Escherichia coli and persistent diarrhea
Schultsz, C.

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Chapter 9

General discussion

Detection methods for diarrhoeagenic E. coli

To meet the needs of returning travelers, a method was developed for rapid and sensitive detection of enterotoxigenic E. coli (ETEC) in stool samples. The well established association of ETEC with the onset of acute diarrhea of short duration among travelers to (sub)tropical areas, warranted the availability of a sensitive detection method for isolation of ETEC in stool samples of travelers with persistent diarrhea after returning from tropical destinations. Assumptions that the number of ETEC in the stool during gastroenteritis may be small, led to the development of a novel method for detection of diarrheaogenic ETEC in the stool samples. PCR and oligonucleotide DNA-probe for detection of ETEC were compared. The PCR of bacterial nature, obtained from stool cultures grown on a CLED agar plate, was proven to be a rapid and convenient method for detection of ETEC, especially since it does not require DNA extraction and purification from fecal specimens. The PCR is far less laborious and more economical than the use of oligonucleotide probes on stool isolated single colonies or on a membrane. In addition, it has a much higher sensitivity. The detection level was similar using 10^3 to 10^7 CFU/g feces. Therefore, the PCR was applied for the detection of ETEC in subsequent studies in returning travelers.
General discussion

Persistent diarrhea is a torment for patients, but it is a challenge for clinicians and investigators. In this thesis, 3 patient groups with persistent diarrhea were studied: travelers returned from (sub)tropical areas, inflammatory bowel disease (IBD) patients and patients with human immunodeficiency virus (HIV) type 1 infection. Although these patient groups differ from each other in many respects, they have one aspect in common: the etiology of persistent diarrhea in them is largely unknown. The studies described in this thesis were performed to determine whether diarrheagenic \textit{E. coli} may play a role in the etiology of persistent diarrhea.

Routine bacteriological investigations in most clinical microbiology laboratories do not include the detection of diarrheagenic \textit{E. coli}, since the required methods are laborious and time consuming. Therefore, little is known about the frequency of diarrheagenic \textit{E. coli} in stool samples of returned travelers, patients with IBD and HIV-1 infected patients. Whether the presence of such \textit{E. coli} strains is associated with diarrhea is even less clear.

Detection methods for diarrheagenic \textit{E. coli}

To start the studies in returned travelers, a method was developed for rapid and sensitive detection of enterotoxigenic \textit{E. coli} (ETEC) in stool samples. The well established association of ETEC with the onset of acute diarrhea of short duration among travelers in (sub)tropical areas, warranted the availability of a sensitive detection method for studies in patients with persistent diarrhea after traveling to similar destinations. Assuming that the number of ETEC in the stools during prolonged infection may be small, the method should enable detection of low numbers of ETEC in the stool samples. PCR and oligonucleotide DNA-probes for detection of ETEC were compared. The PCR of bacterial sweeps, obtained from stool cultures grown on a CLED-agar plate, was proven to be a rapid and convenient method for detection of ETEC, especially since it does not require DNA extraction and purification from fecal specimens. The PCR is far less laborious and time consuming than the use of oligonucleotide probes on either isolated single colonies or on a sweep. In addition, it has a much higher sensitivity. The detection level was raised from $10^6$ CFU/g feces to $10^2$ CFU/g feces. Therefore the PCR was applied for the detection of ETEC in subsequent studies in returned travelers.
For detection of ETEC with oligonucleotide DNA probes, hybridization of the bacterial sweep material was at least as sensitive as hybridization of 5 single *E. coli* isolates. This finding was extrapolated to the application of polynucleotide DNA probes for detection of other types of diarrheagenic *E. coli*. Therefore, sweep blots were used for the detection of enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC) and diffuse adherent *E. coli* (DAEC) in stool samples from returned travelers and HIV-1 infected patients, using polynucleotide DNA probes, combined with the HEp-2 adherence assays when appropriate. If the screening method yielded positive results, 5 *E. coli* colonies were isolated from the sweep and tested in the HEp-2 adherence assay for phenotypic expression of the adherence pattern. Another method was used for the detection of such *E. coli* strains, as well as shigatoxin producing enterohemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC), in stool samples and rectal biopsies from IBD patients. To enable isolation of single probe positive *E. coli* strains from consecutive stool samples and from rectal biopsies from these patients, 100-300 *E. coli*-like colonies were replica-plated from a MacConkey agar plate to nylon filters, which were hybridized with each of the probes. Subsequently, probe positive colonies could directly be picked from the original MacConkey agar plate. Indeed, although probe-positive colonies were in the majority on most positive filters, less than 10 probe positive colonies were present among the hundreds of negative colonies on some filters. This suggests that this method has enhanced sensitivity when compared with sweep blots. However, we did not directly compare the sensitivities of replica-plating with either the sweep blot or the hybridization of 5 colonies.

The gold standard for detection of adherent diarrheagenic *E. coli*, i.e. EPEC, EAggEC and DAEC, is the HEp-2 adherence assay. Probe positive colonies, or *E. coli* isolates obtained from probe positive sweeps, were therefore tested in the HEp-2 adherence assay for detection of phenotypic expression of HEp-2 adherence. There are several important remarks that can be made about this approach. First, the application of the screening method, using DNA hybridization of sweeps or replica plates, may introduce a bias since it is known, particularly for EAggEC and DAEC, that the commonly applied DNA-probes do not detect all *E. coli* variants with the respective adherence traits. The reported sensitivities of the probes vary between 35 and 90%, depending on the population and the geographic area studied (1-3). It is therefore possible that certain EAggEC and DAEC strains which were present in the stools but which do not hybridize with the DNA-probes used, were missed. However, the frequency of DAEC in 3 patient groups as well as in the control groups studied, was high and it is unlikely
that direct screening of *E. coli* isolates in the HEp-2 adherence test, for example by testing 5 isolates from each stool sample, would lead to significantly higher recoveries in patients and controls. This assumption may be not be valid for EAsggEC. For example, while using polynucleotide DNA probe screening, no EAsggEC was detected in HIV-1 infected patients with and without diarrhea in the Netherlands. In contrast, EAsggEC was significantly associated with diarrhea in HIV-1 infected patients in the USA, using the Hep-2 adherence test (4). The EAsggEC strains detected in the Hep-2 adherence test in the latter study were negative in a PCR for detection of genes encoding adherence fimbriae, with a similar sensitivity as the EAsggEC probe (4).

Second, there has been debate about the performance of the HEp-2 adherence test. While most investigators use a 3 hour assay, others found that a 6 hour assay, which includes a wash step after 3 hours of incubation, results in improved distinction between diffuse and aggregative adherent strains (5,6). To avoid difficulties with the interpretation of the adherence test as much as possible, the 6 hour assay was used in the studies described in this thesis.

Finally, one can argue whether results obtained in the HEp-2 adherence test can be extrapolated to the *in vivo* situation, since HEp-2 cells are not of intestinal origin. However, studies using intestinal epithelial cell culture and continuous cell lines of intestinal origin indicate that the adherence patterns of EPEC and EAsggEC to such cells are similar to the patterns observed with HEp-2 cells (7,8). Nevertheless, it is possible that, when using intestinal epithelial cells lines for detection of adherent *E. coli*, more (or maybe even less) *E. coli* strains in a stool sample appear to be adherent, or that additional categories of adherent *E. coli* can be identified. For example, in a recent study on the occurrence of adherent *E. coli* among patients with Crohn’s disease (CD) using Caco-2 cells, adherent *E. coli* were isolated significantly more often from the ileum from patients with CD than from controls (9). In contrast, in the study in IBD patients described in this thesis, no differences were found in the frequency of adherent *E. coli* between patients with IBD and controls, using DNA-probes and HEp-2 adherence test. However, when considering the type of *E. coli* strains which were isolated most frequently, the results of the 2 studies are similar, i.e. mainly diffusely adherent and hemolysin producing strains.

**Frequencies of diarrheagenic *E. coli* in stool samples**

No association was found between the presence of diarrheagenic *E. coli* and diarrhea in the patient groups studied, except for ETEC, and possibly EAsggEC, in returned travelers with
acute diarrhea. EIEC and EHEC could not be detected in any of the patients. In contrast, DAEC were commonly present in stool samples from each patient- as well as control group studied. Sequential stool samples from patients with IBD showed that these strains are present as part of the resident microflora in the large intestine. This was concluded from the fact that identical strains, as characterized by RAPD analysis, could be detected in stool samples and rectal biopsies from IBD patients, collected after an interval of at least 2 weeks. EAggEC and EPEC were also found in sequential stool samples and rectal biopsies from these patients, and it is likely that in some individuals these strains also form part of the resident intestinal flora. As mentioned in chapter 2, the heterogeneity of DAEC and EAggEC, as demonstrated in volunteer studies, has led to the hypothesis that clones in different geographic areas may vary in the virulence factors they possess. Therefore, the EAggEC strains isolated from returned travelers may indeed be pathogenic, but it is unknown in which respect these strains differ from the ones found in controls, or in individuals who did not travel to “high-risk” areas.

Induction of IL-8 release by diarrheagenic E. coli

The isolation of E. coli, which are capable to adhere to epithelial cells, in returned travelers with diarrhea, patients with IBD, and controls, has led to the suggestion that one of the virulence factors distinguishing pathogenic from commensal strains could be that such E. coli strains have the capacity to induce cytokine release by intestinal epithelial cells through bacterial adherence. It was already shown by other investigators that the adherence of uropathogenic E. coli to bladder and kidney epithelial cells could induce the release of IL-6 and IL-8 by these cells (11-13). Similarly, adherence of Salmonella typhimurium to intestinal epithelial cells induces the production of IL-8, and promotes migration of polymorphonuclear leukocytes along the epithelial monolayer (14). Preliminary experiments with an EAggEC strain, isolated from a patient with ulcerative colitis, showed that this strain induced high levels of IL-8 release by HT-29 cells. This observation was the incentive to the study whether the diarrheagenic E. coli could induce IL-8 release by HT-29 cells. Since relatively few isolates were available from patients with IBD, this study was performed with isolates obtained from the group of returned travelers with acute and persistent diarrhea and their controls.

Although no association was found between diarrhea and the presence of bacteria inducing IL-8 release, two important observations were made. First, no differences in IL-8 inducing capacity were present between the different categories of diarrheagenic E. coli. Second, adherence to the epithelial cells did not seem to be essential for IL-8 release. These
findings led to the conclusion that subpopulations of resident, commensal *E. coli* strains, not
only present in patients with diarrhea but also in controls, are capable of triggering intestinal
epithelial cells to release cytokines in response to bacterial components or secreted proteins.

Lipopolysaccharide (LPS) from Gram-negative bacteria is a well known stimulator of
cytokine release. There is growing evidence that, in addition to LPS, other components of both
Gram-positive and Gram-negative bacteria have the capacity to induce cytokine synthesis (15).
These components may be structural bacterial components, such as outer membrane or fimbrial
proteins, or peptidoglycan, as well as extracellularly secreted products such as toxins or
hemolysins. Together these components have been called modulins (15). The results of the
study described in this thesis are in agreement with this observation. In addition, Wilson et al.
suggest that members of the normal microflora not only can stimulate cytokine synthesis, but
also possess surface components or secrete products with the ability to downregulate the
synthesis of pro-inflammatory cytokines, upregulate the release of anti-inflammatory cytokines
or neutralize the biological activities of pro-inflammatory cytokines (16). Thus, commensal
bacteria may form an important part of the mucosal cytokine network and their presence may
add to the control of inflammation at the mucosal surface.

The spatial distribution of bacteria in the inflamed and normal mucosa

Since no difference was observed in the occurrence of diarrheagenic *E. coli* in patients
with IBD and controls, and adherent strains appeared to be part of the resident flora in the
large intestine of both groups, it was hypothesized that not the mere presence but the
localization of adherent bacteria in the mucosa may be important in the pathogenesis of IBD
and may thus differ between patients with IBD and controls. To study the spatial distribution
of enteric bacteria in rectal biopsies, an *in situ* hybridization (ISH) method was developed,
targeted at the 16S rRNA gene of the kingdom Bacteria. This method was applied to rectal
and colonic biopsies from patients with IBD and controls. Surprisingly, very few to no bacteria
were found to adhere to the rectal epithelium of healthy controls. In contrast, it was shown
that bacteria with varying morphology were associated with the mucus layer of the rectal
mucosa from patients with IBD. As yet, it is unknown whether this association, which may be
the result of underlying changes in mucus composition, for example due to genetic differences
between patients with IBD and controls, causes or is secondary to the inflammation. Strikingly,
similar results were reported by other investigators who studied the presence of intraepithelial
bacteria in patients with colorectal cancer (17). Using a quantitative PCR for detection of 16S
rRNA genes of bacteria, followed by sequence analysis of the amplification products, they found that colonic biopsies of the large majority of asymptomatic (30/31) and symptomatic controls (24/34), with abdominal complaints and normal colonoscopic findings, did not contain any bacteria, in contrast to biopsies of patients with colonic malignancies (17). Similar to the results obtained in IBD patients, the increased presence of bacteria was independent of the biopsy site in the colon and whether this site was close or distant to the actual carcinoma. It is known that in colorectal carcinoma, changes occur in the expression of cellular surface antigens (18), which may promote adhesion of bacteria. This could explain the increased presence of intraepithelial bacteria in patients with colorectal carcinoma. While it is tempting to speculate that in patients with IBD similar changes occur, the question remains whether such changes are primary or secondary to inflammation. However, it has become clear from these studies that we need to change our view on the distribution and localization of commensal bacteria in the large intestine. While it has often been assumed that resident bacteria are trapped in the large intestinal mucus, or adhere directly to the intestinal epithelium through their adhesins, it appears that the mucus in the uninflamed large intestine forms a physical barrier which, under normal conditions, bacteria are unable to penetrate.

**Unifying hypothesis**

The intestine is an extremely large mucosal surface area which is continuously exposed to an enormous antigenic load, consisting mainly of luminal bacteria and food antigens. The mucosal immune system forms a complex defense mechanism, consisting of different components which are acting in concert to exert their function. These components include M-cells, concentrated in the gut associated lymphoid tissue being responsible for the uptake of antigens and micro-organisms (19), B-cells, T-cells, macrophages, being activated upon antigen uptake resulting in production of secretory IgA and the release of pro-inflammatory and down-regulating cytokines (20), and intestinal epithelial cells, which are also capable of antigen-presentation and the production of pro-inflammatory and down-regulating cytokines (21). In the normal physiological state, the antigenic stimulation by luminal contents and the combined defense mechanisms are balanced. However, this balance can be disturbed by various exogenous stimuli, which may lead to intestinal inflammation. Disturbance of the local defense balance not only results in intestinal inflammation, but also in decreased epithelial barrier function, and thus to increased epithelial permeability (22). In turn, increased epithelial permeability permits back-diffusion of luminal contents, which may sustain the inflammatory
reaction. A typical example of such a stimulus which leads to local intestinal inflammation, is represented by enteric infection due to an invasive pathogen, such as *Shigella*. Non-invasive enteric microorganisms may also disturb the local defense balance, for example by activation of epithelial NF-κB through adherence to epithelial cells, leading to increased production and secretion of pro-inflammatory cytokines (21).

Persistent diarrhea in travelers typically develops after a bout of acute diarrhea. Although approximately 40% of individuals traveling to high-risk areas experiences traveler’s diarrhea, only 3% develops persistent diarrhea. In more than 50% of patients with acute or persistent diarrhea no cause is found, even after extensive microbiological investigations. Similarly, first manifestations of IBD often present as an episode of acute infectious diarrhea. In fact, gastrointestinal infections, travel abroad and antibiotic treatment are known precipitating factors in first attacks of IBD, suggesting that changes in the composition of the intestinal flora precipitate the development of inflammation (23). HIV-1 infected patients frequently experience gastro-intestinal infections, for which no pathogen can be identified in up to 46% of patients, and which become persistent in a considerable number of patients (24). In all three patient groups, the acute momentum functions as a disturbance of the local defense in the intestine. In HIV-infected patients, the immune depletion, which is also found locally in the intestinal mucosa, and the presence of HIV itself, which has been shown to activate pro-inflammatory cytokines such as TNFα, can explain why the disturbed balance of defense mechanisms can not be repaired (25). In returned travelers, a new balance eventually is attained, explaining the self-limiting character of persistent diarrhea in these patients. However, it is unclear why certain individuals require an extended period of time for achieving this new balance. In IBD patients, the disturbance of the balance between pro-inflammatory and anti-inflammatory responses is irreparable. Future investigations may provide insight into the underlying factors which are responsible for this apparently irreversible imbalance. Genetic factors may play a role in the pathogenesis of both IBD and persistent diarrhea in returned travelers.
Reference List


