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SHORT COMMUNICATION

Association of Untypeable Enteropathogenic *Escherichia coli* (EPEC) Strains with Persistent Diarrhea in Children from the Region of Lower Silesia in Poland

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Abstract

Enteropathogenic *Escherichia coli* strains (EPEC) carrying the *eae* gene encoding intimin are divided into typical strains producing bundle forming pili, encoded by the *bfpA* gene, and atypical strains lacking the gene. In the study typical and atypical EPEC that did not agglutinated with EPEC polyvalent antisera but carrying virulence factors characteristic to other pathogenic *E. coli i.e.* diffusely adhering and enteroaggregative *E. coli* were isolated from 24 (43.6%) of 55 children > 10 years old with persistent diarrhea. These results indicated that non-typeable typical and atypical EPEC can contribute to chronic intestinal infections in teenagers.

Key words: adherence patterns, typical and atypical enteropathogenic *E. coli*

There are several groups of pathogenic *Escherichia coli* (*E. coli*) strains associated with gastrointestinal infections in humans. Enteropathogenic *E. coli* (EPEC) in contrast to other pathogenic *E. coli* characterize localized adherence pattern (LA) in which compact clusters of bacteria adhere to epithelial cells. The LA pattern results from specific to EPEC pathotype virulence factors involved in adhesion *i.e.* bundle forming pili (BFP) that initiate binding of bacteria to the host cells. The *bfp* operon encoding BFP fimbriae is localized on a 50–70 kDa plasmid called EPEC Adherence Factor (EAF). The binding of EPEC to host cells is crucial to the delivery of effector molecules secreted into the enterocytes *via* a type III secretion system (TTSS) that cause cytoskeleton rearrangement and actin accumulation beneath clusters of adhering bacteria. This specific lesion called 'attaching and effacing' (AE) characterize loss of microvilli and intimate attachment of EPEC to the epithelial cells of the small intestine (Clarke *et al.*, 2003). All genes required to produce AE lesions are localized in EPEC on a large chromosomal pathogenicity island, locus of enterocyte effacement (LEE). The LEE region contains the *eae* operon encoding the outer membrane protein intimin that interacts with translocated intimin receptor Tir delivered into the host cell membrane *via* TTSS. The *eaeA*- and *bfpA*-positive EPEC strains that carry genes involved in LA adherence and produce AE lesions are classified as typical (tEPEC). Unlike tEPEC, the *eaeA*-positive but *bfpA*-negative EPEC are classified as atypical (aEPEC) and display LA-like (LAL), diffuse adherence (DA) or aggregative adherence (AA) patterns (Clarke *et al.*, 2003). EPEC cause profuse watery diarrhea in infants younger than 2 years of age. Generally course of diarrhea caused by EPEC is acute although, there are some reports on the association of these pathogens with prolonged episodes of diarrhea (Afset *et al.*, 2004). Diffusely adhering *E. coli* (DAEC) is a heterogenous group of *E. coli* strains responsible for urinary tract infections and diarrhea in humans. DAEC harbor Afa/Dr family of afimbrial and fimbrial adhesins involved in diffuse adherence (DA) to the epithelial cells (Servin, 2005). Enteroaggregative *E. coli* (EAEC) is a diverse group of *E. coli* associated with acute and chronic diarrhea worldwide. Most EAEC produce virulence factors that are under the transcriptional control of the pAA plasmid-borne *aggR* activator (Cennimo *et al.*, 2007). All EAEC strains share characteristic aggregative adherence (AA) pattern to the epithelial cells distinguishing this group of *E. coli*. (Kaur *et al.*, 2010).

The aim of the study was to search for EPEC strains among 55 *E. coli* strains isolated from intestinal biopsy specimens of children with chronic diarrhea. A fifty five *E. coli* strains were isolated from intestinal biopsy

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Table I
Primer sequences and PCR conditions used in the study.

Gene	PCR primers	PCR	Product size (bp)	References conditions
eaeA	(F) CACACGAATAAACTGACTAAAATG (R) AAAAACGCTGACCCGCACCTAAAT	55°C 20 s, 30 cycles	376	Lindqvist, 1997
bfpA	(F) ATTGGTGCTTGCGCTTGCTGC (R) GCCGCTTTATCCAACCTGGTA	69°C 1 min 30 cycles	326	Gunzburg *et al.*, 1995
hlyA	(F) GCTTGCAAAGCAATCCGCTGCAAATAAA (R) CTGTGTCCACGAGTTGGTTGATTAG	58°C 1 min, 30 cycles	561	Müller *et al.*, 2009
afaD	(F) GTCAGGTGCCGGAATATCAGT (R) CACTCTCCCCTGTGAACTTCA	65°C 30 s, 35 cycles	250	Sobieszczańska *et al.*, 2012
daaD	(F) GGGAGTATAAGGAAGATGATGCG (R) TATTCCTGTGGCACCACACA	60°C 30 s, 35 cycles	437	this study
aggB	(F) GCATATTACCGATGTCCTGCG (R) CCTCTTGATATTAGACATTCACCA	58°C 30 s, 30 cycles	421	Sobieszczańska *et al.*, 2012
aggR	(F) CTAATTGTACAATCGATGTA (R) ATGAAGTAATTCTTGAAT	42°C 1 min, 25 cycles	308	Czeczulin *et al.*, 1999

specimens obtained during endoscopy from 55 consecutive untreated children (mean age 11 years) with persistent diarrhea associated with patchy sites of inflamed small intestine mucosa that were diagnosed in the 2nd Department and Clinic of Paediatrics and Gastroenterology of Medical University of Wrocław, Poland. A reference *eaeA*- and *bfpA*-positive EPEC O26:H11 displaying LA adherence pattern was used as a positive control in all assays. All strains examined were defined as *E. coli* by standard biochemical testing (PLIVA-Lachema Diagnostica). The strains were serogrouped by slide agglutination method employing antisera: O25, O26, O44, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128, O142 (BIOMED, Poland) specific for classical EPEC O serogroups. Hemolytic activity of *E. coli* isolates was assessed on sheep blood agar after 3 h and 24 h of incubation at 37°C. The *in vitro* adherence assay to embryonic human epithelial cell line Int407 was performed according to Cravioto *et al.* (1991). The expression of the *eaeA* gene was evaluated using fluorescent actin staining (FAS) test detecting the accumulation of F-actin at the intimate adherence sites of bacteria. The assay was performed according to Knutton *et al.* (1989). Oligonucleotides used for amplification of the *eaeA, bfpA, hlyA, afaD, daaD, aggB* and *aggR* were synthesized on the basis of published nucleotide sequences or designed for the study in our laboratory (Table I). All PCR amplifications were performed in a DNA-Engine PT200 thermal cycler (MJ Research Waltham, MA, USA).

None of the 55 *E. coli* isolates agglutinated with polyvalent O antisera, although 24 (43.6%) of the isolates showed the presence of the *eaeA* gene encoding intimin (Table II). Only 7 of these 24 *eaeA*-positive strains (29.2%) adhered to the epithelial cells in LA pattern. The remaining 17 *eaeA*-positive strains (70.8%) showed undefined (UD) pattern of adherence *i.e.* diffuse/localized (DA/LA) or localized/aggregative (LA/AA). FAS assay was positive for 19 out of the 24 *E. coli* strains (79.2%) and the reference strain (Fig. 1). The remaining 5 *eaeA*-positive but FAS-negative *E. coli* (20.8%) showed hemolytic activity on blood agar after 3 h of incubation and induced changes in the epithelial cells morphology (Fig. 1). All these 5 hemolytic isolates showed the presence of *hlyA* gene (Table II). *E. coli* α-hemolysin is a pore forming cytolysin targeting to the plasma membrane of red blood cells and a wide range of nucleated host cells, including intestinal epithelial cells. Damage to the cell membranes and the flow of ions triggers the disruption of cellular actin cytoskeleton, thus preventing the accumulation of polymerized F-actin beneath adhering bacteria (Menestrina *et al.*, 2001). Based on the presence of the *bfpA* gene, 18 out of the 24 *eaeA*-positive *E. coli* (75%) were categorized as tEPEC (typical EPEC). The remaining 6 *eaeA*-positive, but *bfpA*-negative strains (25%) were considered as aEPEC (atypical EPEC). Since most 18 *eaeA*-positive strains did not show adherence patterns of EPEC pathotype, therefore the genes encoded adhesins characteristic to diffusely adhering *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC) were evaluated. From a number of different Afa/Dr adhesins associated with DAEC pathotype the *afaD* gene of the *afa* operon encoding the AfaE-I afimbrial adhesin and the *daaA* gene of the *daa* operon encoding F1845 fimbria from a subfamily of Afa/Dr adhesins were selected. As EAEC pathotype characteristic genes, the *aggR* and *aggB* genes were chosen. The *afaD* and *daaA* genes were associated with 10 (41.7%) and 3 (12.5%) of the 24 *eaeA*-positive *E. coli*, respectively. Less commonly, the examined EPEC strains carried EAEC pathotype characteristic genes *i.e. aggB* and *aggR* that were present

Table II
Phenotypic and genotypic characteristics of twenty-four *eaeA*-positive strains of *E. coli*.

E. coli strain	Adherence pattern	№ of strains (%)								
		eaeA	*bfpA*	FAS	*hlyA*	hem[1]	*afaD*	*daaA*	*aggB*	*aggR*
Typical EPEC										
EC35/1	UD	+	+	+	+	–	+	–	+	+
EC37/1	UD	+	+	+	–	–	+	–	–	–
EC41/5	LA	+	+	+	–	–	–	–	–	–
EC42/1	LA	+	+	+	–	–	–	–	–	–
EC44/1	CDT	+	+	ND	+	β	–	–	–	–
EC45/3	UD	+	+	+	+	–	+	–	+	+
EC49/2	LA	+	+	+	+	–	–	–	–	–
EC28/1	UD	+	+	+	–	–	+	–	–	–
EC30/1	UD	+	+	+	–	–	+	–	+	–
EC38/3	UD	+	+	+	–	–	+	–	+	+
EC47/1	LA	+	+	+	–	–	–	–	–	–
EC74P/4	LA	+	+	+	+	–	–	–	–	–
EC25/1	UD	+	+	+	+	–	–	–	+	+
EC36/1	LA	+	+	+	–	–	–	–	–	–
EC84/1	CDT	+	+	ND	+	β	–	+	–	–
EC68P/5	CDT	+	+	ND	+	β	–	–	–	+
EC87/1	UD	+	+	+	–	–	+	+	–	+
EC87P/5	LA	+	+	+	–	–	–	–	–	–
Atypical EPEC										
EC86/1	CDT	+	–	ND	+	β	–	–	–	–
EC50/1	CDT	+	–	ND	+	β	–	–	–	+
EC53P/5	DA	+	–	+	–	–	+	+	+	+
EC48/2	UD	+	–	+	–	–	+	–	–	–
EC43/3	LAL	+	–	+	–	–	–	–	–	–
EC46/1	UD	+	–	+	–	–	+	–	–	–

[1] hemolytic activity was evaluated after 3 h and 24 h of incubation at 37°C; β, clear zone of hemolysis visible after 3 h of incubation; LA, localized adherence; LAL, localized-like adherence; DA, diffuse adherence; UD, undefined mixed adherence *e.g.* localized/diffuse or localized/aggregative; CDT, cell-detaching strain; ND, not determined

in 6 (25%) and 8 (33.3%) of isolates, respectively. Moreover, 6 of the 24 strains (25%) carried both genes specific for DAEC and EAEC pathotype. In contrast, none of the 7 of tEPEC strains (29.2%) that demonstrated LA pattern carried *aggB* and *aggR* genes.

In the study both, tEPEC and aEPEC strains were isolated from biopsy specimens from inflamed intestinal mucosa of children with chronic intestinal disorders accompanied by persistent diarrhea. The atypical strains constituted the minority of these strains. The association of tEPEC and aEPEC strains with intestinal mucosa may indicate their relationship with the inflammation and clinical symptoms or imply secondary colonization of the diseased mucosal tissue. In each of these cases, direct contact of tEPEC or aEPEC with intestinal epithelial cells can cause induction of characteristic histopathological AE lesions in the intestinal mucosa leading to the development of diarrhea. The ability of isolated EPEC to induce AE lesions was confirmed by positive FAS test for all but five hemolytic strains. All isolated EPEC strains were nontypeable with antisera specific for classical EPEC and majority of them carried genes characteristic to other than EPEC pathotypes, indicating that these strains differ somehow from tEPEC and in that respect seem to be more similar to aEPEC. The presence of the *afaD* gene characteristic to DAEC pathotype that encodes both, adhesin and invasin AfaD (Jouve *et al.*, 1997) among more than forty percent of EPEC, suggests that the acquired virulence factor can contribute to the internalization of these strains. Indeed, our preliminary study shows that many of these strains were internalized by the intestinal epithelial cells (data not shown). Moreover, nearly one third of the EPEC strains showed the presence of the *aggR* and *aggB* genes that are characteristic to EAEC pathotype. Yatsujangi *et al.* (2002) have also described EPEC strains isolated from diarrheal patients that represented typical tEPEC serotypes *i.e.* O126:NM and

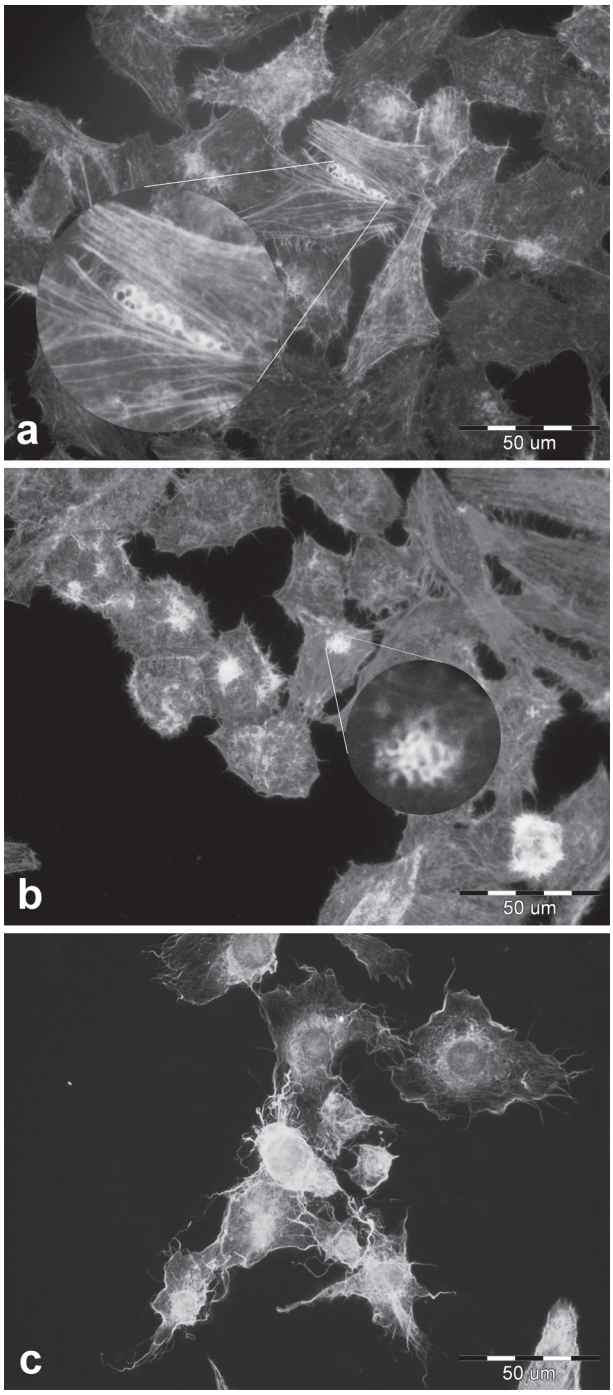

Fig. 1. a) FAS- and *eae*–positive *E. coli* O26: H11 strain. Fluorescence accumulation (AE, attaching and effacing lesion) visible beneath adhering bacteria; b) FAS-positive *E. coli* isolated from child with Crohn's disease. The magnified fragment of picture indicates AE lesion caused by the *eae*-positive *E. coli* strain; c) Epithelial cells damaged by hemolytic *E. coli* strain and stained with FITC-conjugated falloidine. The cells lost their natural shape and become veil-like shaped. Fluorescent microscope. Magnification 40x.

O111:NM and possessed the *aggR* gene, but not *eae* or *bfpA* genes associated with classical EPEC strains.

In conclusion, the results of the study indicated that nontypeable tEPEC and aEPEC strains carrying virulence factors characteristic to DAEC and EAEC pathotypes can contribute to chronic intestinal infections in children. The results of the study also raised the problem of the proper diagnosis of infections caused by nontypeable EPEC strains based only on the serologic determination of EPEC O serogroups, without detection of virulence factors these pathogens.

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