Escherichia coli and persistent diarrhea
Schultsz, C.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 6

Frequency of pathogenic and enteroadherent *Escherichia coli* in patients with inflammatory bowel disease and controls

*Journal of Clinical Pathology* 1997; 50: 573-579
Frequency of pathogenic and enteroadherent *Escherichia coli* in patients with inflammatory bowel disease and controls

C Schultsz, M Moussa, R van Ketel, G N J Tytgat, J Dankert

Abstract

**Aims**—To determine whether inflammatory bowel disease (IBD) is associated with pathogenic or enteroadherent *Escherichia coli*.

**Methods**—A least two stool specimens and one rectal biopsy were taken from 30 patients with IBD and from 20 controls. A large number of *E coli*-like colonies cultured from each stool sample and biopsy was tested, using DNA probes, for the presence of genes encoding shiga-like toxins, invasiveness, attachment-effacement and the ability to adhere to HEp-2 cells. Similarity among isolates from stool samples and rectal biopsies was determined by random amplified polymorphic DNA (RAPD) analysis.

**Results**—Enterohaemorrhagic and entero-invasive *E coli* were not found in samples from either patients or controls. No significant difference in the detection rate of enteroadherent *E coli* between patients and controls was found. Rectal biopsies from 11 of 28 patients with IBD and 4 of 18 controls contained *E coli*, which hybridised with probes for detection of genes encoding diffuse adherence to HEp-2 cells, or encoding P-pili (p = 0.2). Enteroadherent *E coli* isolated from two or three stool specimens from the same patient or control appeared to be identical by RAPD analysis, and are considered to be residents in the colon. Probe positive isolates obtained from stool specimens and corresponding rectal biopsies were always identical on RAPD analysis.

**Conclusions**—*E coli* strains possessing adherence factors reside in the large intestine and adhere to the rectal mucosa, irrespective of the presence of colitis.

(J Clin Pathol 1997;50:573-579)

Keywords: enteroadherent *Escherichia coli*; inflammatory bowel disease; resident flora

Although the aetiology of inflammatory bowel disease (IBD) is not known, evidence from a variety of animal models of intestinal inflammation suggests that the presence of bacteria is required to sustain the inflammatory reaction. Pathogenic *Escherichia coli* strains, present in the colon, may play a role in the pathogenesis of IBD. In several studies, *E coli* possessing properties such as Shiga-like toxin production, hydrophobicity, adhesiveness, or the capacity to bind to matrix proteins were present in a higher proportion of patients with IBD than in controls without colitis.

Bacterial adherence to the epithelial surface or to the mucous layer covering the epithelium is considered a prerequisite for persistence of bacteria in the gut, including pathogenic bacteria such as *Vibrio cholerae* and enterotoxigenic *E coli*. Among the various adhesins of *E coli* mediating binding to epithelial cells, P-pili have been shown to be associated with persistence of *E coli* in the colon. *E coli* strains with the property to adhere to HEp-2 cells and intestinal epithelial cells in vitro have been associated with diarrhoea in studies from different geographic areas. Such *E coli* strains are designated enteroadherent *E coli*, showing either localised adherence, diffuse adherence (DAEC), or aggregative adherence (EAggEC) to HEp-2 cells. The localised type of adherence is characteristic for the classic enteropathogenic *E coli* (EPEC) and is expressed in conjunction with the capacity to produce typical attaching-effacing lesions in various epithelial cells. Although enteroadherent *E coli* are known to produce adhesins and are thought to cause diarrhoea, the pathogenic nature of DAEC and EAggEC is not known and data obtained in volunteer studies were not conclusive as to whether such strains have pathogenic properties.

*E coli* strains isolated from faecal specimens or rectal biopsies, expressing increased adhesion to buccal epithelial cells, HEp-2 cells or to HT-29 cells, have been cultured more frequently among patients with IBD than controls. Although it has been suggested that *E coli* from IBD patients shows more ability to adhere, results from studies on adherence are conflicting. In the majority of these studies, strains isolated from either stool specimens or rectal biopsies were studied, and *E coli* adherence was determined only phenotypically. Test results could vary because of differences between the type of epithelial cells to which the bacteria were allowed to adhere, the duration of the incubation period, the number of isolates tested from each patient, the bacterial growth media used, and observational differences. The variability of these conditions will be reduced if specific methods to detect genes encoding adherence factors in conjunction with phenotypic assays are used. Although only strains will be detected for which the particular adherence factor has been characterised, this approach will yield relevant information...
information about the significance of such adherent \textit{E. coli} in the gastrointestinal tract of patients with IBD.

To study whether the presence of pathogenic or enteroadherent \textit{E. coli} is associated with IBD, we compared the prevalences of pathogenic and enteroadherent \textit{E. coli} in patients with and without IBD. For this purpose, large numbers of isolates from stool specimens and corresponding rectal biopsies were tested using seven DNA probes for specific detection of genes encoding different virulence factors and adhesions. In addition, phenotypic expression of genes encoding adherence was assessed by using the HEp-2 cell adhesion assay. Finally, to determine whether isolates from stool specimens and corresponding rectal biopsies were related, subsets of isolates were characterised by using random amplified polymorphic DNA (RAPD) analysis.

**Materials and methods**

**PATIENTS AND SAMPLE COLLECTION**

Patients who visited our department of gastroenterology for endoscopy were included in the study. Patients were enrolled if they had active (newly diagnosed or relapse) ulcerative colitis or Crohn’s disease. Diagnosis was confirmed by endoscopy and histology. Only patients with a definite diagnosis of ulcerative colitis or Crohn’s disease were included.

From each patient, stool specimens were collected on the day of presentation, two to eight weeks later and, whenever possible, approximately three months later. In addition, from each patient a rectal biopsy was performed at endoscopy on the day of presentation. From patients with Crohn’s disease, an additional biopsy from an ulcerated area was done if the rectum appeared normal.

Control were patients who required endoscopy for reasons other than IBD (for example, irritable bowel syndrome) and who did not complain of diarrhoea. Patients were enrolled as controls if results from endoscopy and histology were normal. From each control, a stool specimen and a rectal biopsy were collected on the day of endoscopy. A second stool specimen was collected two to eight weeks later.

Patients and controls were excluded if they had taken antimicrobial therapy during the previous six weeks, if they had visited tropical areas during the previous year, or if they had visited tropical areas during the previous six weeks, if they had taken antimicrobial therapy during the previous six weeks.

Control were patients who required endoscopy for reasons other than IBD (for example, irritable bowel syndrome) and who did not complain of diarrhoea. Patients were enrolled as controls if results from endoscopy and histology were normal. From each control, a stool specimen and a rectal biopsy were collected on the day of presentation. A second stool specimen was collected two to eight weeks later.

Patients and controls were excluded if they had had antimicrobial therapy during the previous six weeks, if they had visited tropical areas during the previous year, or if they had a history of colon carcinoma. All stool specimens were collected before preparation for endoscopy.

**EXAMINATION OF STOOL SPECIMENS**

Stool specimens were stored at 4°C and processed within 24 hours after collection. All specimens were examined for \textit{Salmonella}, \textit{Shigella}, \textit{Yersinia}, and \textit{Campylobacter} spp using standard identification methods. Stool specimens collected on the day of presentation were also cultured for \textit{Clostridium difficile} and examined for amoeba, ova, and cysts by direct microscopical examination and after concentration (modified Ridley method). All specimens were tested for the presence of \textit{C. difficile} toxin by tissue culture assay.

For detection of pathogenic and enteroadherent \textit{E. coli}, a suspension of a stool specimen was made with an equal volume of phosphate buffered saline (PBS, pH 7.2). After serial 10-fold dilution, 50 μl from each dilution was plated onto a MacConkey agar plate. After growth at 37°C for 18 hours, the plate containing approximately 100–300 separate colonies was selected and replica plated onto six nylon membranes (Hybond-N; Amersham Nederland, Hertogenbosch, The Netherlands). Colonies on each membrane were grown on nutrient agar plates for seven hours, then lysed, denatured, and fixed to the membrane, essentially as described by Sambrook et al. 15

**EXAMINATION OF RECTAL BIOPSIES**

Rectal biopsies were immediately wrapped in paraffin, snap frozen in liquid nitrogen, and stored at -70°C. For examination, biopsies were gently washed twice in PBS, homogenised in 1 ml of Trypticase Soy Broth (TSB) using a Potter apparatus and incubated at 37°C for 18 hours. After serial 10-fold dilution, 50 μl from each dilution was plated onto a MacConkey agar plate, replica plated, and further treated as the stool specimens.

From MacConkey agar plates showing growth of \textit{E. coli}-like bacteria, 10 colonies were randomly picked and identified biochemically as \textit{E. coli} using standard identification methods. These colonies were inoculated on agar slants and stored.

**HAEMOLYSIN PRODUCTION**

Each stored \textit{E. coli} isolated from rectal biopsies was inoculated onto a sheep blood (5% vol/vol) agar plate and incubated at 37°C for 18 hours. Isolates were considered haemolytic if a bright zone was visible surrounding each colony.

**DETECTION OF PATHOGENIC AND ADHERENT \textit{E. coli} WITH DNA PROBES**

Nylon membranes containing approximately 100–300 colonies were hybridised under stringent conditions with six digoxigenin (DIG) labelled polynucleotide DNA probes for detection of genes encoding Shiga-like toxin production (STL1, STL2; enterohemorrhagic \textit{E. coli}, EHEC), the capacity to produce attaching-effacing lesions in epithelial cells (eae; attaching-effacing \textit{E. coli}, AECC), enteroinvasiveness (enteroinvasive \textit{E. coli}, EIEC), diffuse adherence to HEp-2 cells (danC, DAEC), and aggregative adherence to HEp-2 cells (AA, EAggEC) (table 1). Probes were labelled by random primer method and hybrids were detected by alkaline phosphatase labelled anti-DIG antibody, both according to the instruction in the DIG DNA labelling and detection kit (Boehringer Mannheim BV, Almere, The Netherlands). Control strains were included in each hybridisation reaction (table 1). Probes and control strains were kindly provided by B Rowe, Central Public Health Laboratory, London, UK.

From the stored \textit{E. coli} isolates obtained from each rectal biopsy, a suspension in PBS was
Table 1. DNA probes used for detection of pathogenic and enteroinvasive E. coli

<table>
<thead>
<tr>
<th>Plasmid/probe*</th>
<th>Control strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shiga-like toxin 1 producing E. coli</td>
<td>NTP 705</td>
<td>E3757</td>
</tr>
<tr>
<td>Shiga-like toxin 2 producing E. coli</td>
<td>pDEP 24</td>
<td>E3540</td>
</tr>
<tr>
<td>Diffusely adhering E. coli</td>
<td>pSLM852</td>
<td>E72540</td>
</tr>
<tr>
<td>Aggregatively adhering E. coli</td>
<td>pCVD342</td>
<td>E69187</td>
</tr>
<tr>
<td>Attaching-effacing E. coli</td>
<td>pCVD344</td>
<td>E67472</td>
</tr>
<tr>
<td>Interovasive E. coli</td>
<td>H1</td>
<td>E126320</td>
</tr>
<tr>
<td>P-piliated E. coli</td>
<td>PCR-generated</td>
<td>AD110</td>
</tr>
</tbody>
</table>

*Cloned probe fragments were cut from plasmids after digestion with the appropriate restriction enzymes.

Spotted onto nylon membranes, grown for seven hours, lysed, denatured, and fixed to the membrane, and hybridised additionally with a DIG labelled PCR generated polynucleotide probe for detection of pap related sequences encoding P-pili (table 1).

ADHESION TO HEp-2 CELLS

All probe positive isolates from stool samples and rectal biopsies were tested for adherence to HEp-2 cells. In addition, 10 probe negative E. coli strains cultured from each rectal biopsy were grown overnight in nutrient broth containing 1% d-mannose (wt/vol), then equal volumes were pooled and mixed, and 10 µl from this pool was added to the HEp-2 cells.

Adhesion of E. coli to HEp-2 cells was assessed in a six hour assay as described by Scotland et al. Briefly, HEp-2 cells were maintained in Dulbecco’s Minimum Essential Medium (DMEM; Gibco Europe BV, Breda, The Netherlands) containing 10% (vol/vol) fetal calf-serum, at 37°C in 5% CO₂ atmosphere. Cells were transferred to tissue culture plates with 24 wells containing glass coverslips and grown to semi-confluent monolayers. After replacement of the medium with fresh DMEM with 1% d-mannose, cells were inoculated with 10 µl of each probe positive E. coli isolate grown overnight in nutrient broth containing 1% d-mannose or 10 µl of pooled probe negative isolates. Bacteria were allowed to attach for three hours. The cells were washed three times with Dulbecco’s PBS (DPBS, Gibco) to remove non-adherent bacteria and incubated for another three hours. Cells were washed again, fixed with 1% (vol/vol) paraformaldehyde and 0.1% (vol/vol) glutaraldehyde in DPBS for at least 30 minutes and stained with diluted crystal violet. Bacteria were considered adherent if more than 10 bacteria per cell were adhering to at least 40% of the cells. In general, 100 to 200 cells were counted. The pattern of adherence (localised, diffuse or aggregative) was also recorded.

CHARACTERISATION OF ISOLATES BY RAPD

Sets of isolates obtained from stool specimens and corresponding rectal biopsies as well as from consecutive stool specimens, were characterised using RAPD.

Bacteria were grown overnight at 37°C on sheep blood agar plates. Bacteria were washed in PBS and suspended in PBS to a concentration of 3 x 10⁶ bacteria/ml using MacFarlands opacity standard. After heating for 10 minutes, an equal volume of chloroform was added to the suspensions. The lysates were mixed and centrifuged at 12 000 xg for 10 minutes in an Eppendorf centrifuge. Two different PCRs were performed on the pellets to compare the DNA contents of bacteria.

PCR targeting repetitive intergenic DNA sequences

Primers ERIC1R (3’-CAGTAAAAAGGCCGAAAG-GGA-3’) and ERIC2 (5’-AGAGTTAGTGGAGGGAACG-3’) were used as described by Versalovic et al. Briefly, the amplification procedure was performed in a final volume of 50 µl. The reaction mixture contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 25 mM MgCl₂, 100 µM each of deoxyribonucleotide, 100 ng of each oligonucleotide, and 1.25 U Taq polymerase (AmpliTaq, Perkin-Elmer, Gouda, The Netherlands), to which 10 µl of the bacterial suspension was added. The mixtures were processed in a programmable DNA thermal cycler (Perkin-Elmer) and subjected to 35 cycles of amplification. One cycle consisted of one minute at 95°C, one minute at 52°C, and two minutes at 72°C.

Ten microlitres of amplified sample were electrophoresed in a 1% agarose gel with a molecular mass marker and stained with ethidium bromide. Isolates to be compared were processed simultaneously and electrophoresed on the same agarose gel.

PCR using an arbitrary primer

PCR was performed using arbitrary primer TT, (5’-GGCGAGGGAACG-3’). Conditions were as described above except that 1 µg of oligonucleotide was used. PCR started with two cycles consisting of five minutes at 95°C, five minutes at 40°C, and five minutes at 72°C, followed by 33 cycles consisting of one minute at 95°C, one minute at 40°C, and two minutes at 72°C.

STATISTICAL ANALYSIS

Differences between groups were calculated by using Fisher’s exact or χ² test when appropriate.

RESULTS

In total, 61 stool specimens from 30 IBD patients (20 patients with ulcerative colitis, 10 patients with Crohn’s disease), and 28 stool specimens from 20 controls were cultured (table 2). Rectal biopsies were also collected, except from two patients with ulcerative colitis, and two controls.

Salmonella enteritidis was found in one patient with ulcerative colitis, toxin producing C difficile in one patient with ulcerative colitis, and Plesiomonas shigelloides in one patient with Crohn’s disease. One control had both Campylobacter jejuni and Giardia lamblia. No other enteropathogens were detected.

DETECTION OF PATHOGENIC AND ADHERENT E. COLI WITH DNA PROBES

Five of 61 (8%) stool samples from 30 patients with IBD yielded no growth after culture on MacConkey agar plates whereas all 28 stool samples from 20 controls showed growth of coliform bacteria. The detection rate of patho-
Strains from biopsies obtained from patients with IBD more often hybridised with the pap probe than strains obtained from biopsies from controls (table 3), but this difference was not statistically significant (p = 0.27).

**ADHESION TO HEp-2 CELLS**

From the seven rectal biopsies and corresponding stool samples with probe positive *E. coli* strains from both patients and controls, strains adhered to HEp-2 cells and showed the pattern of adherence as predicted by the probe to which they hybridised in four cases. Strains from one biopsy and corresponding stool sample, positive with the daaC probe, did not show adhesion to HEp-2 cells. None of the pools of probe negative isolates from rectal biopsies showed adhesion to HEp-2 cells. daaC probe positive isolates obtained from stool samples from two controls but not from their corresponding rectal biopsies, showed diffuse adhesion to HEp-2 cells. Probe positive strains from two biopsies (strains from one biopsy were positive with both the daaC and AA probes, strains from the other biopsy were positive with the eae probe) and probe negative strains from one biopsy caused detachment of the cells from the glass cover slips, which made it impossible to observe any pattern of adhesion.

**HAEMOLYSIN PRODUCTION**

Strains from seven biopsies produced haemolysin as detected on sheep blood agar plates. These seven were equally distributed among patients and controls. Haemolysin production was associated with the presence of genes encoding P-pili in four cases. All strains causing cell detachment in the HEp-2 cell adhesion assay produced haemolysin.

**RAPD ANALYSIS**

Using RAPD analysis, probe positive isolates from consecutive stool specimens from three patients were characterised. Isolates from consecutive samples from each patient appeared to be identical. As the median period between collection of first and second stool samples from patients and controls was five weeks (range two to 28 weeks) and between collection of second and third stool samples was seven weeks (range two to 16 weeks), identical strains isolated from consecutive stool specimens were considered to be residents in the colon.

RAPD analysis of probe positive *E. coli* strains obtained from a stool sample and corresponding rectal biopsy from one patient with ulcerative colitis showed that the isolates obtained from both materials were identical (fig 2).

In two controls, isolates in stool specimens were probe positive but only probe negative isolates were present in corresponding rectal biopsies. RAPD analysis of two probe positive isolates from stool and 10 probe negative isolates from the corresponding rectal biopsy of one of these controls showed that the probe positive and negative isolates were different.
Figure 1  Detection of enteroadherent E coli in stool samples and biopsies of four patients with ulcerative colitis (UC), two patients with Crohn's disease (CD), and five patients without inflammatory bowel disease (C) in time. Triangles, strains positive after hybridisation with a probe for detection of diffusely adhering E coli; Circles, strains positive after hybridisation with a probe for detection of aggregatively adhering E coli; Squares, strains positive after hybridisation with a probe for detection of attaching-effacing E coli. Isolates obtained from stool samples are indicated by closed symbols, isolates obtained from rectal biopsies are indicated by open symbols.--- No probe positive strains after hybridisation with any of the probes; NA, not available.

Discussion

Various studies have addressed the hypothesis that virulent or adherent E coli strains contribute to the pathogenesis of IBD in an unexplained manner.5-5 13 14 20 No consistent data have been shown in such studies, in which a variety of tests was used and isolates from either stool specimens or rectal biopsies were examined. We used DNA probes to detect E coli carrying genes encoding virulence factors and adhesins. In addition, a large number of colonies was tested from both stool specimens and corresponding rectal biopsies.

We did not observe significant differences in the prevalence of pathogenic or adherent E coli strains in either stool specimens or rectal biopsies from patients with IBD and controls. No VTEC strains were detected by DNA hybridisation, in contrast with some studies7 but in concordance with others.24 Similarly, no enteroinvasive strains were found.

DAEC and P-piliated E coli were the most frequently encountered probe positive E coli. Similar to our results, Hardtke et al.14 found that the majority of adherent isolates from patients with IBD and controls showed diffuse adhesion to HEp-2 cells. The DNA probe we used detects genes shared by a family of genetically related adhesins mediating diffuse adhesion, the Dr family.25 26 These adhesins include the Dr haemagglutinin, AFA I, AFA III, and F1845 adhesin, and recognise the Drt blood group antigen as their receptor.27 This receptor is present on a large number of epithelial cells, including colonic epithelial cells.28 The pathogenic nature of DAEC has not been elucidated.11 Studies from different geographic areas show discordant results concerning their association with diarrhoea. In our study, identical DAEC, as shown by RAPD, could be detected in stool specimens from patients with IBD and controls with time, indicating that DAEC form a part of the resident flora of some adults, irrespective of the presence or absence of colitis (fig 1). Identical strains were also present on corresponding rectal biopsies, suggesting that these strains were truly adherent.

E coli possessing P-pili adhere to colonic epithelial cells in vitro by binding to the Galα1-4Galβ containing receptor.29 Weld et al30 and Tullus et al31 showed that P-pili expressing E coli are residents in the colon at infancy, in contrast with transient strains that express P-pili less frequently. Although we only hybridised isolates obtained from single rectal biopsies with the probe for detection of pap related genes, we assume that the P-pili encoding E coli strains, isolated from rectal biopsies, are also resident strains.

The mechanisms underlying the association between resident bacteria and the mucosal sur-
face have not been elucidated. Adhesion of bacteria to epithelial cells is thought to occur through interactions between bacterial adhesins and cellular receptors at the initial site of infection. Bacterial association with intestinal mucin may also occur through interactions with bacterial outer membrane components. 58 Adlerberth et al. 41 showed that the Dr haemagglutinin, P, type 1, and S fimbrial adhesins mediate binding of E. coli to both colonic and ileal enterocytes. Expression of these adhesins may contribute to the establishment of E. coli in the intestine. We did not study the occurrence of E. coli possessing type 1 fimbriae or S fimbriae. Therefore, such strains may have been present among the DNA probe negative E. coli isolated from stool specimens and rectal biopsies. EAggEC were isolated from stool samples and rectal biopsies from three of 28 patients with IBD but were absent in both stool samples and rectal biopsies from 20 controls. EAggEC have been associated with persistent diarrhoea in children in developing countries. 39, 40 As the number of patients with EAggEC in our study was low, the association of EAggEC with IBD remains unclear. E. coli isolates obtained from three rectal biopsies caused detachment of the HEP-2 cell monolayer on the glass coverslip. This phenomenon has also been described by other investigators and appeared to be related to haemolysin production. 29, 30 Recently, it was shown that such strains are not associated with diarrhoea. 31 An association with IBD is also not very likely as haemolysin producing strains were equally distributed among patients and controls in our study. Although both patients and controls carried adherent E. coli in the large intestine at a similar rate, we cannot rule out the possibility that the mere presence of adherent bacteria in the colon, either E. coli or other species, contributed to the inflammation in patients with active IBD. Shen et al. reported that in vitro adherence of E. coli from patients with colitis to matrix proteins was higher than from control strains. Bacteria may adhere to a damaged mucosal surface through increased exposure of receptors or through non-specific interactions when matrix proteins are exposed. 32 Bacteria adhering to matrix proteins may thus contribute to an inflammatory response by exposing the mucosal immune system to bacterial antigens while bypassing the epithelial barrier. Adherence of some bacteria to epithelial cells has been shown to lead to the release of cytokines by the cells and to induce transepithelial migration of neutrophils. 33, 34 Therefore, functional changes of the epithelial cells to which such bacteria have bound may also contribute to the inflammatory reaction seen in IBD. We have found no significant differences in the presence of true pathogenic E. coli in faecal samples or rectal biopsies from patients with IBD and controls. E. coli strains possessing Dr adhesins, and probably those possessing P-pili, are resident in the large intestine of some individuals and adhere to the rectal mucosa, irrespective of the presence or absence of colitis.

We thank P. Fockens for assistance with sample collection and B. de Vos and L. van Alphen for assistance with RAPD analysis. We thank W. van Loon for technical assistance and M. de Jong for critically reviewing the manuscript.


