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Detection of Shiga Toxins, Intimin and Enterohemolysin in *Escherichia coli* Strains Isolated from Children in Eastern Slovakia

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ABSTRACT. Fifty *Escherichia coli* strains isolated from stool samples of 51 healthy children, 143 strains isolated from stool samples of 327 children with diarrhea and 24 strains isolated from stool samples of 21 children with suspected hemolytic uremic syndrome were examined for the presence of Shiga toxin-producing *E. coli* virulence factors (shiga toxin 1 and 2, intimin and enterohemolysin) and their genes. Vero-cell assay and latex agglutination were used for detection of Shiga toxin 1 and 2, TSB agar with washed erythrocytes was used for detection of enterohemolysin; genes encoding shiga toxin 1 and 2, intimin and enterohemolysin were detected using multiplex PCR. The presence of *E. coli* strains harboring genes encoding shiga toxin 1 and 2 (12 strains), intimin (34 strains) and enterohemolysin (12 strains) was demonstrated.

Karmali *et al.* (1983) recognized that shiga toxin-producing *Escherichia coli* (STEC) are associated with hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans. Since then, an increasing number of outbreaks following consumption of contaminated food have been reported (Ling *et al.* 1999). The STEC strains are the most common cause of bloody diarrhea and HUS throughout the world (Beutin *et al.* 1998). In some European countries, the STEC strains were identified as an important cause of watery diarrhea (Pulz *et al.* 1999); however, some other authors found asymptomatic carriage (*e.g.*, Pradel *et al.* 1999).

HUS, as the most important complication of the STEC infection, is the direct result of shiga toxin-induced kidney damage. It is the major cause of acute renal failure in children 0–15 years old. It is due to susceptibility of renal glomeruli to shiga-toxin activity. Shiga toxins (Stx) are the major virulence factors in STEC. All cells that are susceptible to Stx express glycolipid globotriaosylceramide (Gb_3), whereas cells that do not express Gb_3 are resistant to the toxins (Ling *et al.* 1998). The children's glomerular cells have substantially more gb3 receptors on their cell membrane than adults have (Lingwood 1994). The toxin binding explains the epidemiology of renal pathology which may follow STEC infection. From the clinical and epidemiological viewpoints the capacity to determine rapidly whether a patient with diarrhea is infected with STEC is extremely important (Paton and Paton 1999).

Several members of the Stx family have been characterized. The most significant include shiga toxin 1 (Stx1) and shiga toxin 2 (Stx2) (Lingwood 1996). The natural hosts of STEC are farm and wildlife ruminants. Epidemiological studies have repeatedly identified cattle as the principal reservoir of this human pathogen (Armstrong *et al.* 1996). In continental Europe, STEC has been found in cattle in 10 countries (Čížek *et al.* 1999); poultry and poultry products can also be a source of STEC foodborne disease in man (Pilipčinec *et al.* 1999). It has been recognized for a number of years that the STEC strains causing human disease may belong to a broad range of the O serogroups. However, a subset of these (particularly O157 and O111) appear to be responsible for the majority of serious cases (those complicated by HUS) (Paton and Paton 1999).

As the incidence of STEC in Slovakia has not yet been systematically studied we decided to search for the presence of STEC in infant population suffering from diarrhea, HC and HUS.

MATERIALS AND METHODS

Bacterial strains. E. coli strains were isolated from stool specimens of 399 children (50 healthy children, 327 children with diarrhea and 21 children with suspected HUS). Strains were isolated using selective media (McConkey agar and glucitol-McConkey agar with supplement consisting of cefixime and potassium tellurite).

Serotyping. E. coli strains isolated from fecal samples were serotyped using 8 polyvalent and 43 monovalent antisera (*Denka Seiken*, Japan). Screening for *E. coli* O157:H7 was performed by culture on glucitol-McConkey agar with supplement (SMAC) and confirmed using latex agglutination kit O157 (*Oxoid*, UK).

Detection of shiga toxins. All *E. coli* strains were tested for the presence of Stx using Vero-cell assay and latex agglutination assay (*Oxoid*, UK). Cytotoxicity of Stx was tested on Vero cells cultured in RPMI-1640 medium with 5 % fetal calf serum, penicillin (100 000 U/mL) and streptomycin (100 g/L) at 37 °C in an atmosphere with 5 % CO_2 (*Heracell*, Germany). Culture supernatants obtained from polymyxin-treated bacterial cells were applied on confluent Vero cell monolayer and cytotoxicity was evaluated after 1, 2 and 3 d (the test was adapted from the original assay of Beutin *et al.* 1998). Treatment with polymyxin B was used to gain free Stx from bacteria for the methods mentioned above.

Detection of enterohemolysin. Detection of the enterohemolytic phenotype was performed on TSB agar containing washed sheep erythrocytes (Schmidt and Karch 1996).

PCR. The presence of shiga toxin-1 and -2 genes (*stx1, stx2*), intimin gene (*eaeA*) and enterohemolysin gene (*ehx*) was determined in bacterial lysates obtained after 1-d cultivation on blood agar using multiplex PCR (Paton 1998). Samples (2 μL) of every bacterial lysate were amplified in 50 μL reaction mixture containing 200 μmol/L deoxynucleoside triphosphates, approximately 250 nmol/L of each primer and 1 U of platinum *Taq* polymerase (*Gibco BRL*, UK) in 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl and 1.5 mmol/L $MgCl_2$. The samples were subjected to 35 PCR cycles (Paton 1998). PCR reaction mixtures (10 μL) were electrophoresed on 2 % agarose gels and stained with ethidium bromide; results were read by 1D Image Analysis *Kodak* software program (Fig. 1).

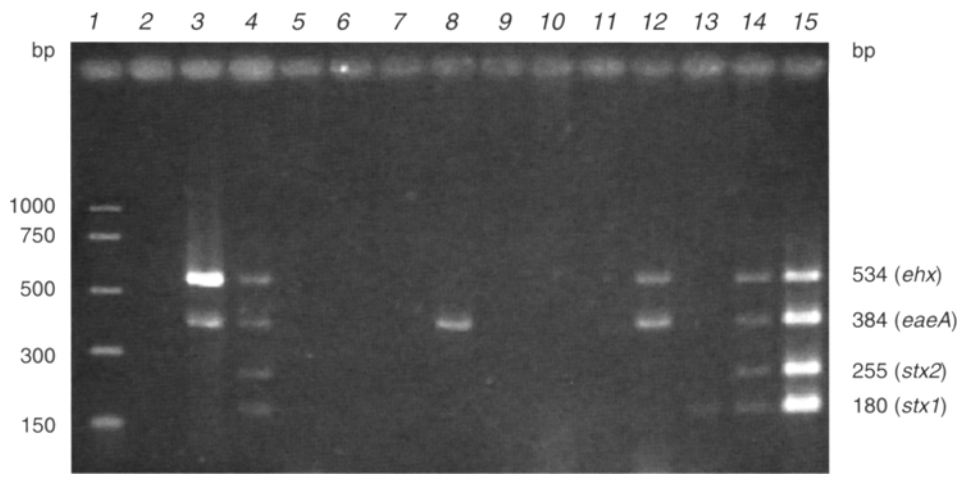

Fig. 1. Multiplex PCR; detection of genes encoding EaeA, Ehx, Stx1 a Stx2; *1* – PCR marker, *2* – *E. coli* C600Rif (negative control); *3–14* – clinical samples, *15* – *E. coli* O157:H7 EDL933 (positive control).

RESULTS

Two-hundred seventeen *Escherichia coli* strains isolated from stool specimens of healthy children (50), children with diarrhea (327) and children with suspected HUS (21) were serotyped and investigated for the presence of Stx (Table I) as the main virulence factor associated with HC and HUS, and other accessory virulence factors, intimin and enterohemolysin (cf. Bogyiová *et al.* 2002).

The 76 of a total 217 *E. coli* strains characterized belonged to 27 different serogroups. Among strains isolated from stools of healthy children 7 strains were classified to O44, 4 strains to O125, 1 strain to O126, 2 strains to O146, one strain to O166 and 36 strains were not serotyped by the antisera available. There were O4 (n = 1), O6 (4), O7 (1), O18 (1), O26 (2), O27 (1), O44 (8), O55 (5), O115 (1), O125 (2),

O128 (2), O127 (4), O142 (1), O153 (2), O157 (1), O158 (1) serogroups and 96 strains were not serotyped among *E. coli* strains isolated from stools of children with diarrhea. Among strains isolated from children with suspected HUS the following O antigens were detected: O1 ($n = 2$), O2 (1), O3 (1), O5 (1), O8 (1), O23 (1), O124 (1), O125 (1), O127 (4), O144 (1), O157 (1) serogroups; 9 strains were nontypable.

We did not find any glucitol-negative strain among the 217 isolated strains, although we have confirmed two *E. coli* O157 strains. We did not detect any shiga-toxin activity either by the Vero-cell assay or by latex agglutination. All 12 *ehx* gene-positive strains showed colonies with the typical enterohemolytic phenotype.

Table I. PCR analysis of isolated *Escherichia coli* strains in a group of children with gastroenteritis (diarrhea) and hemolytic uremic syndrome

No. of isolates	Gene[a]			
	stx1	*stx2*	*eaeA*	*ehx*
gastroenteritis				
65	–	–	–	–
6	–	–	+	–
2	+	–	+	–
1	+	+	–	–
2	–	–	+	+
3	+	+	+	–
1	+	+	–	+
1	–	+	+	+
9	+	+	+	+
hemolytic uremic syndrome				
14	-	-	-	-
6	-	-	+	-
1	-	-	+	+

[a]*stx* – shiga-toxin, *eaeA* – intimin, *ehx* – enterohemolysin.

There were 12 (6 %) strains (Table II) positive for *stx1* and/or *stx2*, 34 (16 %) strains contain *eaeA* gene and 12 (6 %) were positive for enterohemolysin gene, all four virulence-factor genes were found in 8 strains. Ten of 12 (83 %) *stx*-positive strains were *eaeA*-positive and nine of 12 (75 %) *stx*-positive strains harbored *ehx* gene.

Twenty-one children had typical signs of HUS; however, 24 *E. coli* strains isolated from the stools did not possess any *stx* genes. There was one strain with *eaeA* gene and *ehx* gene, and six strains with *eaeA* gene. We did not find any Stx activity in Vero-cell assay or in latex agglutination test.

DISCUSSION

E. coli strains (19 %) were *stx1*- and/or *stx2*-positive, 26 % *eaeA*-positive and 14 % *ehx*-positive. Our *stx*-positive strains were positive for *eaeA* gene sequence in 88 % and for *ehx* gene sequence in 65 %. On the other hand, Beutin *et al.* (1994) found that STEC which were positive for *stx1* and/or *stx2* were frequently positive for *eaeA* sequences (in 92 %) and for production of enterohemolysin (88 %).

As reported by some authors (Boyce *et al.* 1995; Čížek *et al.* 1999; Willshaw *et al.* 2000), *E. coli* O157 usually does not ferment glucitol but we isolated two glucitol-positive O157 *E. coli* strains; similar findings were described by Bielaszewska *et al.* (2000), Sobieszczanska *et al.* (2000) and Coia (1998). After

Table II. Detection of virulence factors and their genes in isolated *E. coli* strains

Clinical status[a]	No. of strains	Ehx[b]	Gene			
			ehx	*eaeA*	*stx1*	*stx2*
HUS	24	1	1	7	0	0
GE	143	11	11	26	12	7
Healthy	50	0	0	1	0	0
Total	217	12	12	34	12	7

[a]HUS – hemolytic uremic syndrome; GE – gastroenteritis.
[b]Enterohemolytic phenotype

a two-year survey we cannot recommend the use of SMAC for the detection of *E. coli* O157 to be a sufficient diagnostic tool; we found only glucitol-positive *E. coli* strains. For routine laboratory work, the use of the SMAC culture medium seems to be useful for detecting *E. coli* O157 but not in the detection of glucitol-fermenting *E. coli* O157 and non-O157 STEC (Pulz *et al.* 1999). Thus it is more effective to search for production of Stx and to identify the *stx*-gene sequences. Positive detection of *stx1* or *stx2* genes confirms the presence of STEC; valuable additional information on infecting strains can be obtained by testing for the presence of genes encoding putative accessory virulence factors, such as intimin or the plasmid-encoded

hemolysin (Paton and Paton 1999). Clinical laboratories may need to implement serotype-independent methods to avoid underdiagnosis of STEC-mediated bacterial gastroenteritis (Fey *et al.* 2000).

Twelve strains harboring *stx* genes without detection of Stx activity either on Vero-cells or on latex agglutination test correlate with the clinical status of children because none of them had complications (HC or HUS, all patients had uncomplicated gastroenteritis). On the other hand, we obtained 21 stool samples from patients with suspected HUS but we did not find any STEC strain; all samples but one from HUS-suspected patients were negative for O157.

Our analysis showed the presence of STEC strains – 3.7 % in the group of 327 children with gastroenteritis. It is in good agreement with Swaiger *et al.* (1999) who found 5.6 % STEC strains in a group of 296 patients with diarrhea. According to Siegfried *et al.* (1999) in our geographical region other *E. coli*-O serogroups and probably other microorganisms rather than *E. coli* O157 could be involved in the pathogenesis of HUS which is supported also by our recent results. Detection of genes should be accompanied and compared with observations of the phenotype because not all the genes can be expressed in the studied strains as was shown here.

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REFERENCES

ARMSTRONG G.L., HOLLINGSWORTH J., MORRIS J.G.: Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiol.Rev.* **18**, 29–51 (1996).

BEUTIN L., ALEKSIC S., ZIMMERMANN S., GLEIER K.: Virulence factors and phenotypical traits of verotoxigenic strains of *Escherichia coli* isolated from human patients in Germany. *Med.Microbiol.Immunol.* **183**, 13–21 (1994).

BEUTIN L., GLEIER K., ZIMMERMANN S.: Comparative evaluation of different diagnostic methods for the detection of shiga-toxin producing strains of *Escherichia coli* (STEC) in human clinical stool specimens, in *Verocytotoxigenic* E. coli *in Europe*. Teagasc, The National Food Centre, Dublin (Ireland) 1998).

BEUTIN L., ZIMMERMANN S., GLEIER K.: Human infections with shiga toxin-producing *Escherichia coli* other than serogroup O157 in Germany. *Emerg.Infect.Dis.* **4**, 635–638 (1998).

BIELASZEWSKA M., SCHMIDT H., LIESEGANG A., PRAGER R., RABSCH W., TSCHAPE H., ČÍŽEK A., JANDA J., BLÁHOVÁ J., KARCH H.: Cattle can be a reservoir of sorbitol-fermenting shiga toxin-producing *Escherichia coli* O157:H-strains and a source of human diseases. *J.Clin.Microbiol.* **38**, 3470–3473 (2000).

BOGYIOVÁ E., SIEGFRIED L., KMEŤOVÁ M., ŠÁNDORČÍNOVÁ Z., LIPTÁKOVÁ A., BIROŠ E.: Occurrence and genetic association of selected virulence factors in clinical *Escherichia coli* isolates. *Folia Microbiol.* **47**, 73–77 (2002).

BOYCE T.G., SWERLOW D.L., GRIFFIN.P.M.: *E. coli* O157:H7 and the hemolytic uremic syndrome. *New Eng.J.Med.* **333**, 364–368 (1995).

ČÍŽEK A., ALEXA P., LITERÁK I., HAMRÍK J., NOVÁK P., SMOLA J.: Shiga toxin-producing *Escherichia coli* O157 in feedlot cattle and Norwegian rats from a large-scale farm. *Lett.Appl.Microbiol.* **28**, 435–439 (1999).

COIA J.: Clinical, microbiological and epidemiological aspects of *Escherichia coli* O157 infection. *FEMS Immunol.Med.Microbiol.* **20**, 1–9 (1998).

FEY P.D., WICKERT R.S., RUPP M.E., SAFRANEK T.J., HINRICHS S.H.: Prevalence of non-O157:H7 shiga toxin-producing *Escherichia coli* in diarrheal stool samples from Nebraska. *Emerg.Infect.Dis.* **6**, 605–608 (2000).

KARMALI M.A., STEELE B.T., PETRIC M., LIM C.: Sporadic cases of hemolytic uremic syndrome associated with fecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* **1**, 619–620 (1983).

LING H., BOODHOO A., HAZES B., CUMMINGS M.D., ARMSTRONG G.D., BRUNTON J.L., READ R.J.: Structure of the shiga-like toxin I B-pentamer complexed with an analogue of its receptor Gb_3. *Biochemistry* **37**, 1777–1788 (1998).

LING H., PANNU N.S., BOODHOO A., ARMSTRONG G.D., CLARK C.G., BRUNTON J.L., READ R.J.: A mutant shiga-like toxin Iie bound to its receptor Gb_3: structure of a group II shiga-like toxin with altered binding specificity. *Structure* **8**, 253–264 (2000).

LINGWOOD C.A.: Role of verotoxin receptors in pathogenesis. *Trends Microbiol.* **4**, 147–153 (1996).

LINGWOOD C.A.: Verotoxin-binding in human renal sections. *Nephron* **66**, 21–28 (1994).

PATON A.W., PATON J.C.: Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eae*, enterohemorrhagic *E. coli ehxA*, rfb_{O111}, and rfb_{O157}. *J.Clin.Microbiol.* **36**, 598–602 (1998).

PATON A.W., PATON J.C.: Direct detection of shiga toxigenic *Escherichia coli* strains belonging to serogroups O111, O157 and O113 by multiplex PCR. *J.Clin.Microbiol.* **37**, 3362–3365 (1999).

PILIPČINEC E., TKÁČIKOVÁ L., NAAS H.T., CABADAJ R., MIKULA I.: Isolation of verotoxigenic *Escherichia coli* O157 from poultry. *Folia Microbiol.* **44**, 455–456 (1999).

PRADEL N., FORESTIER C., SIROT D., JOLY B., LIVRELLI V.: Detection and characterization of verotoxin-producing *Escherichia coli* in human stools, bovine feces, and foods: a one year survey in France. *Acta Clin.Belg.* **54**, 1–37 (1999).

PULZ M., VOGELSANG E., FUCHS T., HINZ S., FRISCH W., WINDORFER A., KARCH H.: Prevalence and molecular characterization of shiga toxin-producing *Escherichia coli* (STEC) in asymptomatic children. *Acta Clin.Belg.* **54**, 37 (1999).

SCHMIDT H., KARCH H.: Enterohemolytic phenotypes and genotypes of shiga toxin-producing *Escherichia coli* O111 strains from patients with diarrhea and hemolytic-uremic syndrome. *J.Clin.Microbiol.* **34**, 2364–2367 (1996).

SCHWAIGER M., GRIF K., PIERARD D., KARCH H., ALLEBERGER F.: Shiga toxin-producing *Escherichia coli* was the third most frequent bacterial cause of diarrhea in Austria during July and August of 1998. *Clin.Microbiol.Infect.* **5**, 645–647 (1999).

SIEGFRIED L., KMEŤOVÁ M., PUZOVÁ H., ROSOCHA J., LIPTÁKOVÁ A., SEHNÁLKOVÁ H.: Characteristics of *Escherichia coli* strains isolated in cases of hemolytic uremic syndrome. *Trans.Univ.Košice* **5**, 67–69 (1999).

SOBIESZCZANSKA B.M., GRYKO R., MALEK C.W.: Isolation of verotoxigenic strains of *Escherichia coli* O26 in Poland. *Clin.Microbiol.Infect.* **6**, 227–229 (2000).

TOZZI A.E., FISHER I.S.T.: Epidemiology of VTEC infections in Europe: enter-net and other data. *Acta Clin.Belg.* **54**, 34 (1999).

WILLSHAW G.A., CHEASTY T., SMITH H.R., O'BRIEN S.J., ADAK G.K.: Verocytotoxin-producing *E. coli* (VTEC) O157 and other VTEC from human infections in England and Wales: 1995–1998. *J.Med.Microbiol.* **50**, 135–142 (2000).