The prevalence of mucosa-associated diffusely adherent *Escherichia coli* in children with inflammatory bowel disease

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;  
D – writing the article; E – critical revision of the article; F – final approval of the article

Abstract

**Background.** The relationship of diffusely adherent *Escherichia coli* (DAEC) with pediatric inflammatory bowel disease (IBD) has not been previously studied. Diffusely adherent *E. coli* are a common cause of long-lasting childhood diarrhea and we postulated that they may induce inflammation of the intestinal mucosa, contributing to the development of IBD in susceptible children.

**Objectives.** The aim of the study was to investigate the relationship between DAEC and pediatric IBD, including Crohn’s disease (CD) and ulcerative colitis (UC). Diffusely adherent *E. coli* isolates were also assessed regarding their pathogenicity.

**Material and methods.** Diffusely adherent *E. coli* were screened among 130 *E. coli* strains isolated from intestinal biopsy specimens from 26 children with IBD using polymerase chain reaction (PCR) with primers specific to the pathotype and adherence assays to HEp-2 cells. Diffusely adherent *E. coli* were further analyzed for their ability to adhere to and invade polarized Caco-2 cells. The immunomodulatory effect of DAEC on the secretion of tumor necrosis factor α (TNF-α) by human monocyte-derived macrophages (MDM) was assessed using an immunoenzymatic assay.

**Results.** Diffusely adherent *E. coli* were recovered from 18 (69.2%) of the 26 intestinal biopsy specimens from both CD and UC patients. Most DAEC isolates carried AfaE3 adhesin, adhered to and were internalized by Caco-2 cells, and induced secretion of elevated levels of TNF-α.

**Conclusions.** The study demonstrated the internalization of DAEC by intestinal epithelial cells and their ability to induce secretion of increased level of TNF-α in a Caco-2/macrophage compartmentalized culture. This indicated that the pathovar should be considered a pathobiont inducing inflammation of the intestinal mucosa in pediatric patients with IBD.

**Key words:** childhood inflammatory bowel disease, diffusely adherent *E. coli*, AfaE3 adhesin

Cite as


DOI

10.17219/acem/94149

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Inflammatory bowel disease (IBD) is a chronic inflammation of the digestive system. The principal types of IBD include Crohn's disease (CD) and ulcerative colitis (UC). The currently prevailing view is that genetic background combined with intestinal dysbiosis and a related aberrant immune response contribute to IBD pathogenesis.1–3

*Escherichia coli* (E. coli) has been implicated in the etiopathogenesis of the ideal form of CD in adults, and many independent studies have demonstrated an increased number of *E. coli* bacteria in the intestinal mucus layer of patients with CD in comparison with healthy individuals.4–6 Investigating the composition of mucosa-associated microflora in colonoscopic biopsy specimens from children with newly diagnosed CD, Conte et al. found *E. coli* bacteria in 74% of the samples examined.6

The relationship between diffusely adherent *E. coli* (DAEC) and IBD has not been studied extensively, although Darfeuille-Michaud et al. reported the isolation of *E. coli* strains hybridizing with the *daaC* probe specific to DAEC from adult patients with CD.1 Prorok-Hamon et al. found that mucosa-associated *afaC*-positive *E. coli* strains were more common in CD and colorectal cancer than in UC and the controls.7 Diffusely adherent *E. coli* causes urinary tract infections in humans and diarrhea in children aged from 18 months to 5 years, but is also isolated from healthy children and adults.8,9 It has been suggested that carrying DAEC may predispose to chronic inflammation of the intestinal mucosa and the development of CD.9

Diffusely adherent *E. coli* are divided into 2 main classes: *Afa/Dr*-positive (*Afa/Dr)* strains that express *afa*, *dra* and *daa* operons encoding *Afa/Dr* adhesins, and *Afa/Dr*-negative (*Afa/Dr)* strains. The subclass of *Afa/Dr*–diarrhoea-associated DAEC strains expressing AIDA-I adhesin represent a subgroup of atypical enteropathogenic *E. coli* (EPEC).8 The *Afa/Dr* group is further divided into 2 subgroups: *Afa/Dr*DAEC possessing *AfaE3*, *Dr* and *F1845* adhesins binding to hDAF, CEACAM-1, CEA, and CEACAM6 cell receptors; and *Afa/Dr* DAEC presenting *AfaE1* and *Dr*-I adhesins that bind to hDAF but not to CEACAMs.9 The adherence of *Afa/Dr* strains to differentiated, polarized epithelial cells through membrane-bound receptors such as hDAF, CEACAMs and CEA induces structural and functional lesions in the epithelial barrier that generate an increase in paracellular permeability, transepithelial migration of polymorphonuclear leukocytes and the production of pro-inflammatory cytokines, i.e., interleukin (IL)-8, tumor necrosis factor α (TNF-α) and IL-1β.10–12

In the present study, we searched for DAEC among 130 *E. coli* isolates obtained from intestinal biopsy specimens from 26 children with IBD. Diffusely adherent *E. coli* isolates were assessed with regard to their pathogenicity, i.e., their ability to adhere to and invade polarized epithelial intestinal cells, the type of adhesins involved in their adherence to intestinal mucosa and their ability to induce TNF-α from monocyte-derived macrophages (MDM) co-cultured with Caco-2 cells infected with DAEC.

### Material and methods

#### Biopsy specimens

Biopsy specimens were obtained from 26 consecutive children and adolescents (mean age 11.1 years, ranging from 7 to 18 years) with IBD (17 patients with CD and 9 patients with UC) diagnosed at the Department and Clinic of Pediatrics and Gastroenterology of Wroclaw Medical University (Poland). Biopsies from inflamed intestinal mucosa were collected from each patient for routine histopathologic examination and punch biopsies from the terminal ileum or colon were obtained for culture. The Ethics Committee of Wroclaw Medical University approved the study. The specimen for culture was washed in saline and immediately dispersed onto MacConkey (MCA) agar to isolate *E. coli* strains. Five lactose-positive colonies were isolated from every biopsy and defined as *E. coli* using standard biochemical testing. In total, 130 *E. coli* strains were screened for DAEC. Two *E. coli* reference strains, i.e., C1845 DAEC13 and non-pathogenic *E. coli* K-12, were included in the study as positive and negative controls, respectively.

#### Polymerase chain reaction assay

The genes screened with polymerase chain reaction (PCR) assay included the *afaC* gene present in the *afa*–3, *afa*–7 and *afa*–8 operons of the *Afa/Dr* adhesins, the *afaBC* gene of the conserved region of the *afa* operons and the genes encoding *afaE1*, *afaE2* and *afaE3* adhesins.14 In addition, the *daaE* gene encoding the adhesion of the C1854 strain was investigated. *Escherichia coli* isolates were assigned to one of the 4 phylogenetic groups (A, B1, B2, and D) using a multiplex PCR-based method.15

#### Cell cultures

The human epithelial HEp-2 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with heat-inactivated 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution (penicillin 100 U, streptomycin 100 µg/mL, amphotericin B 25 µg/mL). HEp-2 cells were seeded at a density of 4 × 10⁴ cells per well on 24-well plates with round glass coverslips (1 cm in diameter) in each well, and cultured for 24 h at 37°C in a humidified atmosphere with 5% CO₂. Caco-2 cells (ATCC HTB-37™; ATCC, Manassas, USA) were maintained in a minimal essential medium (MEM) with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and antibiotics (penicillin 100 U, streptomycin 100 µg/mL). Caco-2 cells were seeded at a density of 5 × 10⁵ cells per well and cultured for 10 days to differentiate. Twenty-four hours before the assays, the cells were washed 3 times with phosphate-buffered saline (PBS; pH 7.2), and MEM with 2% FBS and D-mannose (1%, w/v) without antibiotics was added.
In the co-culture experiments, Caco-2 cells were seeded at a density of $2 \times 10^5$ cells onto polyethylene terephthalate (PET) insert membranes (0.3 cm$^2$, 0.4 μm pore diameter) and cultured for 10 days post-confluence. The integrity of the Caco-2 cell monolayers was checked by measuring transepithelial electrical resistance (TEER) using a Millicell ERS-2 ohm meter (Merck Millipore, Darmstadt, Germany). Caco-2 cell monolayers with TEER $\geq 500$ Ω cm$^2$ were used for experiments. Human monocytic cell line THP-1 (ATCC TIB-202TM) was routinely cultured in RPMI-1640 medium with 10% FBS. THP-1 cells were differentiated into monocyte-derived macrophages (MDM) with 200 nM phorbol-12-myristate 13 acetate (PMA) in 24-well plates. After 2 days, the PMA-supplemented medium was removed, the cells were washed twice, supplemented with 2% FBS and allowed to rest in fresh PMA-free culture medium for 3 days to acquire the phenotypic characteristics of macrophages.

**Bacterial adhesion and invasion assays**

Qualitative mannose-resistant adherence assays to HEp-2 cells and quantitative mannose-resistant adherence and invasion assays to Caco-2 cells were carried out as described by Cravioto et al.\textsuperscript{16} Overnight *E. coli* cultures at 37°C in Luria broth (LB) were used to infect epithelial cells at a multiplicity of infection (MOI) of 50 bacteria per cell. In the adherence assay to HEp-2 cells, after 3 h of incubation with *E. coli* isolates, infected epithelial cells were washed to remove unbound bacteria, fixed with 70% methanol and stained with Giemsa stain. To assess the pattern of adherence, glass slides were examined under light microscopy (BX50; Olympus Corp., Tokyo, Japan). To quantify the total number of cell-associating bacteria (surface-adherent and internalized) in the adherence assay to Caco-2 cells, after 3 h of incubation, cells infected with *E. coli* strains were washed and lysed with 1% Triton X-100. The collected lysates were serially diluted in PBS and plated on MCA for assessment of bacterial colony-forming units (cfu). The invasion of *E. coli* was performed in the same way as the adherence assay, but after 3 h of incubation and washing away unbound bacteria, a culture medium containing gentamycin (100 μg/mL) was added for an additional 1 h to kill off adherent bacteria. Then the cells were washed and lysed with Triton X-100 as described above. Invasion indices $\geq 0.1$% were considered significant. Each assay was repeated at least 3 times.

**Co-culture of Caco-2 and THP-1 cells**

A co-culture model was used to analyze the immunomodulatory effect of DAEC on the secretion of TNF-α. Caco-2 cells grown on inserts were infected apically with *E. coli* at the MOI = 10 for 3 h. After incubation, the Caco-2 cell monolayers were washed and a culture medium containing 100 μg/mL of gentamycin was added to both apical and basal compartments for 1 h to kill adherent bacteria. After that, a culture medium containing 20 μg/mL of gentamycin to avoid bacterial overgrowth was added to the apical compartments of the inserts with Caco-2 cell monolayers, which were transferred to 24-well plates with MDM cultures. After 16 h of incubation, the culture medium from the basal compartment was harvested for quantification of TNF-α using a Quantikine® ELISA Human TNF-α Immunoassay (R&D Systems Europe, Ltd., Abingdon, UK).

**Statistical analysis**

Pearson’s χ$^2$ test and Student’s t-test p-values ≤0.05 were considered statistically significant, and the Pearson correlation coefficient ($r$) was used to analyze the results.

**Results**

**Adherence pattern**

Qualitative mannose-resistant adherence to HEp-2 cells was the first criterion to evaluate the diffuse adherence (DA) pattern of *E. coli* isolated from the biopsy specimens. Diffusely adherent *E. coli* were recovered from 18 (69.2%) of the 26 biopsies. There was no difference in the distribution of DAEC among CD and UC patients: they were isolated from 12 (70.6%) out of 17 children with CD and 6 (66.7%) out of 9 children with UC (p = 0.8).

**Distribution of Afa/Dr DAEC genes among E. coli isolates**

*Escherichia coli* were screened for DAEC-specific genes, i.e., *afaC* and *afaBC*. The *afaBC*-positive and *afaC*-positive (Afa/Dr) strains were isolated from 14 (53.8%) of the 26 patients with IBD. The *afaBC*-negative but *afaC*-positive DAEC isolates (Afa/Dr) were obtained from 3 (17.6%) patients with CD and 1 patient with UC (11.1%). Screening for AfaE subtypes among the Afa/Dr+ DAEC indicated that they all carried only 2 different subtypes of AfaE adhesins, i.e., AfaE2 and AfaE3, which were associated with 2 (14.3%) and 12 (85.7%) isolates, respectively (p = 0.0002). The AfaE3 adhesin was carried by 8 (88.9%) and 4 (80%) Afa/Dr+ from CD and UC, respectively (p = 0.7; Table 1). None of the examined *E. coli* carried the *daae* gene, except for the reference C1845 strain.

**Phylogroup distribution**

Ten (55.5%) and 8 (44.4%) of the 18 DAEC belonged to the B2 and D phylogroups, respectively. There was no statistically significant difference between CD and UC isolates in the prevalence of the B2 and D phylogroups (p = 0.4).

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**Table 1**

<table>
<thead>
<tr>
<th>Phylogroup</th>
<th>CD (n=12)</th>
<th>UC (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2</td>
<td>10 (83.3%)</td>
<td>2 (33.3%)</td>
</tr>
<tr>
<td>D</td>
<td>2 (16.7%)</td>
<td>4 (66.7%)</td>
</tr>
</tbody>
</table>

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Quantitative adherence and invasion assays to Caco-2 cells

The mean adherence levels of DAEC isolated from the UC and CD patients were $2.8 \pm 0.7 \times 10^6$ cfu/mL and $4.5 \pm 1.3 \times 10^6$ cfu/mL, respectively ($p = 0.08$), whereas the mean invasion indices for DAEC from UC and CD were $1.9 \pm 0.2\%$ and $0.8 \pm 0.1\%$, respectively ($p = 0.054$). The non-pathogenic *E. coli* K12 strain was not internalized by differentiated Caco-2 cells (Table 1; Fig. 1). There was a negative correlation between the adherence and invasion levels among the DAEC from UC ($r = -0.66$), but a positive correlation among those from CD ($r = 0.67$).

**Immunomodulatory effect of *E. coli* on monocyte-derived macrophages co-cultured with Caco-2 cells**

To determine effect on TNF-α secretion by MDM a co-culture of the epithelial cells and macrophage-like cells...
was performed. Infection of Caco-2/MDM compartmentalized culture with Afa/Dr+ and Afa/Dr− DAEC isolates induced the secretion of elevated levels of the cytokine in comparison with the control level (Caco-2 cells co-cultured with MDM without bacteria; Fig. 1). The mean levels of TNF-α secreted by MDM after infecting Caco-2 cells with DAEC from CD and UC were comparable: 247 ± 22 pg/mL and 202 ± 11 pg/mL, respectively (p = 0.26). In general, all but 2 (88.9%) DAEC isolates induced secretion of TNF-α at levels similar to or greater than the reference C1845 strain. The level of TNF-α released after infection of Caco-2 cells with the Escherichia coli K-12 C600 strain was negligible. These results indicated that the infection of Caco-2 cells co-cultured with MDM with Afa/Dr DAEC induced the secretion of TNF-α independently of strain origin (UC vs CD), the level of adherence and invasion, and the affiliation to Afa/Dr+ or Afa/Dr− DAEC. There was no correlation between the adherence or invasion levels and TNF-α secreted by DAEC from UC and CD (r = 0.1 and r = 0.2, respectively).

Histopathology

The histopathologic findings in the biopsy specimens were graded according to the scoring system proposed by Geboes et al., in which grade 0 corresponds to normal mucosa; grade 1 indicates inflammation in the lamina propria with slight invasion of plasma cells and/or lymphocytes and granulocytes; grade 2 denotes moderate or severe inflammation with destructive crypt abscesses, abundant lymphocytes and granulocytes in the lamina propria; and grade 3 means severe inflammation in the lamina propria, partial granular atrophy, crypt dilation, and microgranulomas.17 Acute inflammation included cryptitis, crypt abscesses and neutrophils in the lamina propria and in the epithelium, whereas chronic inflammation was assumed when chronic inflammatory infiltrate consisting of mononuclear cells was present in the lamina propria but there was no crypt destruction or epithelial ulceration (Table 2; Fig. 2). Acute and chronic inflammation were present in 9 (50%) of the 14 Afa/Dr+ and 4 Afa/Dr− DAEC-positive biopsies. There was no correlation between the isolation of Afa/Dr+ DAEC or Afa/Dr− DAEC and acute or chronic inflammatory lesions.

Discussion

Adherent-invasive E. coli (AIEC) is implicated in the etiopathogenesis of ileal CD in adults; however, the role of AIEC in the pathogenesis of childhood CD is not as clear as among adult patients. According to a study published by Conte et al., AIEC-like isolates of an aggregative adherence pattern were identified in children with CD as well as in non-IBD controls.6 Escherichia coli colonizes infants in the first months of life and is present in the intestines of humans throughout life. However, diet and the composition of intestinal microbiota affect the quantitative ratios

<table>
<thead>
<tr>
<th>Biopsies findings</th>
<th>Number of biopsies (%)</th>
<th>acute (n = 9)</th>
<th>chronic (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial damage</td>
<td></td>
<td>8 (88.9)*</td>
<td>0</td>
</tr>
<tr>
<td>Architectural changes</td>
<td></td>
<td>3 (33.3)</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>Mononuclear cells in lamina propria</td>
<td></td>
<td>9 (100)</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td>Polymorphonuclear cells in lamina propria</td>
<td></td>
<td>9 (100)</td>
<td>5 (55.5)</td>
</tr>
<tr>
<td>Neutrophils in epithelium</td>
<td></td>
<td>7 (77.8)*</td>
<td>0</td>
</tr>
<tr>
<td>Erosion or ulceration</td>
<td></td>
<td>8 (88.9)*</td>
<td>0</td>
</tr>
<tr>
<td>Crohn’s disease (n = 12)</td>
<td></td>
<td>5 (41.7)</td>
<td>7 (58.3)</td>
</tr>
<tr>
<td>Ulcerative colitis (n = 6)</td>
<td></td>
<td>4 (66.7)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Mean level of TNF-α [pg/mL]</td>
<td></td>
<td>199.7 ± 22.6</td>
<td>233.7 ± 18.7</td>
</tr>
<tr>
<td>Mean level of adherence [×10⁶ cfu per well]</td>
<td></td>
<td>3.9 ± 1.2</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>Mean invasion index [%]</td>
<td></td>
<td>1.6 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>AfaE2 (n = 7)</td>
<td></td>
<td>0</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>AfaE3 (n = 7)</td>
<td></td>
<td>7 (100)</td>
<td>5 (71.4)</td>
</tr>
</tbody>
</table>

* – number of DAEC-positive samples; a – number of Afa/Dr+ strains carrying AfaE2 or AfaE3 adhesins; cfu – colony-forming units; * statistically significant difference (p ≤ 0.01).
of various *E. coli* pathovars, leading to the predominance of one over another. Martinez-Medina et al. demonstrated that a high fat and high sugar Western diet led to dysbiosis in mice, with a particular increase in the *E. coli* population. Microbiotas are different in children and adults, and pathogenic *E. coli* (i.e. DAEC) are common in children’s intestinal microflora. Mansan-Almeida et al. showed that DAEC isolated from children were remarkably different from DAEC isolated from adults in terms of the diversity of adhesins and virulence factors. Moreover, they found that DAEC from children had a greater ability to colonize the gastrointestinal tract.

Bacterial adhesins are known virulence factors that permit pathogens to colonize the intestinal mucosa. Adherent-invasive *E. coli* is characterized by mannose-sensitive adherence to epithelial cells through type 1 fimbriae. Most studies examining *E. coli* from patients with IBD have focused on *E. coli* isolates adhering through mannose-sensitive adhesins, which are common among pathogenic and non-pathogenic *E. coli* strains. In the present study we preselected *E. coli* isolates using an adherence assay to HEp-2 cells in the presence of mannose to eliminate the influence of type 1 fimbriae on the adherence of *E. coli*.

The results of the study indicated that DAEC were isolated from 18 (69.2%) of the 26 children. Screening for AfaE subtypes indicated that AfaE3-positive strains represented 85.7% of the Afa/Dr+ isolates. According to other studies, AfaE3-positive DAEC were isolated at a much lower frequency. Zhang et al. isolated afaE3-carrying *E. coli* from 12% of 787 isolates from urinary tract infections and diarrhea cases. Mansan-Almeida et al. detected afaE3-positive DAEC in 2% of children with diarrhea and healthy controls, respectively, and in 7.4% and 6.7% of adults with diarrhea and controls, respectively. In a study of human diarrheal isolates, Le Bougenec et al. detected DAEC carrying afaE1, afaE3 and afaE5 subtypes with similar frequency (21.4%). Those investigators suggested that there might be an association between the subtype of AfaE adhesins and the physiological site of infection caused by afaE-positive strains. In their study they found that the AfaE8 subtype is predominant in sepsis patients but absent from diarrhea-associated strains. Similarly, Zhang et al. demonstrated that the AfaE5 subtype occurred 3 times as often among Afa/Dr+ DAEC causing urinary tract infections than in fecal strains. The predominance of the AfaE3 subtype among Afa/Dr+ strains demonstrated in the present study raises the question if the result simply reflects frequent colonization of the gastrointestinal tract of children in our region with AfaE3-positive DAEC, or if this subtype of adhesins is actually connected with childhood IBD. Hence, further studies are necessary.

Receptors for the AfaE3 adhesin, like DAF and CEACAM6, are exposed on the apical membrane of intestinal epithelial cells, providing a docking site for colonization of the intestinal mucosa by Afa/Dr+ DAEC. Interestingly, both of these molecules are upregulated in patients with CD, so they may promote enhanced adherence of DAEC. In addition, CEACAM engagement triggers endocytosis of bacteria into epithelial cells. Internalization of microorganisms through interaction with CEACAMs prevents exfoliation of the epithelium, favoring persistent infection. Indeed, in young children, diarrhea caused by Afa/Dr+ DAEC can become persistent. Furthermore, it has been demonstrated that infecting cultured epithelial Caco-2 cells with Afa/Dr+ DAEC increased the expression of major histocompatibility complex (MHC) class I-related MICA, a molecule that is expressed to a greater extent on the colonic epithelium of patients with CD.

In patients with UC, there is no difference in the expression level of the CEACAM6 molecule compared to patients with CD, although, like patients with CD, DAF expression is enhanced on colonic epithelial cells of patients with UC in relation to the severity of the mucosal inflammation. Thus, upregulated expression of DAF, CEACAM6 and MICA may predispose pediatric patients with IBD to colonization by Afa/Dr+ DAEC.

Acute IBD is characterized by increased expression of pro-inflammatory cytokines, e.g. TNF-α, which can modulate DAF expression, favoring the adherence of Afa/Dr+ DAEC to the intestinal mucosa. Both CD and UC exhibit elevated levels of TNF-α. In our study, we investigated whether the adherence and/or invasion of DAEC to epithelial cells induces secretion of TNF-α by MDM cocultured with epithelial cells. The results indicated that all but 1 Afa/Dr+ and all but 1 Afa/Dr- DAEC induced secretion of elevated levels of TNF-α independently of the strain origin (CD vs UC) or IBD activity (acute vs chronic). This indicates that Afa/Dr DAEC colonizing intestinal epithelium can promote their own adherence and invasiveness via induction of TNF-α release, which in turn modulates the expression of cellular receptors for these pathogens.

**Conclusions**

In summary, the results of the present study indicated that although DAEC was the most prevalent pathotype among the *E. coli* associated with biopsy specimens from pediatric patients with IBD, Afa/Dr+ strains were not specifically associated with CD or UC. Moreover, the study revealed that Afa/Dr+ DAEC from children with IBD most commonly presented the AfaE3 subtype of adhesins. The study demonstrated the internalization of Afa/Dr isolates by intestinal epithelial cells and their ability to induce increased secretion of TNF-α from monocytes/macrophages co-cultured with epithelial cells, which indicated that DAEC should be considered one of the pathobionts responsible for inducing inflammation of the intestinal mucosa in childhood IBD.
References


