Genotyping of Fimbrial Adhesins in *Escherichia coli* Strains Isolated from Slovak Piglets Suffering from Diarrhea

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ABSTRACT. One-hundred sixty *Escherichia coli* isolates obtained from piglets with diarrhea from different parts of Slovakia were examined for the presence of genes coding for F4, F5, F6 and F41 fimbrial adhesins, and hemolytic activity. According to polymerase chain reaction tests 74 (46 \%) *E. coli* isolates were positive for primers that detected genes coding for fimbrial adhesins. Of these 74 isolates, 64 were positive for genes encoding for F4\textsuperscript{+}, four for F5\textsuperscript{+}, five for F6\textsuperscript{+}, and one for both F41\textsuperscript{+} and F5\textsuperscript{+} adhesins.

Many strains of *Escherichia coli* are part of the nonpathogenic facultative flora of intestinal tract of humans and other mammals. Some of them are capable of inducing diseases of the gastrointestinal and urinary tracts or may affect the central nervous system (Balows \textit{et al.} 1991). Such effects are ascribed to entero-toxigenic *E. coli* (ETEC) which cause serious economic losses in farm animal herds and are widespread in newborns (Kaper \textit{et al.} 1988) in both developed and developing countries. This is a consequence of a wide range of transmission possibilities of these pathogens including direct contact, food, drinks, environment, atmosphere and others (Ondrašovič \textit{et al.} 1997). Epidemiology and clinical symptoms of the disease are similar in various animal species but the majority of strains are species-specific (Zhu \textit{et al.} 1994). They differ particularly in the type of the expressed surface “adherence” antigen (adhesin or pilus). These micro-organisms produce two main types of virulence factors – fimbrial adhesions and enterotoxins (Martins \textit{et al.} 2000).

Adhesin is considered to be a primary component of pathogenicity of entero-toxigenic strains of *E. coli* which allows them to adhere to small-intestine enterocytes (Donnenberg 1995). The first adhesin described, obtained from field isolates of *E. coli* capable of producing diarrheal diseases in piglets, was the K88 antigen (Ørskov \textit{et al.} 1961). Later on serological methods were used to differentiate between three different types of K88 antigens, K88ab, K88ac, and K88ad (Bijlsma \textit{et al.} 1987). In addition, Dykes \textit{et al.} (1985) differing in two subtypes of the K88ab antigen, viz. K88ab1 and K88ab2.

In addition to K88 fimbrial antigens, we recognize additional factors of virulence – fimbrial adhesions and enterotoxins (Martins \textit{et al.} 2000). The diagnostics of ETEC strains has developed from the tests on intestinal loops of piglets (Evans \textit{et al.} 1973) through immunological tests (latex agglutination, ELISA) (Thorns \textit{et al.} 1989, 1992; Cryan 1990) to DNA tests including the PCR (Stacy-Phipps \textit{et al.} 1995; Holoda \textit{et al.} 1998; Osek 1999; Alexa \textit{et al.} 2001; Bogyiová \textit{et al.} 2002).
The aim of our study was to determine the prevalence of ETEC strains expressing one of the mentioned adherence antigens in three different territories of Slovakia by the polymerase chain reaction (PCR). In parallel, the strains were examined for their ability to express hemolysins.

MATERIALS AND METHODS

Reference strains. PCR diagnosis was done using the following reference strains: *E. coli* 298:K88+ (F4), 329:K99+ (F5), 318:987P+ (F6), and 320:K88ac+, *E. coli* 491:K88ad+ were kindly supplied by Dr. J. Osek (National Veterinary Research Institute, Pulawy, Poland); *E. coli* G491:K88ac+, *E. coli* M1:K88ab+ (Holoda and Mikula 1994). An apathogenic laboratory strain of *E. coli* HB101 was used as negative control (Ausubel et al. 1989).

Field isolates. The screening examination included field isolates of *E. coli* strains obtained from piglets suffering from diarrhea (1–28-d old), originating from nonvaccinated herds from the territory of Bratislava, Nitra and Košice (all Slovakia). The strains were isolated from intestinal contents of dead animals or from rectal swabs of piglets with diarrhea at the Departments of Bacteriology (State Veterinary Institutes in Bratislava and Nitra, Slovakia) and the Department of Food Hygiene and Technology (Institute of Microbiology and Immunology, University of Veterinary Medicine, Košice, Slovakia) in the period 2001–2002. The samples were inoculated directly on MacConkey agar (*Oxoid*, England) and the colonies obtained were identified by standard biochemical procedures (Holoda et al. 2001). The solitary colonies intended for PCR examination were inoculated on LB agar and cultivated at 37 °C overnight.

Preparation of samples for the PCR determination. One colony grown on the LB agar was resuspended in 100 μL saline. After centrifugation (12 000 g, 30 s), the sediment was resuspended in 100 μL deionized water and incubated at 100 °C for 10 min. Second centrifugation (14 000 g, 5 min) provided a supernatant of which was subjected to PCR examination (2 μL aliquot). Samples with negative PCR results were used to isolate genomic DNA by the Miniprep method (Ausubel et al. 1989). The DNA was analyzed again by the PCR.

Reaction conditions for the PCR. The primers used are shown in Table I.

| Target gene | Oligonucleotide sequence | Amplified product, bp | Reference |
|-------------|--------------------------|-----------------------|-----------|
| F4          | 5’-GCT GCA TCT GCT GCA TCT GGT ATG G-3’ | -a                    | Holoda et al. 2003 |
|             | 5’-CCA CTG AGT GCT GGT AGT TAC AGC C-3’ |                       |           |
| F5          | 5’-TGC GAC TAC CAA TGC TCC TTC TG-3’ | 450                   | Ojeniyi et al. 1994 |
|             | 5’-TAT CCA CCA TTA GAC GGA GC-3’ |                       |           |
| F6          | 5’-TCT GCT CTT AAA GCT ACT GG-3’ | 333                   | ditto     |
|             | 5’-AAT TCC ACC GTT TGT ATC AG-3’ |                       |           |
| F41         | 5’-GAT GGA CTT TCA TCA TCT TTG AG-3’ | 431                   | ditto     |
|             | 5’-AGT CCA TTC CAT TTA TAG GC-3’ |                       |           |

The PCR reaction was done by AmpliTaq DNA polymerase (*Perkin Elmer*) in an amount of 1.0 U, 2.5 mmol/L dNTPs, PCR buffer solution *Perkin Elmer* with MgCl2 in a total volume of reaction mixture equal to 50 μL. Additional reaction conditions for PCR corresponded to the references in Table I. With every PCR determination, the DNA isolated from the respective reference strain was used as positive control and DNA from *E. coli* HB101 as negative control.

Agarose gel electrophoresis. The PCR products were identified by agarose gel electrophoresis using 1.5 or 2 % agarose gel in electrophoretic TAE buffer solution (*Oxoid*, England) supplemented with sheep erythrocytes up to a concentration of 5 %, and cultivated at 37 °C for 18 h. The hemolytic activity was evaluated visually.
Genotyping. One-hundred sixty isolates of *E. coli* were collected from samples of intestinal contents from nonvaccinated herds. Genetic variants of fimbrial adhesins F4, F5, F6 and F41 of all isolates were differentiated using PCR. The presence of genes encoding expression of individual fimbrial adhesins was determined.

RESULTS

Seventy-four isolates contained genes coding for some of the investigated adherence adhesins. These 74 positive cases made it possible to obtain 64 PCR-amplified products indicating the presence of F4+ genes, four F5+ genes and five F6+ genes. In one field isolate we found the presence of a gene coding for both F41 and F5 adhesin. None of the isolates contained only the gene encoding F41 adhesin.

Analysis PCR amplification products of F4+ (Fig. 1) by restriction endonucleases revealed that two out of 64 F4+ (K88+) positive isolates showed the characteristics of genotype K88ab and 62 of genotype K88ac. None of the isolates could be classified as K88ad genotype.

The products of amplification of DNA reference strains were specific in all examinations while amplification of DNA obtained from *E. coli* HB101 provided in all cases negative results.

Hemolytic activity. Cultivation on blood agar of 160 field isolates of *E. coli* showed that 117 of them (73 %) exhibited β-hemolysis; it was detected in all 64 isolates positive for F4+. Isolates that provided positive PCR results for genes coding for other adherence adhesins (F5, F6, F41) were all nonhemolytic. Absence of hemolysis was also observed in a strain which harbored genes coding for both F5 and F41 adhesin (Table II).

DISCUSSION

Enterotoxigenic *E. coli* strains (ETEC) are the frequent cause of acute diarrhea on pig farms. A common feature of these strains is the expression of one or more adherence adhesins (F4, F5, F6, F41, or combination of them) and the subsequent production of enterotoxins. This results in the development of profuse diarrhea which has frequently fatal consequences. The diagnosis of ETEC strains has long relied on the detection of enterotoxins. ELISA tests, radioimmunoassay tests (Cryan 1990) and LT (heat-labile *E. coli* enteroxin) polynucleotide probe with enzymic, nonisotopic detection systems (Rademaker et al. 1993) have been used for this purpose. The effectiveness of serological tests is affected by the level of expression of antigen and the quality of the antisera used. In the recent period diagnosis of these strains has been carried out increasingly with the help of the PCR method as a quite sensitive and specific method with primers for detection of genes coding for enterotoxin (Stacy-Philips et al. 1995) or adherence antigens (Ojeniyi et al. 1994; Osek 1999; Alexa et al. 2001; Holoda et al. 2002; Vu-Khac et al. 2003).

We found a prevalence of F4 adherence antigen in ETEC strains isolated from piglets suffering from diarrhea originating from nonvaccinated farms located in three different Slovak territories (east, central, west). In 74 (46 %) of the total number of isolates we detected the presence of genes coding for some adherence antigen. Of these 74 isolates 87 % were F4-positive (40 % of the total number). Also Hampson
and Nagy and Fekete (1999) detected F4 adherence antigen as the most frequent in ETEC strains isolated from piglets with diarrhea from various countries. Analysis of F4+ amplified products by restriction endonucleases showed unambiguously that F4ac was the predominant variant – as 62 of the 64 F4 positive isolates were F4ac (97%). A high incidence of F4 adhesin in ETEC strains was also observed by Wilson and Francis (1986) (48%), Westerman et al. (1988) (71%), Osek and Svennerholm (1991) (56%) and Ojeniyi et al. (1994) (31%). Also Alexa et al. (2001) reported that 233 of 238 F4-positive isolates in Czechia were F4ac (98%).

Wilson and Francis (1986), Garabal et al. (1997) and Kwon et al. (1999) emphasized a high prevalence of the F6 adherence antigen in ETEC strains. We found only five to be positive for F6 (3.1%). A low prevalence of ETEC strains in isolates from piglets with diarrhea which expressed F6 adherence antigen was reported in Sweden (Soderlind et al. 1988) and United Kingdom (Wray et al. 1993).

High prevalence of ETEC strains expressing F5 and F41 fimbrial adhesions was described in Spain (Garabal et al. 1997) and Sweden (Soderlind et al. 1988). On the other hand, strains expressing F5 and F41 adherence antigens were isolated rarely in the United Kingdom (Wray et al. 1993) and in Poland (Osek and Truszczynsky 1992) which is in agreement with our results (F5-positive were recorded only in four cases, i.e. 2.5%) and none of the isolates coded for F41 alone. One field isolate showed positivity for two adhesins (F5 and F41). These observations support the earlier finding (Morris et al. 1980) that adhesins of F41 types were most often found in association with F5.

The results of our study and published data indicate that the frequency of occurrence of individual types of adherence antigens is related to geographical location.

All F4-positive isolates formed β-hemolysis on blood agar (see Table II). This corresponds to the findings of Wittig et al. (1994) who reported that all ETEC strains F4+ or F18+ adhesins were capable of producing hemolysin. They assumed the presence of pathogenicity DNA islands on plasmids or on chromosomes which can explain the correlation of the linkage gene clusters coding for hemolysin and fimbriae. Frydendahl (2002) suggested the hemolytic activity as a possible marker for pathogenic potential as defined by the presence or absence of virulence factor genes. Fifty-three of the remaining field isolates exhibited hemolytic activity but none of them was positive for the presence of any of the adherence antigens investigated in this study.

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### Table II. The occurrence of fimbrial adhesins and hemolytic activity in E. coli strains isolated from suckling piglets with diarrhea

| Fimbrial adhesin | Number of isolates | Hemolytic activity |
|------------------|--------------------|-------------------|
|                  |                    | +     | –     |
| F4ab             | 2                  | 2     | 0     |
| F4ac             | 62                 | 62    | 0     |
| F4ad             | 0                  | 0     | 0     |
| F5a              | 4                  | 0     | 4     |
| F6               | 5                  | 0     | 5     |
| F41a             | 0                  | 0     | 0     |
| F41 + F5         | 1                  | 0     | 1     |
| Fb               | 86                 | 53    | 33    |
| Total number     | 160                | 117   | 43    |

*Individual.

*F4, F5, F6, and F41 negative.

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