MICROBIAL PATHOGENESIS

Virulence properties of *Escherichia coli* faecal strains isolated in Poland from healthy children and strains belonging to serogroups O18, O26, O44, O86, O126 and O127 isolated from children with diarrhoea

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Four hundred and twenty-seven *Escherichia coli* isolates from 427 cases of infantile diarrhoea in Poland, belonging to serogroups O18, O26, O44, O86, O126 and O127 and 150 *E. coli* isolates from 52 healthy children were examined for selected virulence properties. The presence of the plasmid pAA, a plasmid encoding enterohaemolysin, the genes encoding intimin (*eae*), bundle-forming pili (*bfp*), Shiga toxins I and II (*stxI*, *stxII*) and cytotoxic necrotising factor types 1 and 2 (*cnf1*, *cnf2*) was investigated by PCR. Adhesion to HEp-2 cell monolayers was also tested and selected strains were investigated for the presence of P-fimbriae and haemolytic activity. Typical enteropathogenic *E. coli* isolates (typical EPEC, strains possessing *eae* and *bfp*, but not *stx*) were not found. The particular classes of *E. coli* among 427 isolates from ill children were: atypical EPEC (*eae*⁺ *bfp*⁺ *stx*⁻), 21.3%; Shiga toxin-producing *E. coli* (STEC), 0.7%; diffusely adherent *E. coli* (DAEC), 4%; enteroaggregative *E. coli* (EAEC), 16.9%; necrotoxic *E. coli* type 1 (NTEC1), 0.2%; and cell-detaching *E. coli* (CDEC), 29%. With the exception of STEC, all the above classes of *E. coli* were found among the isolates from healthy children which comprised: atypical EPEC 8.0%, DAEC 6.7%, EAEC 17.3%, NTEC1 14.0% and CDEC 40.0%. Cell detachment (CD) was significantly associated with 3-h haemolytic activity. There was also strong correlation between haemolytic activity (Hly) and the presence of P-fimbriae. No correlation was found between the presence of the *cnf1* gene and CD, Hly or P-fimbriae.

Introduction

Enteropathogenic strains of *Escherichia coli* (EPEC) are important causes of infantile diarrhoea in the developing world [1]. Since their first description in the 1940s, the principles of defining EPEC have evolved from epidemiological observations that isolates belonged to certain serogroups, then that certain O:H types correlated with diarrhoea, and that isolates possessed well-characterised pathogenicity markers. EPEC are identified by their ability to cause effacement of microvilli and intimate adherence between the bacterium and the epithelial cell (A/E lesion) with

polymerised actin accumulation and pedestal-like structures forming beneath adherent bacteria [2, 3]. The locus of enterocyte effacement (LEE) pathogenicity island contains the *eae* gene, encoding intimin, a bacterial outer-membrane protein (OMP) involved in the intimate bacterial attachment to the gut mucosa. Other properties of EPEC include localised adherence (LA, bacteria form characteristic microcolonies on the surface of the cells) mediated by the EPEC adherence factor (EAF) plasmid-encoded bundle-forming pili (*bfp*) gene [4, 5]. However, the EAF plasmid was not detected among many EPEC isolates. Thus, typical EPEC are *eae*⁺, possess the EAF plasmid and do not produce Shiga toxins [6]. Atypical EPEC, irrespective of their adherence phenotype, are *eae*⁺ and neither possess EAF plasmid nor produce Shiga toxins. However, many Shiga toxin-producing *E. coli* (STEC) also produce A/E lesions mediated by a homologue of the EPEC LEE pathogenicity island [7, 8]. Further-

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more, STEC produce Shiga toxin I or II (*stx* genes), or both [9, 10], and many possess a plasmid that encodes the so-called enterohaemolysin [11].

Other types of *E. coli* that have been associated with diarrhoea have been characterised by their adherence pattern and effect on tissue culture cells. Thus, diffusely adherent *E. coli* (DAEC) are bacteria that are dispersed over the surface of the cell [12] and possess none of the virulence factors known to be characteristic of other diarrhoeagenic *E. coli*. DAEC seems to be a heterogeneous group of strains composed probably of true pathogens that cause infantile diarrhoea (especially among children aged 2–5 years) and non-pathogenic bacteria [13]. Enteraggregative *E. coli* (EAEC) adhere to tissue culture cells and coverslips in a characteristic stacked brick-like configuration [12]. Many EAEC strains possess a 60-MDa aggregative adherence plasmid (pAA) [14]. Like DAEC, EAEC seem to be heterogeneous and have been found in healthy people, but there are an increasing number of reports of their association with diarrhoea [15]. Cell-detaching *E. coli* (CDEC) strains cause detachment of HEp-2 cell monolayers from coverslips. CDEC were significantly associated with diarrhoea in Australian aboriginal children [16], although another study in children did not find an association [17]. The results of the latter study showed a correlation between cell detachment (CD) and α -haemolysin production. It was shown later that the CDEC isolates of the Australian study produced cytotoxic necrotising factor 1 (CNF1) [18]. CNF1 or CNF type 2 (CNF2) are virulence markers of necrotoxic *E. coli* (NTEC). NTEC that produce CNF1 (NTEC1) are strains with well-documented extra-intestinal pathogenicity [19]. Although found in the faeces of healthy children, they were isolated in some cases as a putative agent of diarrhoea [18, 19]. CNF1- and α -haemolysin-encoding genes (*cnf1* and *hly*) are clustered together on a pathogenicity island [20]. The close association of both factors with P-fimbriae, an adhesin of the uropathogenic *E. coli*, has also been reported [19].

In Polish regional sanitary laboratories, stool specimens of children aged up to 2 years with diarrhoea are routinely investigated for the presence of *E. coli* strains belonging to 15 selected serogroups regarded as EPEC. Of these serogroups, the most frequently isolated are O18 (28%), O26 (8.2%), O44 (12.6%), O86 (7.1%), O126 (4.8%) and O127 (5.1%) (total, 66%). The aim of this study was to determine the prevalence of the genetic markers of EPEC, STEC, EAEC and NTEC among isolates from children with diarrhoea and belonging to the six EPEC serogroups mentioned above. Adherence pattern or cell detachment were also tested. Selected isolates were also tested for haemolytic activity and the presence of P-fimbriae. Isolates from stool specimens of healthy children were also included in the study.

Materials and methods

Bacterial isolates

A total of 427 *E. coli* isolates was received from laboratories in different areas of Poland: O18 isolates, 108; O26, 66; O44, 97; O86, 60; O126, 48 and O127, 48. They were isolated from the faeces of 427 children aged up to 2 years with diarrhoea during the period 1993–1999. Their identity was confirmed at the National Institute of Hygiene by classical biochemical tests. The O antigen was confirmed by a latex agglutination kit (Biomex Kraków, Poland). The kit can detect serogroups O18, O25, O26, O44, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128 and O142; its specificity and sensitivity have been described elsewhere [21, 22]. The assay was performed with overnight bacterial cultures in peptone water according to the manufacturer's instructions. To remove the K antigens, the culture was heated at 100°C for 15 min before testing.

Another 150 isolates from 52 healthy children of comparable age were collected in different areas of Poland. Firstly, 10 *E. coli*-like colonies isolated on MacConkey agar were tested with the latex agglutination kit for the presence of EPEC serogroups. Three isolates were then identified biochemically and were investigated further. In six cases, only two *E. coli* isolates per child were obtained. All isolates were stored as agar stabs.

Shiga toxin-producing *E. coli* strain EDL933, enteropathogenic strain E2348/69, enteroaggregative strains DMi155, DEF47, D4140/86 (all provided by Professor H. Karch, Institute für Hygiene und Mikrobiologie der Universität Würzburg, Germany), diffusely adherent strain 3431 (provided by Professor M. Schmidt, Zentrum für Molekularbiologie der Entzündung, Institut für Infektiologie, Münster, Germany) and necrotoxic 1 or 2 strains FVL2, FVL21, FVL25, CCB21, CCB37, B62 (provided by Dr J. Blanco, Facultade de Veterinaria, Universidade de Santiago de Compostela, Lugo, Spain) were used as controls.

Adhesion and cell detachment test

Briefly, a modification of the adhesion and cell detachment test [23] was performed with monolayers of HEp-2 cells grown on circular coverslips in 7-ml trac bottles (Bibby Sterilin Ltd, UK). Bacteria were grown in L broth with D-mannose (Sigma) 1% for 18 h at 37°C without shaking. From each bacterial culture, 40 μ l containing *c.* 2×10^7 bacteria were incubated with HEp-2 monolayers in the presence of D-mannose 1% for 5 h at 37°C in air with CO₂ 5%. Coverslips were then washed three times with phosphate-buffered saline (PBS), fixed with ethanol 70% and stained with Giemsa 10%. Coverslips were mounted on glass slides and examined by oil immersion light microscopy.

Selected isolates were also tested for the pattern of adherence after 6 h with a washing step after 3 h [24]. LA was quantified by selecting microscope fields at random, counting 500 cells, and recording the number of cells with adherent microcolonies (more than five bacteria). An adhesion index was defined as the percentage of cells with adherent microcolonies.

PCR-based assays

The primers and sequences detected by them are listed in Table 1 [25–30].

DNA was extracted from bacteria with the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Amplification was performed in a total volume of 50 μ l, containing 5 μ l of 10 $\times$ concentrated buffer (100 mM Tris-HCl, pH 8.3 and 500 mM KCl), 2 mM MgCl₂, 200 μ M of each deoxynucleosidetriphosphate, 50 pmol of each primer (IDT) except EP, CNF1 and CNF2 (250 pmol), and AmpliTaq[®] DNA Polymerase (Perkin Elmer) 1.25 U. DNA was amplified in a Mastercycler 5330 (Eppendorf). After preliminary denaturation at 95°C for 2 min, DNA was denatured at 94°C for 1 min, then the primers were annealed and extended at 64°C (EP, SLT I, SLT II) or at 55°C (SK, pCVD432, CNFs) for 1.5 min. The MFS, CNF1 and CNF2 primers were annealed at 56°C for 1 min and extended at 72°C for 1 min. After 30 cycles, terminal extension was performed at 72°C for 10 min. The amplification products were subjected to submarine gel electrophoresis on High Resolution Agarose 1% gels (Sigma) and visualised by staining with ethidium bromide.

Restriction endonuclease analysis

Restriction endonuclease analyses of all PCR-amplified products were performed. According to the PCR product sequences [10, 29–34], the following DNA

fragments should be obtained after digestion (bp): SK primer/*Nci*I endonuclease – 8, 418, 455; SK/*Pst*I – 248, 250, 383; EP/*Hha*I – 12, 94, 218; EP/*Hpa*II – 131, 193; SLT I/*Aha*I – 6, 157, 356, 375; SLT I/*Hind* III – 377, 517; SLT II/*Eco*RV – 86, 393; MFS/*Mva*I – 42, 124; pCVD432/*Dra*I – 271, 359; CNF1/*Eco*RV – 87, 227, 229; CNF1/*Mva*I – 32, 77, 434; CNF2/*Eco*RV – 229, 314; CNF2/*Mva*I – 109, 434.

All analyses were performed according to Sambrook *et al.* [35] with digestions for 1 h at 37°C.

P-fimbriae and haemolysin assay

The latex reagent for detection of P-fimbriae was prepared as described elsewhere [36]. Latex particles were coated with Glycoprotein P1 containing P-fimbriae receptor α -D-galactopyranosyl-(1-4)- β -D-galactopyranose (antigen provided by Professor S. Kaluzewski, National Institute of Hygiene, Warsaw, Poland). The strains were grown overnight on CF agar. The tests were performed with a 10¹⁰/ml suspension of live bacteria in NaCl 0.9% and results were read after 3 min.

The haemolysin assay was performed on Columbia agar containing sheep blood (bioMérieux, France) 5%. Plates were examined after 3 h and 24 h for zones of haemolysis.

Statistical analysis

SYSTAT 8.0 was used to calculate the p value (Pearson χ^2 or Fisher exact test) and Phi coefficient for each pair of the following properties: presence of *cnf1* gene, P-fimbriae, haemolytic activity and cell-detachment activity. In the case of isolates from healthy children, only single isolates representing certain phenotypes/genotypes per child were taken for the analysis.

Table 1. Primers used and factors detected by PCR

Primers	Nucleotide sequences (5'–3')	Factor detected by primers	Size of PCR amplified product (bp)	Ref. no.
SK 1	CCC GAA TTC GGC ACA AGC ATA AGC	Intimin	881	25
SK 2	CCC GGA TCC GTC TCG CCA GTA TTC G			
EP 1	AAT GGT GCT TGC GCT TGC TGC	Bundle-forming pili	324	26
EP 2	GCC GCT TTA TCC AAC CTG GTA			
SLT I – 1	CAG TTA ATG TGG TGG CGA AG	Shiga toxin I	894	27
SLT I – 3	CTG CTA ATA GTT CTG CGC ATC			
SLT II – 1	CTT CGG TAT CCT ATT CCC GG	Shiga toxin II	479	27
SLT II – 2	GGA TGC ATC TCT GGT CAT TG			
MFS 1F	ACG ATG TGG TTT ATT CTG GA	STEC plasmid	166	28
MFS 1R	CTT CAG GTC ACC ATA CAT AT			
pCVD432/start	CTG GCG AAA GAC TGT ATC AT	pAA	630	29
pCVD432/stop	CAA TGT ATA GAA ATC CGC TGT T			
CNFs-A	CTG GAC TCG AGG TGG TGG	CNF1 and CNF2	533	30
CNFs-B	CTC CTG TCA ACC ACA GCC			
CNF1-A	GAA CTT ATT AAG GAT AGT	CNF1	543	30
CNF1-B	CAT TAT TTA TAA CGC TG			
CNF2-A	AAT CTA ATT AAA GAG AAC	CNF2	543	30
CNF2-B	CAT GCT TTG TAT ATC TA			

Results

With the latex agglutination kit the following serogroups were identified among isolates from healthy children: O18 (14 isolates from 6 children), O25 (6/2), O26 (1/1), O44 (2/1), O127 (4/2) and O128 (2/1). The remaining 121 isolates belonged neither to the serogroups listed above nor to serogroups O55, O86, O111, O114, O119, O124, O125, O126, O142. The results of isolates from ill children performed in regional sanitary laboratories were confirmed.

The results of the PCR and HEP-2 assay are shown in Tables 2 and 3. Each PCR product gave fragments of the expected sizes after restriction endonuclease digestion (Fig. 1).

Among 427 *E. coli* isolates from children with diarrhoea, atypical EPEC accounted for 91 isolates, STEC1 2, STEC2 1, DAEC 17, EAEC 72 and NTEC1 1 (Table 2). The necrotoxic strain and 26 EAEC isolates (36.1% of all EAEC) caused HEP-2 cell detachment, simultaneously. CDEC accounted for 124 isolates. Of the 91 atypical EPEC isolates, 63.7% were serogroup O26, 34.1% were O127 and 2.2% were O86.

The 72 EAEC consisted of serogroups O86 (47.2%), O44 (38.9%), O126 (12.5%) and O26 (1.4%), and 51 isolates possessed the plasmid pAA. The 17 DAEC belonged to serogroups O127 (35.3%), O126 (29.5%), O86 (17.6%) and O44 (17.6%). All three STEC belonged to serogroup O26 and the only NTEC1 isolate belonged to serogroup O126. The 124 cell detaching isolates belonged to serogroups O18 (71.8%), O86 (16.9%), O44 (10.5%) and O126 (0.8%).

With the exception of STEC, all the above classes of *E. coli* that were found among isolates from children with diarrhoea were also present among isolates from the 52 healthy children. Of the 150 *E. coli* isolates from healthy children, 12 isolates were atypical EPEC, 10 were DAEC, 26 were EAEC, 21 were NTEC1, and they were isolated from 6, 4, 11 and 10 children, respectively. All 21 NTEC1 isolates and 18 EAEC isolates from 8 children caused HEP-2 cell detachment. The 60 CDEC were isolated from 27 children. Of the 26 EAEC, 23 isolates from 10 children possessed plasmid pAA. Among isolates of particular *E. coli* classes, 91.7% of atypical EPEC, 88.5% of EAEC, all DAEC, 90.5% of NTEC1 and 73.3% of CDEC isolates were untypable in the latex agglutination test. The rest

Table 2. Phenotypes and genotypes of isolates from faeces of children with diarrhoea that were positive in at least one of the properties tested by PCR and HEP-2 assay

PCR assay	HEP-2 assay	Number of isolates	Serogroups	Classified as
<i>eae</i> , pSTEC, <i>stx I</i>	ND	2	O26	STEC 1
<i>eae</i> , pSTEC, <i>stx II</i>	ND	1	O26	STEC 2
<i>eae</i> , pSTEC	ND	33	O26	Atypical EPEC
<i>eae</i>	ND	23	O26 (20), O86 (1), O127 (2)	Atypical EPEC
<i>eae</i>	LA	3	O127	Atypical EPEC
<i>eae</i>	DA	3	O127	Atypical EPEC
<i>eae</i>	None	29	O26 (5), O86 (1), O127 (23)	Atypical EPEC
pAA	AA	27	O26 (1), O44 (2), O86 (16), O126 (8)	EAEC
pAA	AA, CD	24	O44 (7), O86 (17)	EAEC, CDEC
<i>cnfI</i>	CD	1	O126	NTEC1, CDEC
	AA	19	O44 (17), O86 (1), O126 (1)	EAEC
	AA, CD	2	O44	EAEC, CDEC
	DA	17	O44 (3), O86 (3), O126 (5), O127 (6)	DAEC
	CD	97	O18 (89), O44 (4), O86 (4)	CDEC

eae, intimin-encoding gene; pSTEC, enterohaemolysin-encoding plasmid of STEC; *stxI*, *stxII*, genes encoding Shiga toxins I and II, respectively; pAA, plasmid of EAEC; *cnfI*, gene encoding cytotoxic necrotising factor type 1; CD, cell detachment; ND, adherence not defined; AA, aggregative adherence; DA, diffuse adherence; LA, localised adherence.

Table 3. Phenotypes and genotypes of isolates from faeces of healthy children that were positive in at least one of the properties tested by PCR and HEP-2 assay

PCR assay	HEP-2 assay	Number of children/ isolates	Serogroups (number of children/isolates)	Classified as
<i>eae</i>	LA	1/3	ND	Atypical EPEC
<i>eae</i>	None	5/9	O26 (1/1), ND (4/8)	Atypical EPEC
pAA	AA	3/8	O25 (1/3), ND (2/5)	EAEC
pAA	AA, CD	7/15	ND	EAEC, CDEC
<i>cnfI</i>	CD	10/21	O18 (1/2), ND (9/19)	NTEC1, CDEC
	AA, CD	1/3	ND	EAEC, CDEC
	DA	4/10	ND	DAEC
	CD	10/21	O18 (5/12), O44 (1/2), ND (4/7)	CDEC

eae, intimin-encoding gene; pAA, plasmid of EAEC; *cnfI*, gene encoding cytotoxic necrotising factor type 1; CD, cell detachment; AA, aggregative adherence; DA, diffuse adherence; LA, localised adherence; ND, not determined.

Fig. 1. Restriction endonuclease analysis of the PCR-amplified DNA. **M**, PCR marker (Sigma); **1**, EP/*HhaI*; **2**, EP/*HpaII*; **3**, SK/*PstI*; **4**, SK/*NciI*; **5**, SLT I/*AluI*; **6**, SLT I/*HindIII*; **7**, SLT II/*EcoRV*; **8**, MFS/*MvaI*; **9**, pCVD432/*DraI*; **10**, CNF1/*EcoRV*; **11**, CNF1/*MvaI*; **12**, CNF2/*EcoRV*; **13**, CNF2/*MvaI*.

of these isolates belonged to the following serogroups: atypical EPEC – O26 (1), EAEC – O25 (3), NTEC1 – O18 (2), and CDEC – O18 (14) and O44 (2). Together with non-typable isolates, three *E. coli* O25, four O127 and two O128 were negative in all tests.

Three healthy children had simultaneous infection with *E. coli* isolates with different properties: one child was infected with *eae*⁺ and aggregative pAA⁺ strains, a second child was infected with *cnfI*⁺, CD⁺ and aggregative pAA⁺, CD⁺ strains, and the third child was infected with *cnfI*⁺, CD⁺ and diffusely adhering strains.

Twenty-five selected intimin-encoding isolates were tested for their adherence pattern after 6 h, with a washing step after 3 h. In the case of the nine non-adhering isolates in the 5-h assay (one O26, one O86 and seven O127 isolates) and two localised-adhering isolates (one untypable and one O127 isolate) the results obtained in the 6-h assay were identical. However, different results were obtained for the remaining 14 isolates. These differences are presented in Table 4. The 14 strains, characterised previously as non-adherent or diffuse adherent or not adhering in a defined way, showed reduced LA indexes in the 6-h assay.

All 150 isolates from 52 healthy children and all serogroup O18 and O86 isolates from children with diarrhoea were tested for haemolytic activity and the presence of P-fimbriae. Table 5 shows the prevalence

Table 4. Comparison of adherence results of 6-h method with those of 5-h method

Isolate no.	Serogroup	6-h assay	LA index	5-h assay
1	NT	LA*	1	0
2		LA*	1	0
3	O26	LA	3.6	0
4		LA	6.8	0
5	O86	LA*	2.6	ND
6		LA*	4.8	ND
7		LA*	13	ND
8		LA*	15	ND
9		LA*	20	ND
10		LA	2	ND
11		LA*	1.2	0
12		LA	1	ND
13		LA	3	DA
14		LA	4	DA

NT, non-typable; DA, diffuse adherence; LA, localised adherence; ND, adherence not defined.

*Localised adherence in which microcolonies were accompanied by few individual bacteria dispersed on the cell monolayer.

of both properties, cell-detachment activity (HEp-2 assay) and presence of the *cnfI* gene (PCR). All 21 of the 60 O86 isolates from ill children, which caused cell detachment (CD⁺), also formed clear zones of haemolysis around the bacterial colony after incubation for 3 h (Hly⁺) and were P-fimbriate (P⁺). Among 108 *E. coli* O18 isolates from ill children, 87 were CD⁺, Hly⁺, P⁺ strains. Other isolates were CD⁺, Hly⁺ (2), Hly⁺, P⁺ (2), P⁺ (3) and 14 had none of the properties sought. All 14 of the *E. coli* O18 isolates from 6 healthy children were CD⁺, Hly⁺, P⁺ and 2 additionally encoded cytotoxic necrotising factor 1 (*cnfI*⁺). Among 136 non-O18 isolates from 48 healthy children the

Table 5. Prevalence of 3-h haemolytic activity, cell detachment, P-fimbriae and cytotoxic necrotising factor 1 (CNF1) among selected *E. coli* isolates

<i>E. coli</i> isolates	Number of children/ strains	Properties
O86 isolates from children with diarrhoea	4/4	CD, Hly, P
	17/17	CD, (AA), Hly, P
O18 isolates from children with diarrhoea	87/87	CD, Hly, P
	2/2	CD, Hly
	2/2	Hly, P
	3/3	P
O18 isolates from healthy children	1/2	<i>cnf1</i> , CD, Hly, P
	5/12	CD, Hly, P
Untypable isolates from healthy children	9/19	<i>cnf1</i> , CD, Hly,
	6/13	CD, (AA), Hly, P
	2/3	CD, Hly, P
	2/5	CD, (AA), Hly
	1/1	CD, Hly
	4/10	P
	1/3	CD
O44 isolates from healthy children	1/2	CD

cnf1, gene encoding cytotoxic necrotising factor type 1; CD, cell detachment; Hly, 3-h haemolytic activity; P, presence of P-fimbriae; AA, aggregative adherence pattern.

particular phenotypes/genotypes accounted for: *cnf1*⁺, CD⁺, Hly⁺, 19 (from 9 children); CD⁺, Hly⁺, P⁺, 16 (from 8 children); P⁺, 10 (from 4 children); CD⁺, Hly⁺, 6 (from 3 children) and CD⁺, 5 (from 2 children).

Statistical analysis showed the strongest correlation between CD and Hly (Phi >0.9). The presence of P-fimbriae also correlated well with Hly and CD (Phi >0.8 and 0.7, respectively). Correlation between *cnf1* and Hly, CD or P was not found (−0.3 < Phi <0.2). Among the O86 isolates from ill children, CD and Hly, CD and P or Hly and P correlated strictly (Phi = 1). These pairs of properties were also correlated in the case of the O18 isolates from ill children (Phi >0.9, 0.7 and 0.8, respectively). In the remaining isolates from healthy children the only correlation found was between Hly and CD (Phi >0.9). For *cnf1* and CD or Hly, the Phi value was *c.* 0.5 and for the remaining pairs of properties (P/CD, P/*cnf1*, P/Hly) the *p* value was >0.05.

Discussion

In this study *E. coli* isolates from faeces of children with diarrhoea and from healthy children were investigated for the presence of selected markers of EPEC, STEC, DAEC, EAEC, NTEC and CDEC, because doubt still exists as to the pathogenic role of some of these classes. In the present study, all classes of *E. coli* were found among both healthy and ill groups of children, with the exception of STEC. However, the isolates from the ill group of children consisted of strains that were seropositive in the latex agglutination kit. In contrast, isolates from healthy children were collected irrespective of the serogrouping results, and 77.8% of these isolates, which were positive in at least one of the tests, were serologically untypable with the latex agglutination kit. Thus, comparison of the prevalence of the *E. coli* classes

among healthy and ill children is not possible. To assign the pathogenic role to the particular classes of *E. coli*, a much bigger selection of untypable and, particularly, typable strains from healthy children needs to be examined. Because many EPEC, EAEC and DAEC isolates from healthy children did not belong to the putative EPEC serogroups, untypable isolates from children with diarrhoea should also be included in a future study.

Typical EPEC strains (*eae*⁺, *EAF*⁺, *stx*[−]) were not found in the present study. This is in agreement with studies in other European countries [37–41], in which most of the isolates that encoded intimin did not hybridise with the EAF probe. In this study, the presence of the *bfp* gene was tested, for which a good correlation with the presence of EAF sequence has been reported [26, 42]. Thus, it seems that in Poland – as in the UK, Italy and France – atypical EPEC strains are more prevalent than typical EPEC. This is supported by the failure to detect the EAF sequence among any of the O26 isolates tested in this study and among selected strains belonging to remaining serogroups tested (data not shown). Although isolates from ill children belonged to serogroups most prevalent in Poland, further study of isolates from other putative EPEC serogroups is needed to determine the prevalence of both atypical and typical EPEC.

Of the 106 isolates encoding intimin, only 6 adhered to HEp-2 cells in a localised way, 3 adhered in diffuse way and 59 adhered in an undefined way (adherence in which a few individual bacteria were dispersed on the cell monolayer or bacteria formed small aggregates of <5 cells on the surface of <1% of HEp-2 cells); 38 isolates did not adhere to the tissue cells. Earlier studies on the adherence pattern of *eae*⁺*EAF*[−] isolates gave variable results and depended on the method used, because many isolates did not adhere when the 3-h

method was used [39, 43, 44]. However, most atypical EPEC isolates adhered in a localised way when incubated for 6 h with a washing step at 3 h [37, 38, 40, 41, 45–48]. Such adherence was termed localised-like to distinguish the adherence pattern from that of typical EPEC strains, which can be detected after incubation for 3 h, and is characterised by the presence of more compact microcolonies of bacteria on most cells [48]. Of the 25 intimin-encoding isolates tested in the 6-h adherence assay in this study, all strains described previously as diffusely adhering (2 isolates) or not adhering in a defined way (7 isolates) and 5 of the 14 strains that were non-adherent after incubation for 5 h adhered in a localised-like way when incubated for 6 h with a washing step at 3 h. Previous localised adherence of the other two isolates was confirmed. Of the nine non-adherent atypical EPEC many were *E. coli* O127, which were described previously [37, 38]. Cantey and Moseley [49] showed that proximity of the bacterial cell to the target cell is necessary for the expression of the *eae* gene cluster and that any adhesive function that enables cell-to-cell contact is sufficient for *eae* expression. They also hypothesised that the putative adhesin – contrary to those encoded by EAF plasmid – may function *in vivo* but not in tissue culture cells. Thus, *eae*⁺ non-adherent isolates could possess adhesins capable of recognising the intestinal mucosal surface but not the cultured cells used in the laboratory assay for adhesion.

Many *E. coli* O26 isolates produce Shiga toxins [50] and in the geographically adjacent Czech Republic O157 and O26 STEC isolates are most prevalent [51]. According to Szych *et al.* [52] and the National Institute of Hygiene (unpublished data), in Polish sanitary laboratories during the period 1997–2000, there were only 93 *E. coli* O157 isolates from people with diarrhoea and among them only 5 isolates produced Shiga toxins (all were O157:H[−] serotype). In the Polish study by Sobieszczńska *et al.* [53], all four *E. coli* O26 isolates tested carried the *stxII* gene. In contrast, of the 66 O26 isolates from children with diarrhoea, only 3 STEC strains were identified in the present study. However, the Shiga toxins encoding prophage has been shown to be unstable [54], and some O26 strains may have been Shiga toxin-producing originally, but lost the prophage during subcultivation in the different laboratories and during storage for several months on agar stabs. Thus, further study based on fresh O26 isolates is needed to clarify the prevalence of STEC among O26 strains in Poland.

Many isolates belonging to O serogroups 44, 86 and 126 were classified as EAEC on the basis of the results of the PCR and adhesion tests. This is in agreement with the results of other studies [37, 38, 55–57].

The results of some previous studies have shown that serogrouping is still a useful diagnostic tool for presumptive identification of diarrhoeagenic *E. coli*

[37, 38]. However, of the six serogroups investigated in this study only 42.8% of isolates from children with diarrhoea possessed markers of STEC, EPEC, EAEC or DAEC. Even excluding the O18 serogroup, which is not considered to be enteropathogenic in most countries, 57.4% of the isolates possessed virulence markers. However, in a particular serogroup, the participation of positive strains differed as follow: O126 – 31.2%, O44 – 36.1%, O86 – 71.7%, O127 – 77.1% and O26 – 93.9%. Thus, in serogroups O86, O127 and, especially, O26 there is a good probability that *E. coli* identified as diarrhoeagenic on the basis of serogrouping are actually putative pathogens. Further studies are now needed to assess the value of serogrouping as a diagnostic tool in the case of isolates belonging to the remaining EPEC serogroups. However, because EPEC, DAEC and EAEC were detected among untypable isolates from healthy children, it is necessary that such *E. coli* isolates from symptomatic children should be tested for the presence of virulence markers when putative EPEC serogroups are not found.

Earlier studies implicated haemolysin production as a cause of cell detachment [17]. As a result of the co-localisation of the *P*, *Hly* and *CNF1* encoding genes on a pathogenicity island [20], these factors could also be associated. Indeed, results obtained from total isolates tested confirmed statistically significant correlation between the presence of cell-detachment activity, haemolytic activity and P-fimbriae. However, these properties were not associated with the presence of the *cnf1* gene.

Gomes *et al.* [58] described enteroaggregative CDEC. In the present study most of the EAEC isolated from healthy children but a minority or none of the EAEC among particular serogroups isolated from ill children caused HEp-2 cell detachment and haemolysis. These findings confirmed the conclusion of Gomes *et al.* that haemolysin should not be regarded as a virulence marker of EAEC.

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