Abstract

Molecular relationship between enterotoxigenic Escherichia coli strains (ETEC) isolated from piglets with neonatal diarrhoea which expressed the same or different fimbrial/toxic virulence markers was determined using the insertion element IS3 PCR-based method (IS-PCR). The dendrograms were created from a matrix of band matching using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) analysis. The genetic analysis of all isolates tested revealed that bacteria possessing the same virulence-associated factors can be classified to different clonal groups. The obtained results show preliminary epidemiological information about the molecular relationship between ETEC strains originating from suckling piglets with diarrhoea. IS-PCR can provide a better knowledge of the genetic relatedness and clonal origin of ETEC as well as should lead to great progress in molecular epidemiology.

Key words: piglets, diarrhoea, E. coli, IS-PCR, genetic relationship.

Escherichia coli is an important intestinal pathogen causing diarrhoea in humans and animals (12, 19). 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) (19). Among them, ETEC is a predominant bacterial agent of neonatal and postweaning diarrhoea in piglets (17). Bacteria of this group produce at least one of two categories of enterotoxins: heat-labile LT and heat-stable ST. Each of these toxins comprises at least two variants: LT1 and LTII, and ST1 and STII (18, 23). LT1, ST1, and STII have been found in both human and pig ETEC whereas LTII is only sometimes reported from E. coli strains of pig origin (5, 24). Moreover, most porcine ETEC possess one of the pilus adherence factors: F4, F5, F6, F17, F18 or F41 (10). A proper characterization and determination of relatedness among pathogenic E. coli strains is a prerequisite for epidemiological investigation. Several phenotyping methods for determination of serotypes (O, K, and H antigens) as well as fimbrial and toxic factors have been used (1, 3). However, most diarrhoeic E. coli isolates, producing the same fimbrial and toxic virulence markers, represent genetically related clones. Therefore, molecular biology-based methods are needed to differentiate the outbreak strains (20).

Several assays have been used for genetic investigation of porcine E. coli isolates. These tests are based either on restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) (2, 7, 14, 16) 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, 9, 13, 21, 26, 30). Among them, the test based on IS-PCR is a good, sensitive molecular method to analyse the degree of genetic relatedness or variability among different ETEC strains.

The purpose of this study was to determine, by the use of the IS-PCR method, the genetic relationship between ETEC strains isolated from neonatal porcine diarrhoea that express the same or different fimbrial/toxic virulence markers.

Material and Methods

Bacterial strains. E. coli strains (n = 240) were isolated from pigs with diarrhoea. Rectal swabs were taken from piglets (1-14 d. old) and plated on MacConkey’s agar (Oxoid). Those identified as E. coli
using the API-20E biochemical system (bioMerieux, France) were tested by PCR with the primers specific for the universal stress protein gene (uspA) as described previously (7). After 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 as described previously (6). The genes encoding heat-labile I (LTI), heat-stable I and II (STI and STII) enterotoxins were identified by PCR (28). For the further analysis of genetic relatedness of the enterotoxigenic E. coli strains, the isolates were divided into three different groups. Eleven strains from each group were taken for examinations with the IS-PCR method. Characteristics of the E. coli isolates used in the study are shown in Table 1.

### Table 1
Characteristics of E. coli strains used in the study

<table>
<thead>
<tr>
<th>Group</th>
<th>Name in the pattern</th>
<th>Toxin profile</th>
<th>Fimbrial phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1A-11A</td>
<td>LTI</td>
<td>F4</td>
</tr>
<tr>
<td>B</td>
<td>1B-11B</td>
<td>LTI+STI</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>1C-5C</td>
<td>LTI+STII</td>
<td>F4</td>
</tr>
<tr>
<td></td>
<td>6C-11C</td>
<td>STI</td>
<td>F5</td>
</tr>
</tbody>
</table>

**Preparation of genomic DNA.** Isolation and purification of genomic E. coli DNA was performed from 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. For the IS-PCR test, total 10 ng of the bacterial DNA was used.

**PCR amplifications.** DNA template (2.5 µl) was added to the PCR mixture consisting of: 2.5 µl of PCR buffer (10 mM Tris-HCl [pH 8.8], 50 mM KCl, and 0.8% Nonidet P40), 2.5 µl of each deoxynucleotide (dNTPs; final concentration 0.2 mM), 3 µl of 25 mM MgCl₂ (final concentration of 3 mM), 1 U of the Taq DNA polymerase (Fermentas, Lithuania), 0.5 µM of PCR primers (IS3A: CACCTAGCCCGTGTCCA, IS3B: CGCTTAGGCGCTGTCCA) (25) and water to final volume 25 µl. The PCR was as follows: after initial DNA denaturation at 94°C for 5 min, 35 cycles (94°C for 1 min, 72°C for 2 min, 36°C for 1 min) and the final extension step was done for 10 min at 72°C. All PCR amplifications were performed in a thermal cycler (PTC-100; MJResearch, USA). The amplified PCR products (10 µl aliquots) were visualized by gel electrophoresis in 1% agarose gel (Type 1, low EEO, Sigma Chemicals, USA) in Tris-Acetate EDTA (TAE) buffer at 100 V. The gels were stained with ethidium bromide (5 µl/ml) for 1 min, washed in distilled water, analysed under UV light (300 nm) and photographed using the Gel Doc 2000 documentation system (Bio-Rad). The size of the obtained amplicons was compared to the 100 bp DNA ladder (Fermentas).

**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 enterotoxigenic E. coli isolates from suckling piglets with diarrhoea was determined by the use of IS-PCR analysis with the primer pair IS3A and IS3B. These primers and amplification conditions used in the study allowed generation of several weaker or stronger DNA bands varied from 100 bp to ca 2000 bp in size (Fig. 1). The cluster analysis of IS-PCR profiles performed by the UPGMA method revealed that the E. coli strains possessing both LTI and F4 virulence markers belonged to two major genetic groups. One cluster comprised 9 isolates whereas the other group consisted of the remaining 2 strains. The molecular similarity of these two profiles was lower than 55% as shown by the Dice analysis. The highest genetic correlation was observed between strains 7A and 8A, 2A and 6A, 3A and 4A (100%). On the other hand, ETEC strains 9A and 11A, belonging to the same virulence profiles (LTI/F4), generated two separate clusters with a very low percentage of genotypic similarity (Fig. 2).

![Fig. 1. Agarose gel electrophoresis showing IS-PCR fingerprints generated by eleven E. coli LTI/F4-positive strains isolated from suckling piglets with diarrhoea. Lane M: 100 bp DNA ladder.](image-url)
**Fig. 2.** The dendrogram outlining the clonal relationship of the ETEC LTI/F4-positive isolates, generated using Dice coefficient and UPGMA analysis of IS-PCR fingerprints. The degree of similarity (%) is shown on the scale.

**Fig. 3.** Agarose gel electrophoresis showing IS-PCR fingerprintings generated by eleven *E. coli* LTI/STI-positive strains isolated from suckling piglets with diarrhoea. Lane M: 100 bp DNA ladder.

**Fig. 4.** The dendrogram outlining the clonal relationship of the ETEC LTI/STI-positive isolates, generated using Dice coefficient and UPGMA analysis of IS-PCR fingerprints. The degree of similarity (%) is shown on the scale.
Fig. 5. Agarose gel electrophoresis showing IS-PCR fingerprintings generated by eleven *E. coli* strains with different virulence markers isolated from suckling piglets with diarrhoea. Lane M: 100 bp DNA ladder.

IS-PCR analysis of 11 ETEC isolates possessing LTI and STI virulence markers revealed that these strains produced 11 distinct DNA fragment profiles. The patterns of *E. coli* strains 3B, 4B, 5B, 6B, 7B, 8B, 9B, and 11B displayed weaker or stronger amplicon of 650 bp. On the other hand, this DNA band was not observed among strains 1B, 2B and 10B (Fig. 3). The cluster analysis of the IS-PCR profiles revealed that LTI/STI-positive strains belonged to three major genetic groups. One group comprised isolates 3B, 4B, 5B, 6B with a high degree of similarity (80%) and the strain 7B with a lower genetic relatedness (70%). The second clonal group consisted of the 1B, 2B, 9B, and 11B strains. Moreover, the isolate 8B generated the separate cluster with a very low percentage of similarity (<40%) to other strains of this group (Fig. 4).

Further IS-PCR analysis of the enterotoxigenic *E. coli* isolates was performed to determine the genetic relationship between ETEC strains expressing different fimbrial/toxic virulence markers. The obtained results revealed that 11 ETEC strains belonging to group C produced 11 distinct DNA fragment profiles. Each PFGE pattern had several bands within the 1000 and 1300 bp range, common for all isolates tested and the additional 2500 bp amplicon observed in strains 6C and 7C (Fig. 5). The cluster analysis of IS-PCR profiles performed by the UPGMA method revealed that the *E. coli* strains tested belonged to three different genetic groups with an average degree of similarity (70%). Two isolates (4C and 5C), with the same virulence properties (LTI/STI/F4), generated identical profiles. Moreover, they were closely related to strain 2C (90% of similarity). The remaining LTI/STI/F4-positive strain 3C was closely related to the second clonal group which comprised STI/F5-positive isolates (strains 6C, 7C and 11C). It was also observed that two STI/F5-positive strains (8C and 9C) were closer related to the isolates possessing diverse virulence markers (LTI/STI/F4-positive strains 1C, 2C, 3C, 4C) than to ETEC expressing the same virulence properties. Moreover, one STI/F5-positive isolate (10C) generated a separate cluster with a similarity below 80% to other *E. coli* tested (Fig. 6).
Discussion

Several reports on genetic investigation of *E. coli* bacteria were published before. The authors utilized tests that were based either on restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) (2, 8, 14, 16) or arbitrary amplification of DNA sequences with PCR (AP-PCR) (4, 9, 13). Among them, IS-PCR used as a molecular method for subtyping of *E. coli* strains due to its high discriminatory power and a good reproducibility (15). In the previous study, the genetic relatedness of *E. coli* producing Shiga toxins that were isolated from cattle and humans, were examined by the methods based on arbitrary primer amplification of DNA sequences and by pulsed-field gel electrophoresis of chromosomal DNA digested with *XbaI* endonuclease. It was shown that PFGE with the *XbaI* restriction endonuclease was the most powerful tool to reveal genetic relationship among *E. coli* O157 strains isolated form different sources (27). On the other hand, Davis et al. (8) found that in the absence of epidemiological data similarity coefficients from a single-enzyme PFGE are poor measures of relatedness of *E. coli* 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 (22, 29). Other researchers have found that only combination of results obtained with several typing methods, including AP-PCR and PGFE, improve the discriminatory power of the molecular differentiation tests used (11, 14).

All ETEC isolates tested in the present study were regularly recovered in the faeces of the diarrhoeic animals and possessed fimbrial and toxic virulence markers which are responsible for diarrhoea in pigs (6, 28). The genetic analysis of these isolates revealed that bacteria possessing the same virulence-associated factors can be classified as different clonal groups, e.g. strains 9A and 11A possessing the same virulence profiles (LTI/F4), generated two separate clusters with a low percentage of genotypic relatedness. The very low percentage of similarity was also observed between strain 8B and the remaining isolates of group B (<40%). These results are in agreement with the previous studies of Weiner et al. (27). It was observed then, that *E. coli* strains possessing the same virulence-associated factors can be classified, using UPGMA method, as belonging to different clonal groups. On the other hand, the highest genetic correlation was noted between 3 pairs of LTI/F4-positive strains (7A and 8A, 2A and 6A, 3A and 4A, respectively). Moreover, LTI/STII/F4-positive isolates 4C and 5C generated identical fingerprints.

In spite of a limited number of strains, the obtained results show that the IS-PCR method is a quick and convenient tool for the detection of molecular relationship between *E. coli* strains originating from sucking piglets with diarrhoea. Therefore, the information obtained with this technique can provide a better knowledge of the genetic relatedness and clonal origin of ETEC strains as well as should lead to great progress in molecular epidemiology.

References