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# Characterisation of selected virulence markers in clinical *Escherichia coli* strains associated with haemolytic uremic syndrome and enteritis

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In the present study we analysed haemolytic phenotype, relation to O groups as well as the presence of genes encoding enterohaemolysin, intimin and Shiga toxin in clinical *Escherichia coli* strains associated with haemolytic uremic syndrome and enteritis. We concluded that the presence of enterohaemolytic genotype was associated with intimin gene. Our results supported the finding that both enterohaemolytic phenotype and genotype can be employed as an easily detectable marker for screening of enterohaemorrhagic in clinical samples.

Key words: *Escherichia coli*, enterohaemolysin, intimin, enteritis, haemolytic-uremic syndrome.

Abbreviations: EHEC, enterohaemorrhagic; HUS, haemolytic-uremic syndrome; Stx, Shiga toxin.

## Introduction

Strains of *Escherichia coli* that cause diarrhoea are classified into six groups: enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC), enteroaggregative (AeggEc) and enterodiffusive (DAEC) (Nataro & Kaper, 1998). These groups of *E.coli* strains are characterised according to their specific virulence determinants responsible for the typical clinical, pathological and epidemiological features. The most important ones include toxins, adhesins and haemolysins.

Enterohaemorrhagic *E. coli* has emerged as

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a significant cause of food-borne diseases during the past few years. EHEC are dangerous human pathogens, which include predominant serogroup *E. coli* O157:H7. However, there are also other non-O157 serogroups responsible for a variety of symptoms and development of severe, potentially life-threatening illness. In humans, EHEC are associated with watery or bloody diarrhea, hemorrhagic colitis, and systemic toxemic complications, such as haemolytic-uremic syndrome (HUS) (Čížek et al., 1999).

Enterohaemorrhagic strains of *E. coli* have two unique characteristics: they can produce toxins and colonize the intestinal tract of susceptible individuals. The defining virulence determinant of EHEC is Shiga toxin (Stx) (Coia, 1998). Members of the Stx family are cytotoxins grouped according to both biological activity and antigenic characteristics to two distinct groups known as Stx1 and Stx2. They are compound toxins, comprising a single catalytic 32-kDa A subunit and a multimeric B subunit (7.7-kDa monomers) that is involved in the binding of the toxin to specific glycolipid receptors present on the surface of target cells (Paton & Paton, 1998a,b). In humans, the Stx binds to the globo-triaosylceramide surface receptor (gb3), which is present, e.g., on endothelial and renal human cells.

The *stx* genes carried by EHEC strains are, with one possible exception (*stx2e*), encoded on bacteriophage genome integrated into the bacterial chromosome (Kimmitt et al., 2000). A single EHEC strain may express Stx1 only, Stx2 only, or both toxins, or even multiple forms of Stx2 (Nataro & Kaper, 1998).

Many EHEC strains can produce intimin, an outer membrane protein involved in adhesion of bacteria to the intestinal mucosa. Intimin is a 97-kDa protein responsible for attachment-effacement (A/E) lesions similar to those produced by enteropathogenic *E. coli* (Beutin, 1999). All of the genes necessary for intimin expression in EPEC are located on a 35.5-kb "pathogenicity island" termed the locus for enterocyte effacement (LEE). The mechanism whereby EHEC strains generate A/E lesions is less well characterized but it is essentially analogous to that for EPEC. EHEC strains displaying the A/E phenotype have a LEE homologue, which contains a copy of *eaeA* gene, whose protein product (934 amino acid residues) exhibits 83% amino acid identity to EPEC intimin (Paton & Paton, 1998a).

Production of enterohaemolysin has also been described as a virulence factor. EHEC-haemolysin (enterohaemolysin) is a pore-forming cytotoxin, which is cytolytic to human and animal cells. It belongs to the group of toxins characterised by glycine-rich repeats. The toxin was discovered by Beutin et al. (1989), who observed that a high proportion of EHEC strains (89% of those tested) had a novel haemolytic phenotype distinct from that associated with the *E. coli* $\alpha$-haemolysin. The term EHEC-hemolysin was used to distinguish it from the $\alpha$-haemolysin to which it is related, but not identical (Burland et al., 1998). Strains producing EHEC-haemolysin are not haemolytic on standard blood agar but produce small, turbid haemolytic zones on washed sheep blood agar after overnight incubation (Paton & Paton, 1998a). The operon encoding the enterohaemolysin phenotype was found to be located on the large virulence plasmid. The genes are organized in an operon structure and share approximately 60% sequence homology with *E. coli* $\alpha$-haemolysin (Beutin, 1999). The EHEC-haemolysin operon is present in almost all EHEC of serogroup O157 and less frequently in other EHEC serotypes (Pierard et al., 1997).

In the present study we investigated the production of enterohaemolysin and $\alpha$-haemolysin, presence of genes encoding intimin and shiga toxin, and relation to O groups aimed to detect EHEC among clinical *Escherichia coli* strains isolated from stools of patients with clinical diagnosis of haemolytic-uremic syndrome and diarrhoea.

## Material and methods

We investigated a total of 167 *E. coli* strains. 24 *E. coli* strains were isolated from stools of patients with haemolytic-uremic syndrome (HUS) (group I) and 143 *E. coli* strains were isolated from stools of patients with enteritis (group II). The samples were inoculated on Sorbitol McConkey agar plates and incubated for 24 h at 37 °C. Sorbitol negative colonies (5–10) were subjected to biochemical tests to confirm species identification. Sorbitol fermenting colonies (5–10) were also examined. The colonies were further investigated for their enterohaemolytic phenotype and genotype, *eaeA* genotype and *stx1* and *stx2* genotype.

Serotyping of *E.coli* strains was performed by standard method with 8 polyvalent *E. coli* antisera and 42 monovalent antisera (Denka Seiken, Japan).

*E. coli* strains were streaked onto agar plates containing defibrinated washed sheep erythrocytes as described previously by Beutin et al. (1989). The *E. coli* strains were grown on tryptose blood agar base (Difco Laboratories, USA) containing sheep erythrocytes washed three times in phosphate-buffered saline, pH 7.2. The enterohaemolysin agar plates were read for haemolysis after 3 h incubation (indicating only $\alpha$-haemolytic phenotype) and after overnight incubation (indicating enterohaemolytic phenotype).

Evaluation of the plates after 3 and 18 to 24 h of incubation allowed optimal assessment of the haemolytic phenotype (either α-haemolysin or enterohaemolysin). After overnight growth at 37 °C, the enterohaemolytic phenotype was detected by the occurrence of small, turbid zones of haemolysis.

A multiplex PCR was used for the detection of genes encoding Shiga toxins 1 and 2 (*stx1* and *stx2*), intimin (*eae*A), and enterohaemolysin A (*ehx*). Crude DNA extracts were used as DNA templates for multiplex PCR as described by PATON et al. (1993). The *ehx, eaeA, stx1* and *stx2* genes were detected by PCR using the specific primers as described by PATON & PATON (1998b). Using primers for stx1 and stx2 specific gene regions for A subunit coding region including stx2 variants were amplified. Amplicon size for stx1 is 180 bp, for stx2 is 255 bp. Primer pair for eaeA is conserved between EPEC and STEC and its size is 384 bp. Amplicon size for primer pair encoding specific sequence of enterohaemolysin gene is 534 bp. After amplification (PATON & PATON, 1998b), specific DNA fragments were resolved by gel electrophoresis using 2% (w/v) agarose. Gels were stained with 0.5 mg of ethidium bromide per mL, visualized with UV illumination, and imaged with KODAK system.

*E. coli* K12 $Rif^R$ 330/74 from The Czechoslovak National Collection of Type Cultures (Prague, Czech Republic) was used as negative control. *E. coli* O157:H7 EDL 933 *stx1+*, *stx2+*, *eaeA+* and *ehx+* was used as positive control. The strain was obtained from Dr. M. SOBIESZCZANSKA (Department of Microbiology, University of Wroclaw, Poland).

Table 1. Serogroups of *E. coli* strains associated with HUS and enteritis.

| Clinical diagnosis | Serogroup[a] | No. of strains |
|---|---|---|
| HUS ($n = 24$) | O1 | 2 |
| | O2 | 1 |
| | O3 | 1 |
| | O5 | 1 |
| | O8 | 1 |
| | O23 | 1 |
| | O124 | 1 |
| | O127 | 4 |
| | O144 | 1 |
| | O157 | 1 |
| | ND | 10 |
| Enteritis ($n = 143$) | O4 | 1 |
| | O6 | 4 |
| | O7 | 1 |
| | O18 | 1 |
| | O26 | 2 |
| | O27 | 1 |
| | O44 | 1 |
| | O55 | 4 |
| | O115 | 1 |
| | O128 | 1 |
| | O142 | 1 |
| | O153 | 2 |
| | O157 | 1 |
| | O158 | 5 |
| | ND | 111 |

[a]ND = not detected.

## Results

In our study 167 *E. coli* strains were investigated. All isolated strains were sorbitol positive. The 46 of 167 *E. coli* strains characterized in this study belonged to 24 different serogroups.

Four strains belonged to serogroup O127, two to O1 and one to O2, O3, O5, O8, O23, O124, O144, O157 of 24 strains isolated from patients suspected from HUS. Ten strains were not serotyped by antisera available.

Among the *E. coli* strains isolated from children suffering from enteritis, serogroup O158 was found in five strains, O55 and O6 in the four and O26 and O153 in the two. One isolate belonged to serogroup O4, O7, O18, O27, O44, O115, O128, O142 and O157. Remaining 111 *E. coli* strains isolated from children suffering from eneritis were not typeable by antisera available (Tab. 1).

Haemolytic phenotype was observed in 43 isolates (26%) out of 167 investigated isolates. Twelve strains of *E. coli* exhibited the typical enterohaemolytic phenotype with small, turbid zones of haemolysis occurring after 18 to 24 h of incubation and 31 isolates (19%) were α-haemolytic. Of these α-haemolytic strains, four strains were isolated from patients with HUS and 27 strains from patients with enteritis. One of enterohaemolysin-positive strain was isolated from patient with HUS and eleven were isolated from patients with enteritis. All twelve strains with enterohaemolysin phenotype were positive for *ehx* gene detected by multiplex PCR.

The *eaeA* gene was detected in 33 (19.7%) of 167 *E. coli* strains (Tab. 1).

There was any *stx* gene found among strains from patients with HUS (group I). The *stx* genes were found in twelve strains obtained from patients with enteritis (group II). Nine of 12 *stx* positive strains possessed also the *ehx* gene as well as the *eaeA* gene. One *stx* positive strain possessed *eaeA* gene and two *stx* positive strains possessed neither *ehx* nor *eaeA* gene.

The detection of enterohaemolysin, α-hemolysin, *ehx* gene and *eaeA* gene in 23 strains of *E. coli* isolated from group I and 142 strains of *E. coli* isolated from group II is summarised in Ta-

Table 2. Virulence factors in *E. coli* strains associated with HUS and enteritis.

| Clinical diagnosis | No. of strains | Enterohaemolysin phenotype | *ehx* | α-hemolysin | *eae* |
|---|---|---|---|---|---|
| HUS | 24 | 1 | 1 | 4 | 7 |
| Enteritis | 143 | 11 | 11 | 27 | 26 |
| Total | 167 | 12 | 12 | 31 | 33 |

ble 2. Enterohaemolysin phenotype in correlation with presence of both the *ehx* and *eaeA* genes was detected in one of *E. coli* strains from patient with HUS and in eleven of *E. coli* strains from group II. Two strains from the group I and three strains from the group II were *eaeA* positive and they also produced α-haemolysin. Two strains (8.3%) from the group I and 14 strains (9.8%) from the group II possessed only *eaeA* gene with no haemolysins. Fourteen *E. coli* strains (58%) from the group I and 90 from the group II (63%) showed neither the enterohaemolytic phenotype or genotype, nor α-haemolysin phenotype and *eaeA* sequences.

The enterohaemolytic phenotype was observed in 1 of 24 strains (4.2 %) from patients with HUS and in 11 of 143 strains (7.7 %) from patients with gastroenteritis.

## Discussion and conclusions

EHEC represents a group of very important pathogens capable of causing serious human disease – haemolytic-uremic syndrome. Since little is known about the prevalence of human EHEC among diarrhoeagenic *E. coli* in Slovakia, we have studied 167 *E. coli* strains isolated from cases of HUS and diarrhoea aimed to analyse the presence of some selected phenotype (O antigens, enterohemolysin, α-haemolysin) and genotype (both *eae* and *stx* genes) characteristics.

Current available epidemiological data suggest that among EHEC strains the O157:H7 is the predominant serotype in the USA (Tarr, 1995; Warner, 1996), Canada (Rowe, 1994), United Kingdom and Japan (Nishikawa et al., 2000; Watanabe et al., 1999) but non-O157:H7 serotypes are more common in mainland Europe, Australia and South America (Beutin et al., 1998, Goldwater & Bettelheim, 1995, Paton & Paton, 1999, Schmidt et al., 1999). Our results support the findings reported in some European countries. Among 24 clinical *E. coli* strains isolated from patients suffering from HUS only one strain (4.2%) belonged to O157 (Bielaszewska et al, 2000; Sobieszczanska et al., 2000).

Looking on pathogenic potential of *E. coli* causing HUS, the most important trait is that of shiga toxin production. Of less importance, as presented by Paton & Paton (1998a), is the capability of *E. coli* strains to produce enterohaemolysin. Some authors (Beutin et al., 1989; Schmidt et al., 1996; Nataro & Kaper, 1998), however, demonstrated a high coincidence of both shiga toxin and enterohaemolysin – almost 90%. Compared to data stated above we did not find *stx* gene in any of *E. coli* causing HUS. However, surprisingly we demonstrated up to 12 *E. coli* strains (8.3%) carrying *stx* gene among diarrhoeagenic isolates.

The enterohaemolytic phenotype, which is expressed by many EHEC strains from humans and animals, was found to be associated with the presence of *ehx* gene (Schmidt & Karch, 1996; Schmidt et al., 1999). In our study all twelve strains producing enterohaemolysin were also positive for *ehx* gene. However, some strains carrying the genes for enterohaemolysin were found to be phenotypically haemolysin-negative, whereas others showed enhanced haemolytic activity similarly as presented by Schmidt et al. (1999).

As some authors pointed out there is a strong association between carriage of the *eae* gene and the capacity of EHEC to cause the HUS (Paton & Paton, 1998a; Beutin, 1999). In our study, however, only seven (29.1%) HUS strains and 27 (18.8%) diarrhoeagenic strains carried the *eae* gene. It is of interest that all *ehx* positive isolates were also *eae* positive (Tab. 3), however, other 22 *eae* positive isolates did not carry the *ehx* gene.

We also judged a role of α-haemolysin that is well-known to take part in pathogenesis of extraintestinal *E. coli* infections (Johnson, 1991; Siegfried et al., 1992). In the present work we did not find any interesting correlation of α-hemolysin to other virulence factors.

In conclusion we did not confirm fully the findings presented by other authors that are associated to incidence of virulence factors in *E. coli* strains causing the HUS. This distinctive picture

Table 3. Coincidence of virulence factors in *E. coli* strains associated with HUS and enteritis.

| Clinical status | Enterohaemolysin | *ehx* | α-hemolysin | *stx* | *eae* | No. of strains |
|---|---|---|---|---|---|---|
| HUS ($n = 24$) | + | + | − | − | + | 1 |
| | − | − | + | − | + | 2 |
| | − | − | − | − | + | 4 |
| | − | − | + | − | − | 2 |
| | − | − | − | − | − | 15 |
| Enteritis ($n = 143$) | + | + | − | + | + | 9 |
| | + | + | − | − | + | 2 |
| | − | − | + | − | + | 3 |
| | − | − | + | − | − | 23 |
| | − | − | − | + | + | 1 |
| | − | − | − | − | + | 12 |
| | − | − | − | + | − | 2 |
| | − | − | − | − | − | 91 |

indicates possible differences in geographical distribution of characteristics influencing the pathogenetic potential of *E. coli*.

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