

# Genes of intestinal *Escherichia coli* and their relation to the inflammatory activity in patients with ulcerative colitis and Crohn's disease

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**Abstract** *Escherichia coli* gene *fimA* was the most frequent gene that occurred in the intestine of all investigated groups. All subjects with *fimA* gene had significantly higher values of tumor necrosis factor alpha (TNF- $\alpha$ ) and CRP than those with other *E. coli* genes. There was also a tendency to increased serum interleukin (IL)-6 levels in patients carrying the *fimA* gene; however, no relation was observed to serum IL-8 and IL-10. Patients with Crohn's disease had significantly higher IL-6 than those with ulcerative colitis (UC) and controls. The highest levels of TNF- $\alpha$  were detected in the UC group. There were no significant differences in serum IL-8 and IL-10 between all three groups. The presence of *E. coli* gene *fimA* in the large bowel of patients with IBD is related to the immunological activity of the disease which may be important from the aspect of therapeutic strategy.

Inflammatory bowel diseases (IBD) comprising two forms, Crohn's disease (CD) and ulcerative colitis (UC), are chronically remitting disorders characterized by inflamma-

tion of the gastrointestinal tract. Currently, the etiopathogenesis of these disorders is not completely understood, although chronic relapsing inflammation is considered to be a result from a dysregulated, aberrant immune response to intestinal flora in a context of genetic predisposition (Sanchez-Muñoz et al. 2008; Chamberli and Naser 2006).

Many studies documented an increase in proinflammatory cytokine expression from T lymphocytes, neutrophils, macrophages, and epithelial cells in patients with IBD; however, the results about their clinical significance and relation to clinical activity of CD and UC are controversial (Fantini et al. 2007; Scaldaferrri and Fiocchi 2007). Tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin (IL)-6 seem to be most closely associated with clinical and laboratory activity of the disease (Irving et al. 2005).

There is strong evidence that environmental factors implicated in the pathogenesis of IBD are bacteria and their components. Both CD and UC primarily affect intestinal areas with high bacterial counts. Further evidence supporting a role of enteric flora in the pathogenesis of IBD is that treatment with antibiotics and probiotics was found to be beneficial and could be used to maintain the remission of the disease (Dignass et al. 2004; Macfarlane et al. 2009).

The most prevalent bacteria in patients with IBD are *Enterobacteriaceae*, especially *Escherichia coli* (Guarner and Malagelada 2003). Recently, novel genetic classes of *E. coli* have been considered to be associated with CD and UC. Many genes of *E. coli* were described to be present in the small and large bowel in healthy humans and patients with IBD; however, only some of them are supposed to play a role in the pathogenesis of inflammation. Currently, there are no data about the clinical significance of these genes in the pathogenesis of CD and UC as well as their prevalence in the large bowel of patients with IBD (Dogana and Simpson 2008).

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The aim of this study was to determine the occurrence of pathogenic *E. coli* genes in the large bowel of patients with CD and UC in comparison with control subjects and to assess their relation to clinical as well as laboratory activity of the disease.

## Subjects and methods

### Subjects

Group of patients consisted of 68 subjects with the diagnosis of inflammatory bowel disease, i.e., CD or UC (36 men, 32 women), with mean age of 34.5 years (range, 19–72). Thirty-two patients were diagnosed to have ulcerative colitis (14 men, 18 women; mean age, 37.8 years; range, 21–72) and 36 had Crohn's disease (22 men, 14 women; mean age, 31.1 years; range, 19–63).

The diagnosis of IBD was based on clinical symptoms, laboratory evaluations, and endoscopic finding confirmed by histological evaluation. None of the patients were in complete endoscopic remission. Sixteen patients with CD and 12 patients with UC were treated by antibiotics (quinolones or metronidazole). The clinical activity of CD was based on the Crohn's Disease Activity Index (Best et al. 1976), and the activity of the ulcerative colitis was based on the Ulcerative Colitis Disease Activity Index (Schroeder et al. 1987).

Twenty-eight% ( $n=10$ ) of the patients with Crohn's disease had mild activity, and 72% had moderate activity. None of them had severe activity. Among the patients with ulcerative colitis, 25% ( $n=8$ ) had mild disease activity, 43% ( $n=14$ ) had moderate activity, and 32% ( $n=10$ ) of patients had severe activity, respectively. Control group consisted of 37 subjects (15 male, 22 female; mean age, 52.4 years; range, 21–78) with negative colonoscopy. Histological examinations of the colon specimens were negative in all these subjects.

### Methods

All patients underwent colonoscopic examination after preparation using 3–4 L of the mineral water (Šaratica, Slovakia) with high magnesium content. During the colonoscopy (performed with the colonoscopic device Olympus Q 145 L), specimens of the colon mucosa were collected for histological and microbiological evaluation.

### Microbiological evaluation

Two mucosal specimens were obtained during colonoscopy from the surrounding of maximal ulcerative mucosal lesions. Samples were immediately inserted into a 0.9%

NaCl solution and processed within 1 h after sampling. Bacteria were isolated after washing, and the biopsy specimens were homogenized and inoculated into MacConkey agar and blood agar and cultivated for 24 h at 37°C. Identification of strains was done based on biochemical characteristics using Enterotest 16, Entero-Rapid 24 (Lachema, Czech Republic), and some strains were confirmed by using identification kits BBL Crystal (Becton Dickinson, USA). The virulence of *E. coli* strains was tested by the phenotype expressions and genes detection. Examined genes coded factors of virulence in bacterial genome of tested strains. Polymerase chain reaction (PCR) was used to detect *E. coli* genes. One gene was detected by simplex PCR, while occurrence of other genes was detected by multiplex PCR.

*Preparation of template DNA for PCR* Bacteria were inoculated into LB broth (5 mL), incubated for 24 h at 37°C, and then centrifuged (1,000×g, 10 min). Sediment was resuspended in 200 µL of sterile distilled water, incubated for 10 min in water bath at 100°C, and cooled to laboratory temperature. After an additional 10 min, fragments of lysed cells were removed by centrifugation from the lysate. Supernatant was used as a template DNA for PCR.

Occurrence of the following genes has been estimated: genes coding for toxin production ( $\alpha$ -hemolysin ( $\alpha$ -hly), cytotoxic necrotizing factor (*cnf1*)), genes coded for production of invasins (invasin (*ipaH*), invasin (*ial*)), genes coded for production of aerobactin (aerobactin synthetase (*iucC*), aerobactin (*aer*)) and genes coded for surface properties (fimbrias I-type (*fimA*), afimbrial adhesin (*afa*), S-fimbrias (*sfa*), P-fimbrias (*pap*), fimbrial adhesin (*bfpA*), intimin (*eaeA*)). The presence of genes coding for virulence factors of enterovirulent *E. coli* is an important marker for the above strain identification.)

*PCR detection of genes encoding virulence factors* For demonstration of virulence genes' (*bfpA*,  $\alpha$ -hly, *cnf1*, *sfa*, *pap*, *ial*, *ipaH*, *eaeA*, *fimA*, *iucC*, *aer*) characteristic for enterovirulent *E. coli*, the protocol according to Kuhnert et al. (1997), Yamamoto et al. (1995), and Liptakova et al. (2001) was used; appropriate bacteria encoding each of the virulence genes were included as PCR positive controls. *E. coli* C600 Rif was used as a negative control.

*Detection of PCR products* Specific gene products were detected using electrophoresis. The reaction mixture was analyzed by electrophoresis on 2.0% agarose gels, and the reaction products were visualized by staining with ethidium bromide under UV light.

According to the presence of genes coding for virulence factors, we have included examined *E. coli* into enter-

ovirulent strains. *E. coli* strains without genes of virulence were included into nonpathogenic strains of commensal enteric flora.

*E. coli* strains were not detected in mucosal samples from nine patients. Some other strains, such as *Shigella* sp., *Streptococcus* sp., *Klebsiella* sp., *Morganella* sp., *Serratia* sp., were also found. No bacteria were detected in mucosal samples of 11 patients (Table 1).

**Laboratory evaluations** All patients as well as controls underwent blood sample collection before endoscopy. Laboratory investigation included the measurement of serum cytokines, namely IL-6, IL-8, IL-10 and TNF- $\alpha$  and serum C-reactive protein (CRP). All cytokines were measured by ELISA method (ELISA kits, Thermo Scientific; Pierce Biotechnology, USA). CRP was measured by standard biochemical methods.

**Statistical analysis** Data are presented as means $\pm$ SD. An unpaired *t* test was used for the demonstration of differences between two groups, and  $\chi^2$  test was used to assess differences in the prevalence of evaluated parameters.

## Results

### Immunological evaluations

Patients with CD had significantly higher serum level of IL-6 than those with UC ( $p<0.01$ ) and mildly higher IL-6 than control subjects that reached a borderline significance ( $p=0.05$ ; Table 2). There were no significant differences in serum levels of IL-8 and IL-10 between all three groups. Serum TNF- $\alpha$  was the highest in the UC group ( $p<0.01$ ). We also detected a tendency to higher TNF- $\alpha$  levels in CD group; however, it did not reach the statistical significance in comparison with the control group. Both CD and UC

groups had significantly higher CRP values than controls with the highest levels in UC group.

### Occurrence of *E. coli* genes in colonic mucosa of patients with UC, CD, and control subjects

Gene *fimA* encoding the surface properties was the most frequent gene that occurred in all the investigated groups, including the control subjects (Table 3). UC patients had significantly higher prevalence of *iucC* gene encoding the aerobactin in comparison with CD patients. The invasivity gene *IpaH* was not detected in both CD and UC patients. We did not detect significant differences in the prevalence of other *E. coli* genes among all three groups.

All subjects with the *E. coli* gene *fimA* tended to have increased serum IL-6 levels ( $p=0.05$ ). There were no significant differences in the serum levels of IL-8 and IL-10 in relation to the presence of various *E. coli* genes. All subjects carrying the *E. coli* gene *fimA* had significantly higher values of TNF- $\alpha$  ( $p<0.01$ ). There were no significant differences in serum TNF- $\alpha$  in relation to other *E. coli* genes. Moreover, patients with *fimA* gene had significantly higher CRP than those with other *E. coli* genes ( $p<0.05$ ; Fig. 1).

## Discussion

The relation of inflammatory bowel diseases to the presence of some strains of *E. coli* bacteria has been studied during the last years; however, there are still no data about the relation of the *E. coli* genes to the clinical and laboratory activity of IBD. For that purpose, we evaluated the presence of some of *E. coli* genes in relation to the severity of UC or CD.

Patients with CD and UC had significantly increased CRP serum level, which is in agreement with previous studies that showed that CRP serum levels are related to systemic inflammation. Nevertheless, some studies docu-

**Table 1** Summary of detected bacterial strains in mucosal samples

Bacterial strain	Controls (n=37), %	CD (n=36), %	UC (n=32), %
<i>E. coli</i>	86.5	80.6	75
<i>Shigella</i> sp.	0	2.7	0
<i>Streptococcus</i> sp.	0	2.7	3.1
<i>Pseudomonas</i> sp.	2.7	0	0
<i>Enterococcus</i> sp.	2.7	0	0
<i>Klebsiella</i> sp.	0	0	3.1
<i>Morganella</i> sp.	0	0	3.1
<i>Serratia</i> sp.	0	0	3.1
<i>Proteus</i> sp.	0	0	3.1
Bacteria not detected	8.1	13.9	9.4

**Table 2** Immunological evaluation

Parameter	Controls	CD	UC	Significance		
				Controls vs. CD	Controls vs. UC	CD vs. UC
IL-6	2.98±0.17	4.58±0.80	2.41±0.19	<i>p</i> =0.05	ns	<i>p</i> <0.01
IL-8	12.1±3.21	9.54±1.47	15.6±3.00	ns	ns	ns
IL-10	7.92±2.08	7.32±1.10	7.06±1.40	ns	ns	ns
TNF- $\alpha$	24.5±3.25	47.8±9.09	75.5±19.22	ns	<i>p</i> <0.01	ns
CRP	4.40±0.84	25.5±4.60	41.3±7.27	<i>p</i> <0.01	<i>p</i> <0.01	<i>p</i> <0.05

For numbers of CD, UC samples, and controls, see Table 1

ns not significant

mented that CRP level has less significance in the assessment of clinical activity of IBD (Vermèire et al. 2006; Doua et al. 2006; Florin et al. 2006; Denis et al. 2007).

Bowel inflammation was shown to be characterized by mucosal infiltration with neutrophils which is associated with the release of proinflammatory cytokines (Sansone et al. 1999). We also found significantly higher plasma levels of IL-6 in patients with CD in comparison with those with UC and control subjects. An increase in IL-6 is considered to reflect the clinical activity of IBD because none of the patients were in clinical remission.

This is in agreement with previous studies that found an increased serum IL-6 level in patients with CD as compared to UC and controls (Mudter and Neurath 2007). On the other hand, some other authors found that lamina propria mononuclear cells produce markedly higher amounts of IL-6 in UC patients than in those with CD (Reinecker et al. 1993). Because IL-6 was identified to promote tumor growth, this finding may partially explain an increased occurrence of colonic cancer in UC. We also found higher levels of IL-6 in UC patients as compared to

control subjects which is also in agreement with previous observations.

There were no significant differences in serum levels of IL-8 and IL-10 in all three groups. Our results are in agreement with Nielsen et al. (1997), who reported that IL-10 concentrations did not differ among UC, CD, and healthy control subjects. Controversially, Mitsuyama et al. (2006) found that the concentrations of serum IL-10 increased in patients with UC but not in those with active CD when compared to normal subjects. This controversial finding is difficult to explain. Values of IL-10 might be influenced by the treatment because all of the patients were treated pharmacologically and were in various stages of clinical activity. Moreover, the serum levels of IL-10 may not strictly reflect its mucosal mononuclear production, which has been demonstrated in *in vitro* studies to be increased (Sanchez-Muñoz et al. 2008; Scaldaferrri and Fiocchi 2007).

We also detected a significantly higher serum TNF- $\alpha$  concentration in patients with UC and its mild increase in patients with CD. The results are in agreement with Scaldaferrri and Fiocchi (2007) and Mitsuyama et al.

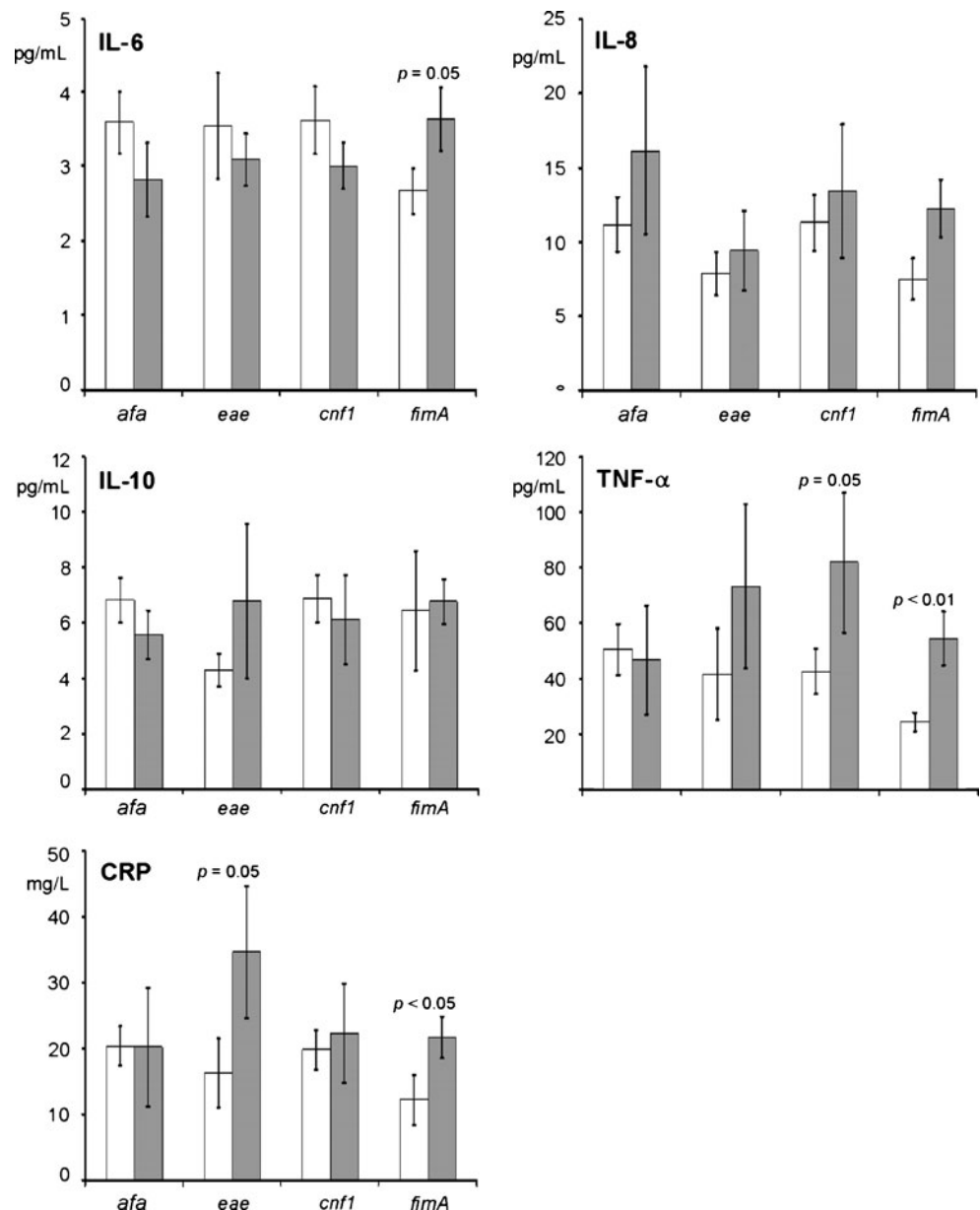
**Table 3** Occurrence of *E. coli* genes in colonic mucosa of patients with UC, CD, and control subjects

<i>E. coli</i> gene	Control	CD	UC	Significance		
				Controls vs. CD	Controls vs. UC	CD vs. UC
<i>ipaH</i>	1	0	0	–	–	–
<i>iucC</i>	17	10	18	ns	ns	<i>p</i> <0.01
<i>fimA</i>	28	23	22	ns	ns	ns
$\alpha$ - <i>hly</i>	0	3	5	ns	ns	ns
<i>afa</i>	2	6	1	ns	ns	ns
<i>aer</i>	16	16	14	ns	ns	ns
<i>cnfI</i>	4	8	10	ns	ns	ns
<i>sfa</i>	7	9	4	ns	ns	ns
<i>pap</i>	10	13	10	ns	ns	ns
<i>bfpA</i>	0	0	0	–	–	–
<i>eae</i>	0	1	5	–	–	ns

For numbers of CD, UC samples, and controls, see Table 1

ns not significant

**Fig. 1** Plasma levels of IL-6, IL-8, IL-10, TNF- $\alpha$ , and CRP in all investigated groups in relation to the presence of most frequently occurring *E. coli* genes (*afa*, *eae*, *cnf1*, *fimA*); white columns negative, gray columns positive



(2006) who demonstrated higher levels of TNF- $\alpha$  in serum as well as in mucosa in patients with IBD, which reflects the clinical activity of the disease.

In our study, the most prevalent gene was the *fimA* encoding the superficial properties of bacteria. It is supposed to participate in initiation and persistence of chronic inflammation by uncontrolled production of proinflammatory cytokines. Our findings are supported by results of Boudeau et al. (2001) who evaluated the relationship between fimbriae I, encoded by the gene *fimA*, and secretion of proinflammatory cytokines; they confirmed that the adherence of fimbriae I initiates the production of proinflammatory cytokines. In healthy humans, the significance of this mechanism is unknown. Genes *eae*, encoding aerobactin, and *iuC*, encoding

areobactinsynthase, were found to occur less frequently. We also demonstrated significantly higher levels of TNF- $\alpha$ , IL-6, and CRP in patients with *E. coli* carrying the gene *fimA*.

*E. coli* is the predominant facultative aerobic inhabitant of human intestine and part of normal enteric flora. There is growing evidence that *E. coli* is more prevalent in patients with IBD in comparison to control subjects (e.g., Martin et al. 2004). For example, some studies demonstrated that CD is associated with invasive *E. coli* strains since 29% of CD patients were infected with intramucosal *E. coli* in comparison with 9% of control subjects (Mitsuyama et al. 2006). Sasaki et al. (2007) showed that *E. coli* isolated from IBD patients induced significantly higher TNF- $\alpha$  expression in macrophage cell cultures in vitro. This is also supported by our study; we showed that some *E. coli* genes,

*fimA* in particular, were associated with the laboratory inflammatory activity, i.e., higher levels of TNF- $\alpha$ , IL-6, as well as CRP.

To the best of our knowledge, this is the first clinical study evaluating the relationship between *fimA* gene of *E. coli* and laboratory markers of inflammation in patients with Crohn's disease and ulcerative colitis, demonstrating that the presence of *E. coli* gene *fimA* is related to the laboratory activity of the disease.

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