

MARKERS OF ENTERIC INFLAMMATION IN ENTEROAGGREGATIVE *ESCHERICHIA COLI* DIARRHEA IN TRAVELERS

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Abstract. As part of a traveler's diarrhea study carried out in Guadalajara, Mexico, and Goa, India, we conducted a case control study to evaluate fecal markers of enteric inflammation in three groups. Forty-five cases of enteroaggregative *Escherichia coli* (EAEC) diarrhea were compared to 56 controls with enterotoxigenic *E. coli* (ETEC) diarrhea, and 126 controls with diarrhea without identifiable pathogens. For EAEC cases we found fecal leukocytes, occult blood, and lactoferrin in 13 (28.9%), 14 (31.1%), and 27 (60.0%) patients, respectively; for ETEC controls they were 15 (26.8%), 16 (28.6%), and 15 (26.8%) respectively; and for patients without identifiable pathogens 19 (15.1%), 34 (27.0%) and 27 (21.4%) were seen for the presence of a positive fecal lactoferrin test in EAEC cases was statistically significant compared to both control groups. The study provides evidence that EAEC infection is associated with an intestinal inflammatory response.

INTRODUCTION

Diarrheagenic *Escherichia coli* are divided into at least six categories including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), and more recently, diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC).¹ The characteristic pattern of attachment of EAEC to human epithelial tissue culture (HEp-2) cells includes prominent clustering of bacteria to cells and glass coverslip described as a stacked-brick configuration.

Enteroaggregative *E. coli* was first described in the early 1980s as a cause of traveler's diarrhea.² Its role as an enteropathogen has been confirmed by volunteer studies.³ A subsequent study found that the virulence of EAEC varied between strains. Volunteers ingesting EAEC strain 042 experienced more severe clinical symptoms than those given EAEC strain 17-2, JM221, or 34-b.⁴ Enteroaggregative *E. coli* strains have been shown to cause acute and persistent (lasting longer than 2 weeks) diarrhea in young children in developing countries.⁵ Enteroaggregative *E. coli* has also been reported as a cause of diarrhea in developed nations⁶ and in AIDS patients, particularly those with low CD4 counts.⁷

The reported clinical symptoms of EAEC infection vary from an acute syndrome of watery diarrhea to a more protracted form causing colicky abdominal cramps or at times dysentery.⁵ Gross blood was present in the stool in as many as 33% of cases in one study.⁵ The pathophysiology of enteric infections caused by EAEC strains has not been fully described. We have evaluated the degree of inflammation that accompanies EAEC infection by examining the markers of intestinal inflammation using a case-control study. If EAEC commonly produces an inflammatory response, antimicrobial therapy may be useful in managing this infection. According to an earlier study, antibiotics can shorten the duration of disease in U.S. travelers with EAEC diarrhea.⁸

MATERIALS AND METHODS

Patients. Stool specimens were collected from U.S. patients 18 years of age and older with traveler's diarrhea liv-

ing in Guadalajara, Mexico as students or from European tourists visiting Goa, India. Informed consent was obtained from all patients prior to enrollment. This study was approved by the Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston.

The study in Mexico was conducted in 1997 and 1998 from June to September; in Goa patients were recruited throughout 1998. All patients aged ≥ 18 years with acute traveler's diarrhea were eligible. Acute diarrhea was defined as the passage of more than two unformed stools in 24 hours and within 72 hours of onset of symptoms, accompanied by at least one other clinical symptom of enteric infection such as nausea, vomiting, abdominal cramps, tenesmus, or mucus or blood visible in the stool. The pretreatment stool specimen was examined for form and for the presence of mucus and gross blood. Samples were submitted to a field site laboratory where a test for fecal occult blood based on guaiac reagents (Hemocult[®], SmithKline Diagnostics, Inc., San Jose, CA) was performed and enteric parasitic and bacterial enteropathogens were sought using previously published methods.⁹ Three colonies of presumptively identified *E. coli* were chosen from a MacConkey agar plate for each stool sample, inoculated into peptone stabs, and taken to the Center for Infectious Diseases, University of Texas-Houston, for further analyses that including detection of toxin genes of ETEC¹⁰ and the HEp-2 cell adherence assay¹¹ to detect the patterns of adherence. An aliquot of stool from each patient was frozen and transported to Houston.

Cases and control groups. An analysis of markers of intestinal inflammation was conducted as a case control study with three study groups selected from the two patient pools. Cases were defined as patients with EAEC as the only recognized pathogen detected in diarrheal stool. We used two control groups: 1) patients with ETEC as the only pathogen found in their diarrheal stool sample and 2) patients with no identified pathogens in their stool. The group without pathogens detected includes a large number with occult ETEC infection.¹² We included patients in the case and control groups who fulfilled the criteria and who had a frozen stool sample available for lactoferrin assay. Eleven patients

TABLE 1

Summary of test results* of different markers of enteric inflammation in cases with enteroaggregative *Escherichia coli* (EAEC) and two control groups

	Gross mucus	Gross blood	Fecal markers of inflammation		
			Occult blood	WBC	Lactoferrin
Cases:					
EAEC (n = 45)	22 (48.9)	2 (4.4)	17 (37.8)	13 (28.9)	27 (60.0)
Control groups:					
EPEC (n = 56)	24 (42.9)	4 (7.1)	17 (30.4)	15 (26.8)	15 (26.8)
No pathogen (n = 126)	54 (42.9)	3 (2.4)	37 (29.4)	19 (15.1)	27 (21.4)
P-value†	0.766	0.310	0.571	0.063	<0.001

* Results are shown as no. (%).

EPEC = enterotoxigenic *E. coli*; WBC = white blood cells.

† P-value was calculated using χ^2 method.

with EAEC diarrhea were not included in the study because the size of the original fecal sample allowed for microbiologic studies only. These patients had similar general characteristics to the cases included in the study.

HEp-2 cell adherence assay. The three *E. coli* strains from each patient were tested for their HEp-2 cell adherence using a previously described tissue culture assay.¹¹ Briefly, bacteria were grown overnight at 35° C in trypticase soy broth with 1% D-mannose. Human epithelial tissue culture cells were grown in minimal essential medium (MEM) in 5% CO₂ at 35°C with antibiotics on 10-mm diameter glass coverslips in 24-well tissue culture plates until approximately 70% confluence. The tissue culture cells were then washed, and MEM containing 1% D-mannose was added. Bacterial suspension (25 mL) was added to each well and incubated at 35° C for 3 hr. The coverslips were washed 3 times in sterile phosphate-buffered saline, fixed with methanol, and stained with Giemsa. The coverslips were then mounted on slides and examined at $\times 1000$ under a light microscope. Enteroaggregative *E. coli* were defined as *E. coli* with diffuse or dense adherence to HEp-2 cells in a stacked-brick pattern.

Fecal leukocytes in stool. Fecal leukocytes were evaluated by smearing a thin layer of the fresh stool sample on a microscope slide, and staining it with trichrome stain reagents. Specimens were examined at $\times 1000$ under immersion oil. A specimen was considered positive if numerous leukocytes were seen on five or more fields. Previous study has shown that the fecal leukocyte test is best done within 24 hours of collection;¹³ therefore, we used only data from fresh samples processed within this time frame.

Fecal lactoferrin. We detected fecal lactoferrin in the frozen stools by the commercial Leuko-Test® kit (TechLab, Blacksburg, VA) following the manufacturer's instructions. A 1:50 dilution of the specimen in the diluent provided was used. The kit contains latex beads coated with antibodies against lactoferrin. The presence of lactoferrin is detected by a positive agglutination reaction of 2+ or more as defined by the manufacturer.

Statistical analysis. Statistical evaluations (prevalence rates, χ^2 , and P-values) were done using Minitab software (version 12, 1998) operating in a Windows environment. We regarded a two-tailed α of 0.05, or a P-value of < 0.05, as significant.

RESULTS

Among the 511 participants, we found EAEC in 129 (25.2%), EPEC in 156 (30.5%), and these were the sole pathogens in 64 (12.5%) and 90 (17.6%) respectively. We retained fecal samples in 45 (EAEC) and 56 (EPEC) cases, respectively, of the populations where these were the sole pathogens, and these samples were included in the study. We found no pathogens in 184 (36.0%) of the patients studied; of these, 126 had available stool samples and were included in the study.

Table 1 shows the results of the different tests of enteric inflammatory markers. In 2 (4.4%) of the EAEC-infected patients, gross blood was identified in stool. This was not significantly different from the two control groups. Gross fecal mucus was found in 22 (48.9%) of EAEC cases but was not statistically significant compared to controls. The finding of occult blood was not statistically significant for the presence of EAEC. The presence of a positive fecal lactoferrin test was statistically associated with EAEC diarrhea where 27 (60.0%) were positive compared with 15 (27.0%) for EPEC patients and 27 (21.0%) for the no pathogen group ($P < 0.001$).

Fecal lactoferrin as a marker of inflammation showed the strongest statistical significance in further analysis ($P < 0.001$), whereas fecal leukocytes as a marker was less strong and did not quite reach statistical significance ($P = 0.063$). Occult blood, gross blood, and gross mucus did not approach statistical significance ($P = 0.571$, $P = 0.310$, and $P = 0.766$, respectively). All combinations of markers of inflammation combined with fecal lactoferrin were statistically significant but did not improve the P-value. No other markers or combinations reached statistical significance in sub-analysis.

DISCUSSION

Recent studies suggest that EAEC strains are important causes of acute and persistent diarrhea. The organism mainly affects children from developing countries, international travelers to these regions, and persons of all ages with AIDS. Enteroaggregative *E. coli* can cause persistent diarrhea and may contribute to malnutrition.¹⁴

Diarrhea is usually classified as inflammatory or non-inflammatory. This separation has important therapeutic im-

plications. Inflammatory diarrheas may be associated with higher mortality and optimally may be studied by stool culture. Several markers of inflammation can be used to study inflammatory diarrhea including fecal leukocytes, occult blood in stool, and fecal lactoferrin.¹⁵ An extensive meta-analysis¹⁵ of the sensitivity and specificity of these different markers of enteric inflammation as a predictor of finding an invasive pathogen concluded that the best diagnostic accuracy function was fecal lactoferrin.

The common finding of fecal leukocytes and lactoferrin in stools of patients with EAEC diarrhea suggests that these patients have a diffuse colonic inflammatory process. *In vitro* studies have confirmed the preferential attachment of EAEC to colonic mucosa¹⁶ and T84 cells that resemble colonic crypt cells.¹⁷ The recognized mechanisms of mucosal damage in EAEC infection include heavy mucus formation,⁴ intimate cell adherence,¹⁸ secretion of toxins (EAST1 and Pet) in at least some strains,^{1,16} and possibly cellular invasion.¹⁹ Several studies have shown that EAEC strains adhere to both the small and large intestines with particularly higher numbers of bacteria adherent to colonic mucosa.¹⁶ In a study of children in Brazil,²⁰ with malnutrition and persistent diarrhea due to EAEC infection, fecal lactoferrin and proinflammatory cytokines IL-8 and IL-1 β were found to be elevated in stool samples. These findings are characteristic of invasive bacterial organisms and suggest the occurrence of intestinal inflammation. The degree of intestinal inflammation is probably lower than that seen with other invasive bacterial pathogens such as *Shigella*, *Salmonella*, and *Campylobacter*, since the presence of fecal leukocytes, a less sensitive test, was not significantly different. Based on results of the present study EAEC diarrhea should probably be considered an inflammatory process.

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