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Virulence Genes Profiles and Phylogenetic Origin of *Escherichia coli* from Acute and Chronic Intestinal Diseases Revealed by Comparative Genomic Hybridization Microarray

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Abstract

The association between *Escherichia coli* virulence factors and chronic intestinal disorders is mostly unknown. The presented study compared the distribution of virulence genes and phylogroups among *E. coli* isolated from chronic intestinal disorders such as Crohn's disease and irritable bowel syndrome (IBS) with strains isolated from patients with acute diarrhea as a control group. The presence of 159 virulence genes corresponding to known *E. coli* pathotypes was determined among 78 *E. coli* archive strains isolated from IBS, acute diarrhea and Crohn's disease using CGH microarray. *E. coli* isolated from IBS demonstrated a mosaic of virulence genes specific to enteropathogenic, enterotoxigenic, enterohemorrhagic *E. coli* strains and *Shigella* species. In contrast, virulence factors and phylogroups distribution among *E. coli* isolated from children with acute diarrhea was similar to extraintestinal *E. coli* strains that probably acquired some virulence genes. The acquisition of virulence genes might have an impact on diarrheagenic potential of these strains. On the other hand, *E. coli* isolated from children with Crohn's disease seem to be similar to adherent-invasive *E. coli* strains (AIEC), as it lack most known virulence genes. The presented study showed that these analyzed groups of *E. coli* strains differed from each other with the respect to the distribution of virulence genes. The differences in gene content support the idea that the participation of *E. coli* in chronic intestinal diseases is mostly related to virulence potential of these strains.

Key words: *E. coli*, DNA microarray, virulence genes

Introduction

There are many *E. coli* pathotypes responsible for gastrointestinal diseases in humans. Most of these pathotypes possess specific virulence factors determining the pathomechanism of the infection, but not necessarily influencing the course of the disease. Enteropathogenic *E. coli* (EPEC) produce intimin responsible for specific histopathological lesions of intestinal microvilli that results in watery diarrhea, whereas enterotoxigenic *E. coli* (ETEC) secrete heat-stable and/or heat-labile enterotoxins causing intestinal net fluid secretion and thus promoting the development of non-inflammatory watery diarrhea similar to that caused by EPEC (Kaper *et al*., 2004). The younger the organism infected with pathogenic *E. coli* strain the bigger the probability of clinically symptomatic disease what is associated with the lack of immunity in young individuals. Thus, most acute intestinal infections caused by pathogenic *E. coli* occur among children (Hunter, 2003).

Crohn's disease, next to ulcerative colitis, is one of the feature of inflammatory bowel disease (IBD) of unknown etiopathogenesis (Moyer, 2005; Abdel-Hady and Bunn, 2004). Recently, the association of adherent-invasive *E. coli* strains (AIEC) with Crohn's disease has been demonstrated. AIEC have been isolated from more than 36% of adult patients with Crohn's disease and from only 6% of healthy individuals (Rolhion and Darfeuille-Michaud, 2007; Darfeuille-Michaud *et al*., 2004). Therefore it is not known whether AIEC can cause intestinal disease only among genetically or immunologically predisposed individuals, or is related to virulence potential of these strains.

IBS is another, probably the most common, chronic intestinal disorder that in contrast with IBD affects only adults (Olden, 2003; Garcia-Rodriguez and Ruigomez, 1999). In our previous study enteroaggregative *E. coli* strains (EAEC) were isolated from more than 80% of patients with diarrheal form of IBS in comparison with only 30% of healthy individuals (Sobieszczańska

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et al., 2007). EAEC are associated with acute or persistent watery diarrhea and have been isolated from children and adults worldwide (Okeke and Nataro, 2001; Weintraub, 2007; Huang *et al*., 2006). The pathotype is defined by its characteristic stacked-brick-like pattern of adherence to epithelial cells (Wientraub, 2007). The pathotype is heterogeneous regarding virulence factors and its ability to cause diarrhea since these strains are isolated from diarrheal and healthy individuals (Kaur *et al*., 2010; Paciorek, 2002).

Most virulence factors of *E. coli* pathotypes involved in acute diarrheas are well characterized. On the other hand, the virulence markers of *E. coli* strains isolated from chronic intestinal diseases are still unknown. The aim of the study was to compare virulence genes profiles and phylogenetic origin of *E. coli* strains isolated from chronic and acute intestinal disorders using CGH microarray.

Experimental

Materials and Methods

***E. coli* strains.** A collection of 78 *E. coli* archive strains was examined. The strains were isolated from three different groups of patients. Two of these groups comprised *E. coli* strains isolated from patients with chronic intestinal diseases, *e.g.* CD and IBS that were compared with *E. coli* strains included in the third group of *E. coli* strains isolated from cases of acute diarrhea. The IBS group included 26 *E. coli* isolates collected in our laboratory from stool specimens of 26 adult patients (age mean age 37 years) with diarrheal form of IBS recognized on the basis of Rome II criteria in the Clinic of Gastroenterology and Hepatology of Medical University, Wrocław, Poland. The Crohn's disease (CD) group comprised 28 *E. coli* isolated from terminal ileum biopsy specimens of 28 children (mean age 1.7 year) with Crohn's disease diagnosed on the basis of clinical presentation, endoscopy examination and histopathology in the Department and Clinic of Pediatrics and Gastroenterology of Medical University, Wrocław, Poland. The third group of 24 *E. coli* strains was collected in our laboratory from 24 stool specimens of children (mean age 2.5 year) with acute diarrhea (AD) hospitalized in the Department and Clinic of Pediatrics and Gastroenterology of Medical University, Wrocław, Poland. None of these *E. coli* strains examined belonged to enteropathogenic *E. coli* (EPEC) as assessed by standard agglutination assay with specific antisera (Biomed, Poland).

Phylogenetic group determination. Affiliation to four main phylogenetic groups A, B1, B2 and D was determined based on an anonymous DNA fragment designated TSPE4.C2, *chuA* gene required for heme transport in EHEC O157:H7 *E. coli*, and *yjaA* gene of unknown function present in nonpathogenic *E. coli* K12 strain by DNA microarray and confirmed by PCR according to Clermont *et al*. (2000).

DNA extraction. Genomic DNA was extracted according to method described by Zhang *et al*. (2005). Briefly, 3 ml of overnight cultures of *E. coli* strains in Luria broth were pelleted by centrifugation at 2000 xg for 20 min and resuspended in 80 µl sonication buffer (50 mM Tris and 10 mM EDTA, pH 7.5 with 100 ng/µl RNase A). The suspension was transfered into a 0.5 ml thin wall PCR tube and treated with sonication using Labo-Plus, Vibra Cell Sonics sonicator. Four treatments of 1 min each at an amplitude setting of 5 were performed. The disrupted cells were centrifuged at 2500 xg for 20 min and incubated at 98°C in a DNA-Engine PT200 thermal cycler (MJ Research Waltham, MA, USA) for 5 min to precipitate out the proteins in the supernatant by heat denaturation. After next centrifugation at 2500 xg for 20 min approximately 40 µl clean and fragmented DNA (purity determined spectrophotometrically – $OD_{260/280}$ ranged from 1.8 to 2.0) was transferred to a new tube. The required size of DNA fragments ranging from 100 bp to 600 bp was controlled by capillary gel electrophoresis.

***E. coli* DNA labeling and hybridization**. A 2 µg of fragmented genomic DNA from each *E. coli* strain was labeled with a fluorescent dye Cy3 (green) whereas DNA from reference strain EDL933 with Cy5 (red) using commercial chemical labeling kit (Universal Linkage System (ULS) array CGH Labeling kit, Kreatech Biotechnology, Amsterdam, Nederland) according to the manufacturer's protocol. The two labeled DNA samples were then mixed and hybridized to the microarray. Hybridization was performed for 16 h at 42°C in a slide hybridization chamber (Hybridization System Maui, France). After washing three times in 0.1 xg SSC buffer (15 mM NaCl, 1.5 mM trisodium citrate, pH 7.0) with 0.1% sodium dodecyl sulphate (SDS) and one washing in 0.1 × SSC buffer without SDS at 37°C for 5 min under agitation, the array was scanned using InnoScan 700 (Microarray Scanner Innopsys, France). The fluorescence emission of Cy5 and Cy3 dyes were measured at 635 nm and 532 nm, respectively. The relative intensity of each the dyes for each of the microarray spots was measured and the relative abundance of each DNA was calculated.

DNA microarray. A DNA microarray developed by Bruant *et al*. (2006) was used to determine the presence of virulence genes among *E. coli* strains. The version of the microarray used in the study was arranged of 234 70-mer oligonucleotide probes specific for 159 virulence genes characteristic for all known *E. coli* pathotypes, 4 positive (*lacY-Ec*, *lacZ*, *tnaA*, and *uidA*) and

5 negative controls (*lacY-Cf*, *Sf0315*, *Sf3004*, *At3g51820*, and green fluorescent protein *gfpmut 3.1*). Probes specific for targeted genes are presented in Table S1 (supplemental material available from the authors). Four independent arrays were printed on the same slide and each nucleotide in the array was printed fourfold. The positive and the negative controls as well as seven printing buffer spots were added in each array. The reference EDL933 strain was used to validate the specificity of the virulence oligonucleotides. Virulence genes detected in EDL933 reference strains are presented in Table I. Microarrays were spotted in the Institute of Bioorganic Chemistry, Polish Academy of Science, using Nanoprint LM60 printing system (Telechem) and Corning Epoxide Slides (cat. No. 40044). 70-mer long DNA probes synthesized by Syngen Biotech (Wrocław, Poland) were diluted in Schott Nexterion Spot buffer to the final concentration 20 μM. Each probe was spotted in four replicates and each slide contained four identical microarrays that were hybridized using compatible MAUI chamber. All hybridization experiments were repeated between two and five times per genome.

Table I
Virulence genes and phylogroups detected in reference *E. coli* strains used in the study

E. coli strain	Pathotype	Virulence genes
Phylogroup		
EDL933	EHEC (O157:H7)	*csgE*, *fimA*, *fimH*, *lpfA*$_{O157}$, *ehx*, *hlyE*
D		*eae*, *eae-gamma*, *paa*, *espA1 and 2*, *espB1*, *tir2*, *stx1A and B*, *stx2A and B*, *astA*, *nleA*, *ccdB*, *efa1*, *espP*, *etpD*, *fliC*, *katP*, *fliC(H7)*, *wzy(O157:H7)*, *rfbE*, *fepC*

Statistical analysis. Genes with differential quantity of DNA were identified using the nonparametric Kruskal-Wallis test. The FDR-adjusted p-value for the selected genes was in the range 0.056 to 0.18.

Results and Discussion

Phylogroups distribution. Most commensal *E. coli* strains belong to A or B1 phylogroups, whereas many pathogenic *E. coli* strains *e.g.* EPEC, ETEC as well as enteroinvasive *E. coli* strains (EIEC) are distributed across all four phylogroups, but are mainly associated with A, B1 and D phylogroups (Ishii *et al.*, 2007; Sahl *et al.*, 2011; Turner *et al.*, 2006). On the other hand, *E. coli* isolates causing extraintestinal infections (ExPEC) not only contain more virulence factors than strains from A and B1 phylogroups, but also derive predominantly from B2 and to a lesser extent D phylogroups (Rolland *et al.*, 1998; Duriez *et al.*, 2001). In our study, only a minority of *E. coli* strains belonged to A and B1 phylogroups (6.4% and 5.1%, respectively). Most *E. coli* isolates belonged to B2 phylogroup (56.4%) independently of the strain origin (Table II). Phylogenetic D group was mainly associated with *E. coli* from AD and IBS (41.7% and 38.5%, respectively) and only a minority (17.8%) of *E. coli* from CD. In general, most *E. coli* strains from CD belonged to B2 group, whereas *E. coli* from IBS and AD derived from both, B2 and D phylogroups. In several studies *E. coli* strains belonging to B2 phylogroup have been associated with IBD (Kotlowski *et al.*, 2007; Petersen *et al.*, 2009), what is in concordance with the results of our study. In contrast to the literature data, the B2 group dominated among all tested *E. coli* examined, indicating that the group is common among *E. coli* associated with acute and chronic intestinal diseases or is widely distributed among clones colonizing intestinal tract of the population living on the area of Lower Silesia in Poland.

Table II
Phylogenetic groups and subgroups distribution among *E. coli* strains examined

	№ of *E. coli* isolates (%)			
	CD n = 28	IBS n = 26	AD n = 24	Total n = 78
Phylogroups A	1 (3.6)	2 (7.7)	2 (8.3)	5 (6.4)
B1	3 (10.7)	0	1 (4.2)	4 (5.1)
B2	19 (67.8)	14 (53.8)	11 (45.8)	44 (56.4)
D	5 (17.8)	10 (38.5)	10 (41.7)	25 (32)

CD, Crohn's disease; IBS, irritable bowel syndrome; AD, acute diarrhea

The comparative analysis of virulence genes distribution among *E. coli* groups examined. We analyzed distribution of virulence genes in every studied group of *E. coli* strains *e.g.* IBS, CD and AD and then groups of *E. coli* strains were compared to each other *e.g.* IBS vs. AD, CD vs. AD. Statistical analysis associated IBS group *E. coli* with 16 virulence genes that can be divided into at least four functional categories: 1. genes encoding adhesins: i) specific to pathogenic *E. coli* strains such as long polar fimbriae (LPF) of EHEC (*lpfA*$_{EHEC}$ gene) and EPEC/EHEC O113 serotype (*lpfA*$_{O113}$ gene), CS18 fimbriae of ETEC (*fotA* gene), AfaE-I fimbriae (*afaE-I* gene) of Dr family adhesins of diffusely adhering *E. coli* (DAEC), and Pic serine protease (*pic* gene) produced by EAEC and *Shigella flexneri* 2a, that degrades mucin and play an important role in mucosal colonization, ii) common among commensal and uropathogenic *E. coli* (UPEC) strains such as P-pili (*papA* and *papC* genes); 2. genes associated with biofilm formation such

as *hra1* gene encoding an accessory adhesin that confers bacterial autoaggregation, enhanced biofilm formation and aggregative adherence to epithelial cells in vitro, *agn43* gene performing function similar to the *hra1* gene, and *shf* gene conferring firm biofilm formation in EAEC O42 reference strain; 3. genes encoding iron acquisition systems such as salmochelin (*iroN* gene) acquired from *Salmonella* spp., yersiniobactin (*irp2* and *fyuA* genes) from *Yersinia* spp., and iut_{EPEC} gene encoding aerobactin associated with ExPEC; 4. genes encoding host immunity evading factors *e.g. iss* gene conferring increased serum survival and gene encoding K1 capsule. Furthermore, 6 (23.1%) *E. coli* strains from IBS showed the presence of *malX* gene, the marker of pathogenicity island (PAI) of UPEC. In addition, more than one third of *E. coli*-IBS carried the *cva* gene encoding microcin V. *E. coli* from CD were associated with only a few genes *e.g.* encoding iron acquisition systems (yersinibactin and aerobactin) and microcin V. *E. coli* from AD were associated with i) genes encoding adhesins ($lpfA_{O113}$, *papA*, *papC*) and iron acqusition system (aerobactin), ii) genes associated with biofilm formation (*agn43* gene), iii) and *iss* gene. Moreover, 4 (16.7%) of these strains showed the presence of genes encoding colicin ColY and enterotoxin EspC (Table III).

Comparative analysis of virulence genes distribution among *E. coli* from chronic intestinal diseases *e.g.* IBS and CD showed only three genes in common *e.g.* genes encoding yersiniobactin, aerobactin and microcin V. *E. coli* from IBS and AD shared eight genes. Three of these genes were associated with adhesins and biofilm formation (LPF, P-pili, and Agn43). Other three genes included those encoding aerobactin and increased serum survival, and microcin V. In addition both, *E. coli* from IBS and AD groups were associated with *pic* and *malX* genes. Comparison of *E. coli* strains from AD and CD demonstrated only two genes in common *e.g.* aerobactin and microcin V encoding genes (Figure 1).

Analysis of the association of phylogroups with virulence genes distribution. In the study all *E. coli*

Table III
Genes significantly associated with *E. coli* strains isolated from irritable bowel syndrome (IBS), Crohn's disease (CD) and acute diarrhea (AD)

Gene	Gene description	№ of *E. coli* isolates (%)		
		CD (n = 28)	IBS (n = 26)	AD (n = 24)
Genes encoding: adhesins/biofilm formation				
$lpfA_{O113}$	Long polar fimbriae[a]	*NS*	12 (46.1)	8 (33.3)
$lpfA_{EHEC}$	Long polar fimbriae[b]	*NS*	10 (38.5)	*NS*
PapA, papC	P pili	*NS*	9 (34.6)	4 (16.7)
fotA	CS18 fimbriae of ETEC	*NS*	10 (38.5)	*NS*
hra1	Heat-resistant hemagglutinin 1	*NS*	11 (42.3)	*NS*
afaE-I	AfaE-I adhesin of Dr family adhesins	*NS*	5 (19.2)	*NS*
agn43	Protein of autotransporter family[c]	*NS*	13 (50)	10 (41.7)
Iron acquisition systems				
iroN	Salmochelin receptor-encoding gene	*NS*	12 (46.1)	*NS*
irp2	Yersiniobactin	9 (32.1)	10 (38.5)	*NS*
fyuA	Yersiniobactin receptor-encoding gene	*NS*	7 (26.9)	*NS*
iut_{EPEC}	Aerobactin receptor	9 (32.1)	11 (42.3)	8 (33.3)
Host immunity evading factors				
iss	Increased serum survival	*NS*	10 (38.5)	8 (33.3)
neuA	K1 capsule	*NS*	5 (19.2)	*NS*
Microcins and colicins				
cva	Microcin/colicin V	6 (2)	9 (34.6)	7 (29.2)
colY	Pore-former colicin ColY	*NS*	*NS*	4 (16.7)
Various function				
pic	Serine protease of EAEC	*NS*	6 (23.1)	6 (25)
espC	Enterotoxin EspC	*NS*	*NS*	4 (16.7)
malX	UPEC PAI[d] marker	*NS*	6 (23.1)	7 (29.2)

[a] gene encoding the major fimbrial subunit of long polar fimbriae LpfA from EHEC O113:H21 strain; [b] gene encoding the major fimbrial subunit of long polar fimbriae LpfA from EHEC O157:H7 strains; [c] gene encoding antigen 43 precursor that confers autoaggregation and biofilm formation; [d] PAI, pathogenicity island; *NS* not associated according to statistical analysis

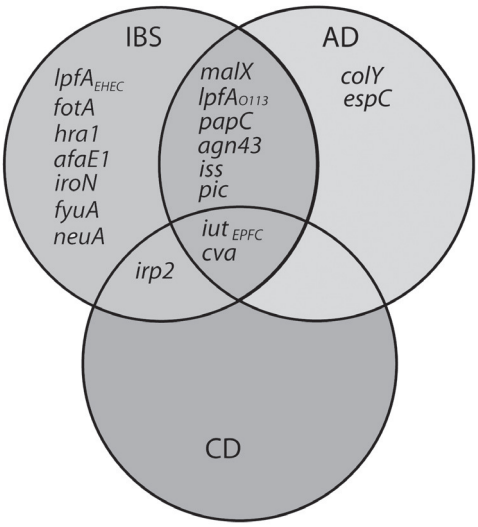

Fig. 1. Virulence genes associated with and shared among *E. coli* from irritable bowel syndrome (IBS), Crohn's disease (CD) and acute diarrhea (AD)

strains from CD, IBS and AD possessing virulence genes, belonged to B2 and D phylogroups. The only exceptions there were two *E. coli* strains. One strain from child with CD that was classified to the B1 phylogroup possessed *irp2* and iut_{EPEC} genes and in one *E. coli* strain of A phylogroup isolated from child with AD $lpfA_{O113}$ gene was present. These results indicated that, with few exceptions, all *E. coli* strains examined corresponded to ExPEC strains that are believed to exert their pathogenic potential only when spread beyond the bowel.

The DNA microarray analysis of virulence genes associated with *E. coli* strains isolated from IBS demonstrated a mosaic of virulence genes specific to pathogenic strains *i.e.* EPEC/EHEC and ETEC, as well as *Shigella* species and EAEC, but also genes common among commensal *E. coli* strains (Doughty *et al*., 2002). The association of *E. coli* from IBS with several different adherence genes may explain their predilection for causing chronic infections and could have an impact on pathophysiology of IBS syndrome. Numerous adhesion-associated genetic determinants may confer augmented adherence and enhanced capability of IBS-associated *E. coli* strains to persistently colonize intestinal mucosa. Furthermore, many of these adhesins could stimulate various receptors in the gut wall, thus inducing an inflammation and visceral hypersensitivity, a frequent finding in IBS patients, as well as an alteration in gut microbiota (Krotkova *et al*., 2006; Ohman and Simren, 2007).

E. coli from AD were mainly associated with determinants characteristic to ExPEC, especially uropathogenic *E. coli* strains (UPEC) that typically express an array of adhesins and iron acquisition systems, but lack of virulence determinants characteristic to diarrheagenic *E. coli* strains (Östblom *et al*., 2011). Moreover, *E. coli* from AD were also associated with genes enhancing advantage in a hostile intestinal milieu *e.g. malX*, *iss*, *colY* and *cva* gene encoding microcin V localized on ColV plasmid in many *E. coli* strains. According to Gilson *et al.* (1987) ColV plasmids are associated with *E. coli* invasiveness and pathogenicity. Moreover, these mobile genetic elements harbor gene encoding aerobactin and increased serum survival (*iss*), as did *E. coli* strains from AD. The virulence determinants associated with diarrheagenic potential of *E. coli* from AD were enterotoxin EspC, a member of autotransporter family of proteins, found in a subset of EPEC (Millies *et al.*, 2001; Vidal and Navarro-Garcia, 2006) and LPF_{O113}. According to Afset *et al.* (2006) the $lpfA_{O113}$ gene was significantly associated with diarrhea among Norwegian children less than 5 years old and has been found exclusively in diarrhea cases. Taking into consideration virulence determinants and B2 and D phylogroups associated with *E. coli* derived from AD we can assume that these isolates correspond to commensal *E. coli* strains that could acquired some virulence genes (*i.e. espC*, *lpfA*) from their diarrheagenic counterparts (Nowrouzian *et al*., 2001).

The peak of incidence of IBD occurs in patients between the ages of 15 and 25 years, but there is an increase in incidence in younger children (Benchimol *et al*., 2009). The association of *E. coli* strains of AIEC pathotype with CD in adult patients is well known, but there is no reports on the characteristics of *E. coli* from childhood-onset CD. The result the study showed that only few virulence determinants were associated with *E. coli* from children with CD (*e.g.* genes encoding aerobactin and microcin V, and *irp2* gene encoding yersiniobactin). In this regard *E. coli* from childhood-onset CD seems to be similar to AIEC.

According to study of Martinez-Medina *et al.* (2009) AIEC from adult patients with CD have demonstrated association with only aerobactin encoding gene, but not with other genes *e.g.* encoding adhesins such as P pili or type 1 fimbriae, what is in concordance with the results of the study. Martinez-Medina *et al.* (2009) have also showed that most of AIEC strains possessed virulence traits characteristic to ExPEC. On the other hand, AIEC differs from ExPEC by their invasion capabilities. Most characteristic feature of AIEC distinguishing these isolates from other *E. coli* strains is their ability to survive and replicate within macrophages. Although in the study *E. coli* strains from children with CD showed some similarity with AIEC, further analysis of the invasive potential of these isolates is necessary.

In summary, the study found that *E. coli* from IBS, CD and AD, apart from some comparability between *E. coli* from IBS and AD, differ from each other indicating that the participation of *E. coli* in chronic intestinal diseases is mostly related to virulence potential of these strains.

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