expressing different or the same fimbrial/toxic virulence markers and isolated from piglets affected with neonatal diarrhoea were determined using the pulsed field gel electrophoresis (PFGE) method after XbaI endonuclease digestion of chromosomal DNA. The strains were classified as enterotoxigenic (ETEC), shigatoxigenic (STEC) and enteroaggregative (EAEC) groups of E. coli according to their virulence properties. After PFGE, the dendrograms were created from a matrix of band matching using the Dice coefficient and the unweighted pair group method with arithmetic mean analysis. The genetic analysis of all isolates tested revealed that bacteria possessing the same virulence-associated factors can be classified as different clonal groups. On the other hand, two pairs of EAEC isolates with different virulence properties generated identical PFGE profiles. The obtained results show preliminary epidemiological information about the molecular relationship between diverse E. coli strains originated from suckling piglets with diarrhoea.

Key words: piglets, diarrhoea, E. coli, genetic relationship, PFGE.

Escherichia coli strains are important causes of diarrhoea in humans and animals (17, 27). E. coli isolates associated with enteric diseases have been classified to different categories, on the basis of their distinct virulence properties: enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enteroinvasive (EIEC), and enteroaggregative (EAEC) (27). Among them, ETEC is a dominant bacterial agent of neonatal and postweaning diarrhoeal diseases in piglets (24). Bacteria of this group are defined as E. coli that elaborate at least one of two categories of enterotoxins: heat-labile LT and heat-stable ST. Each of these toxins comprises at least two variants: LTI and LTI, and STI and STII (26, 35). LTI, STI, and STII have been found in both human and pig ETEC, whereas LTI is only sometimes reported from E. coli strains of pig origin (7, 37). Moreover, most porcine ETEC possess one of the pilus adherence factors: F4, F5, F6, F17, or F41 (14).

Some of E. coli strains, which are called shigatoxigenic E. coli (STEC), may produce another kind of toxin - Shiga toxin (Stx), that is found in two main variants: Stx1 and Stx2 (20, 21, 28, 35). These toxins are usually secreted by enterohaemorrhagic E. coli strains but can also be sometimes detected in isolates of pig origin (17, 20). E. coli strains responsible for oedema disease of pigs usually possess another fimbrial antigen – F18 and may produce Shiga toxin 2 subtype (Stx2e) which is directly responsible for oedema symptoms (3, 12, 17, 22).

EAEC strains, the most recently recognized category of diarrhoeagenic E. coli, adhere to tissue culture cells in an aggregative adherence pattern and are associated with watery diarrhoea in young children in the developing world (27, 34). The strains produce a protein named enteroaggregative E. coli heat-stable enterotoxin 1 (EASt1), encoded by the astA gene (33, 41). Recently, Choi et al. (8) and Yamamoto and Nakazawa (41) reported that EASt1 enterotoxin was present not only in EAEC strains but also in other human and animal (especially pig) diarrhoeagenic E. coli, including ETEC, EPEC, and EHEC strains.

A proper characterization of pathogenic E. coli isolated from suckling piglets with diarrhoea requires application of several phenotyping methods, i.e. determination of serotypes (O, K, and H antigens) as

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well as fimbrial and toxic factors. However, these traits used for the analysis of ETEC bacteria may not represent reliable markers, since different phenotypic variants can be detected in E. coli strains of the same serotype (1, 5). The observation that most E. coli strains isolated from animals with diarrhea belong to a limited number of serotypes and produce the same fimbrial and toxic virulence markers supports the hypothesis that these strains represent genetically related groups (clones) of pathogenic bacteria (29). The observation that most E. coli strains isolated from animals with diarrhea belong to a limited number of serotypes and produce the same fimbrial and toxic virulence markers supports the hypothesis that these strains represent genetically related groups (clones) of pathogenic bacteria (29). The clonal nature of ETEC has been shown in several studies with human enterotoxigenic isolates (2, 31, 36). However, molecular typing methods, including restriction fragment length polymorphism (RFLP) have not been often applied for the analysis and epidemiological characterization of E. coli strains originated from suckling piglets (16, 25, 30, 40). Differentiation of chromosomal DNA based on RFLP and pulsed-field gel electrophoresis (PFGE) is a good and sensitive molecular method to analyse the degree of genetic relatedness or variability among different serotypes as well as among strains of the same serogroup.

The purpose of this study was to determine the genetic relationship between porcine E. coli strains isolated from neonatal diarrhea and expressing different or the same fimbrial/toxic virulence markers.

**Material and Methods**

**Bacterial strains.** E. coli strains (n = 240) were isolated from pigs with diarrhea and characterized previously (10). Rectal swabs were taken from piglets (one to fourteen days old) and plated on MacConkey’s agar (Oxoid). Those identified as E. coli using the API-20E biochemical system (bioMerieux, France) were tested by the PCR test with the primers specific for the universal stress protein gene (uspA). After the isolation, the E. coli bacteria were stored in agar tubes at room temperature and were not subcultured before examination.

**Toxic and fimbrial virulence factors.** Fimbrial antigens F4, F5, F6, F17, and F41 were determined by the slide agglutination test whereas the fedA gene of F18 fimbriae was identified by PCR as described previously (9). The genes encoding heat-labile I (LTI), heat-stable I and II (STI and STII) enterotoxins, EAST1 and Shiga toxin 2e variant were also identified by PCR (38). For the further analysis of genetic relatedness of the E. coli strains, the isolates were divided into three different groups, according to their most prevalent virulence properties: enterotoxigenic (ETEC), shigatoxigenic (STEC) and enteroaggregative (EAEC). Ten to 12 strains from each group were taken for examinations with the RFLP/PFGE method. Characteristics of the E. coli isolates used in the present study are shown in Table 1.

**Preparation of genomic DNA.** Each bacterial strain was grown at 37°C for 18 h with 150 rpm in 10 ml of Luria-Bertani (LB) broth. After centrifugation at 10000 x g for 5 min at 4°C, the bacterial pellet was washed with 10 mM Tris-HCl (pH 8.0) and resuspended in 10 mM Tris-HCl to a final concentration of 1.5 x 10⁹ bacterial cells /ml. Equal volumes of the bacterial suspension and 2% of agarose (Low Melting Point; Sigma, USA), prewarmed to 55°C, were mixed and immediately inserted into block formers (Bio-Rad, Hercules, USA). After solidification, the agarose blocks were then transferred to 3 ml of fresh lysis buffer and incubated for another 20 h at 37°C without agitation. The plugs were then washed three times with 5 ml of 10 mM Tris-EDTA, pH 8.0) containing 1% N-lauroylsarcosine (Sigma) and 0.1 mg/ml proteinase K (Sigma). The agarose blocks were then transferred to 3 ml of the lysis buffer (10 mM Tris-HCl-100 mM EDTA, pH 8.0) containing 1% N-lauroylsarcosine (Sigma) and 0.1 mg/ml proteinase K (Sigma). The agarose blocks were then transferred to 3 ml of Tris-HCl-100 mM EDTA, pH 8.0) containing 1% N-lauroylsarcosine (Sigma) and 0.1 mg/ml proteinase K (Sigma). The agarose blocks were then transferred to 3 ml of fresh lysis buffer and incubated for another 20 h at 37°C without agitation. The plugs were then washed three times with 5 ml of 10 mM Tris-5 mM EDTA (pH 7.5) (TE) buffer for 1 h at room temperature followed by three washings with 10 mM Tris-HCl (pH 8.0). The agarose blocks were then stored in TE buffer at 4°C or directly used for digestion with restriction enzymes.

**Table 1**

Characteristics of E. coli strains used in the study

<table>
<thead>
<tr>
<th>E. coli group</th>
<th>Toxin profile</th>
<th>Fimbrial phenotype</th>
<th>Number of strains</th>
<th>Name in the pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETEC</td>
<td>LTI+STI</td>
<td>F4</td>
<td>3</td>
<td>1A, 2A, 3A</td>
</tr>
<tr>
<td></td>
<td>LTI+STII</td>
<td>F4</td>
<td>3</td>
<td>4A, 5A, 6A</td>
</tr>
<tr>
<td></td>
<td>STI</td>
<td>F5</td>
<td>2</td>
<td>7A, 8A, 9A</td>
</tr>
<tr>
<td>STEC</td>
<td>Stx2e</td>
<td>-</td>
<td>8</td>
<td>1B, 2B, 3B, 4B, 5B, 6B, 7B, 8B</td>
</tr>
<tr>
<td></td>
<td>Stx1+Stx2e</td>
<td>-</td>
<td>2</td>
<td>9B, 10B</td>
</tr>
<tr>
<td>EAEC</td>
<td>EAST1+LTI+STI</td>
<td>F4</td>
<td>4</td>
<td>1C, 2C, 3C, 4C</td>
</tr>
<tr>
<td></td>
<td>EAST1+LTI</td>
<td>F4</td>
<td>3</td>
<td>5C, 6C, 7C, 8C</td>
</tr>
<tr>
<td></td>
<td>EAST1</td>
<td>F4</td>
<td>3</td>
<td>8C, 10C</td>
</tr>
<tr>
<td></td>
<td>EAST1+LTI+STII</td>
<td>F4</td>
<td>2</td>
<td>11C, 12C</td>
</tr>
</tbody>
</table>
Restriction digests and agarose electrophoresis. Digestions of genomic DNA embedded in agarose blocks were carried out using XbaI endonuclease (Fermentas, Vilnius, Lithuania). After 45 min in 200 µl of the respective restriction buffer, the plugs were transferred to the fresh restriction buffer (200 µl) and incubated overnight with 40 U of XbaI at 37°C. PFGE was performed with the CHEF DR II system (Bio-Rad) at 14°C in 0.5X TBE. The agarose blocks were placed in 1.0 % agarose (Bio-Rad Pulsed Field Certified Agarose) gel wells. The gels were then run at pulse ramps from 5 to 40 s for 23 h at a constant voltage of 200 V. After electrophoresis, the gels were stained with ethidium bromide (0.5 µg/ml) for 45 min, destained with distilled water for 30 min and photographed under UV light using the Gel Doc 2000 documentation system (Bio-Rad). A 50-kb lambda PFGE 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). Dendrograms were created from a matrix of band matching using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) analysis.

Results

The genetic relatedness among the E. coli isolates from suckling piglets with diarrhoea was determined by the use of pulsed-field electrophoresis methods. In order to produce chromosomal digests with relatively few fragments, DNA was digested with XbaI endonuclease, which recognized rarely existing codons (TCTAGA).

PFGE analysis revealed that 11 ETEC strains produced 11 distinct DNA fragment profiles. Each PFGE profile displayed several bands within the 50 and 1000 kb range (Fig. 1). The cluster analysis of PFGE profiles performed by the UPGMA method revealed that the E. coli strains tested belonged to two major genetic groups. One cluster comprised 5 isolates whereas the other group consisted of the remaining 6 strains. Similarity of these two PFGE profiles was lower than 70% as shown by the Dice analysis. The highest genetic similarity was observed between strains 9A and 10A (over 95%). These isolates possessed different virulence markers (LTI/STII/F4 and STII/F5, respectively). On the other hand, ETEC strains 10A and 11A belonging to the same virulence profile (STII/F5), generated two separate clusters with a low percentage of genotypic similarity (Fig. 2).

PFGE analysis of 10 STEC isolates revealed that these strains produced 10 distinct DNA fragment profiles. Each PFGE pattern displayed several bands within the 50 and 1000 kb range. All of the examined STEC strains generated a very strong 1000 kb band (Fig. 3). The cluster analysis of the PFGE profiles revealed that STEC strains belonged to two major genetic groups. One group comprised all Stx2e-positive isolates, whereas the other cluster consisted of the remaining 2 Stx1/Stx2e-positive strains. The degree of similarity between both groups was over 70% (Fig. 4).

PFGE analysis performed with PFGE method revealed that 12 EAEC strains which originated from suckling piglets with diarrhoea, produced 12 distinct DNA fragment profiles. Each PFGE profile displayed several bands within the 50 and 1000 kb range. The strains 8C, 9C, 10C possessing both EAST toxin and F4 fimbriae generated as many as 17 bands. On the other hand, the lowest polymorphism was observed in strains 6C and 12C (Fig. 5). The cluster analysis of PFGE profiles performed by the UPGMA method revealed that the E. coli strains tested belonged to two major genetic groups. One cluster comprised 6 isolates, whereas the second one the remaining 4 isolates. The EAST1-positive strains, belonging to the first clonal group, possessed different virulence markers: strains 1C, 3C, 4C: LTI/STII/F4, strain 8C: F4, strains 11C-12C: LTI/STII/F4. The second EAST1 positive clonal group consisted of 6 remaining isolates. Among them, two isolates (7C and 10C with different virulence properties LTI/F4 and F4 only, respectively), 2C and 6C (LTI/STII/F4 and LTI/F4, respectively) generated identical PFGE profiles (Fig. 6).

Discussion

Several reports on genetic investigation of E. coli bacteria were described before. These tests are based either on restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) (4, 11, 19, 23) 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) (6, 13, 18). PFGE is widely used as a molecular subtyping method of E. coli strains due to its high discriminatory power and a good reproducibility (4, 23). In the previous study the genetic relatedness of 12 E. coli O157 strains isolated from cattle and humans were 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 endonuclease. The usefulness of these methods for molecular differentiation of the 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. Davis et al. (11) found that in the absence of epidemiological data similarity coefficients from a single-enzyme PFGE are poor measures of the relatedness of E. coli O157 isolates and even six or more restriction endonucleases are needed to provide a reasonable estimate using this discriminatory method. Moreover, a poor correlation between similarities derived from the PFGE and randomly amplified DNA tests of E. coli O157 was also observed (32). Other researchers have found that only combination of results obtained with several typing methods, including AP-PCR and PFGE, improve the discriminatory power of the molecular differentiation tests used (15, 19).
Fig. 1. PFGE patterns of *Xba*I-digested genomic DNAs of ETEC strains. Lane M - molecular size standard (50 kb λ DNA). Lanes 1A-3A: LTI/STI/F4-positive *E. coli* strains; Lanes 4A-6A: LTI/F4-positive *E. coli*; Lanes 7A-9A: LTI/STII/F4-positive *E. coli*; Lanes 10A and 11A: STII/F5-positive isolates.

Fig. 2. The dendrogram outlining the clonal relationship of the ETEC bacterial isolates, generated using Dice coefficient and UPGMA analysis of PFGE fingerprints obtained with 11 ETEC strains. The degree of similarity (%) is shown on the scale.

Fig. 3. PFGE patterns of *Xba*I-digested genomic DNAs of STEC strains. Lane M - molecular size standard (50 kb λ DNA). Lanes 1B-8B: Stx2e-positive *E. coli* strains; lanes 9B and 10B: Stx1/Stx2e-positive isolates.
**Fig. 4.** The dendrogram outlining the clonal relationship of the ten STEC bacterial isolates, generated using Dice coefficient and UPGMA analysis of PFGE fingerprints obtained with 10 STEC strains. The degree of similarity (%) is shown on the scale.

**Fig. 5.** PFGE patterns of *Xba*I-digested genomic DNAs of EAEC strains. Lane M - molecular size standard (50 kb λ DNA). Lanes 1C-4C: EAST1/LTI/STII/F4-positive *E. coli*; lanes 5C-7C: EAST1/LTI/F4-positive *E. coli*; lanes 8C-10C: EAST1/F4-positive *E. coli*; lanes 11C and 12C EAST1/LTI/STII/F4-positive isolates.

**Fig. 6.** The dendrogram outlining the clonal relationship of the bacterial isolates, generated using Dice coefficient and UPGMA analysis of PFGE fingerprints obtained with twelve EAEC strains. The degree of similarity (%) is shown on the scale.
E. coli strains tested in the present study were all assumed to be a cause of diarrhoea in the suckling piglets because they were regularly recovered as a predominant organism in the faeces of the diarrhoeic animals on the farms. Furthermore, all isolates tested in the study possessed fimbrial and toxic virulence properties which are responsible for diarrhoea in pigs (9, 38). The genetic analysis of all isolates tested revealed that bacteria possessing the same virulence-associated factors can be classified as different clonal groups: ETEC strains 10A and 11A (STI/F5), with two separate clusters with a low percentage of genotypic similarity (under 70%). On the other hand, two pairs of EAEC isolates with different virulence properties generated identical PFGE profiles: strains 7C and 10C (LTI/F4 and LTI/F4, respectively), and 2C and 6C (LTI/STI/F4 and LTI/F4, respectively). It was also observed that all of STEC strains tested generated a common strong 1000 kb DNA band.

In summary, the obtained results show that the PFGE method may be used as a valuable epidemiological tool for the detection of molecular relationship between E. coli strains originating from suckling piglets with diarrhoea.

References
