

Enteroaggregative *Escherichia coli* as a cause of persistent diarrhea: an experimental model using light microscopy

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Escherichia coli enteroagregativa como agente provocador de diarreia persistente: modelo experimental utilizando microscopia óptica de luz

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ABSTRACT

Objective: To examine the interactions of Enteroaggregative *Escherichia coli* strains with small and large intestinal mucosa, in order to detect potential alterations in both regions of the digestive tract.

Methods: Enteroaggregative *Escherichia coli* strains, isolated from stools of infants with persistent diarrhea and the prototype strain 042 (O44:H18), isolated from a child with diarrhea in Lima, Peru (positive control), were analysed by light microscopy after *in vitro* organ culture assay of ileal and colonic mucosa. The interactions between the different enteroaggregative *Escherichia coli* strains and the ileal and colonic mucosa were analysed.

Results: Light microscopy analysis suggested an association of enteroaggregative *Escherichia coli* strains with the epithelium, inducing alterations. These bacteria adhered to both small and large bowel mucosa. The enteroaggregative *Escherichia coli* strains induced alterations in those areas where they were directly interacting with the epithelium. In the ileum, some areas showed a secondary internalization.

Conclusions: The enteroaggregative *Escherichia coli* strains could cause persistent diarrhea inducing alterations in the small intestinal structures, where the digestive-absorptive functions take place. Inflammatory lesions observed

in colons could justify the colitis described in some children infected by enteroaggregative *Escherichia coli*.

Key-words: enteroaggregative *Escherichia coli*; persistent diarrhea; microscopy.

RESUMO

Objetivo: Avaliar interações de amostras de *Escherichia coli* enteroagregativa com tecido intestinal humano, a fim de documentar potenciais alterações em diferentes regiões do trato digestivo.

Métodos: Amostras de *Escherichia coli* enteroagregativa isoladas das fezes de crianças com diarreia persistente e a amostra protótipo 042, isolada de uma criança com diarreia em Lima, no Peru (controle positivo), foram analisadas por microscopia óptica de luz após semeadura em cultura de órgão *in vitro* de fragmentos de mucosa ileal e colônica. Foram analisadas as interações entre as diferentes cepas de *Escherichia coli* enteroagregativa e as mucosas ileal e colônica.

Resultados: A análise por microscopia óptica de luz indicou associação destes micro-organismos com o epitélio, provocando alterações. As cepas estudadas aderiram a ambas as regiões avaliadas (intestino delgado distal e grosso) e causaram alterações, especialmente naquelas áreas onde

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interagiram diretamente com o epitélio. No íleo, algumas regiões mostraram internalização secundária.

Conclusões: Esses agentes podem causar diarreia persistente por meio de alterações no intestino delgado, no qual ocorrem as funções digestivo-absortivas. As lesões inflamatórias descritas na mucosa colônica poderiam explicar a colite mostrada em algumas crianças infectadas por *Escherichia coli* enteroagregativa.

Palavras-chave: *Escherichia coli* agregativa; diarreia persistente; microscopia.

RESUMEN

Objetivo: Evaluar interacciones de muestras de *Escherichia coli* enteroagregativa (EAEC) con tejido intestinal humano, a fin de documentar potenciales alteraciones en distintas regiones del tracto digestivo (intestino delgado distal e intestino grueso) y definir, con eso, su rol en la persistencia del proceso diarreico.

Métodos: Muestras de EAEC aislada de las heces de niños con diarreia persistente y la muestra prototipo 042, aislada de un niño con diarreia en Lima, Perú (control positivo) fueron analizadas por microscopía óptica de luz (ML) después de siembra en cultura de órgano *in vitro* de fragmentos de mucosa ileal y del colon. Fueron analizadas las interacciones entre las distintas cepas de EAEC y las mucosas ileal y del colon.

Resultados: El análisis por ML indicó asociación de estos microorganismos con el epitélio, provocando alteraciones. Las cepas estudiadas adhirieron a ambas regiones evaluadas: intestino delgado distal y grueso y causaron alteraciones, especialmente en aquellas áreas donde interactuaron directamente con el epitélio. En el íleo, algunas regiones mostraron internalización secundaria.

Conclusión: Estos agentes pueden causar diarreia persistente mediante alteraciones en el intestino delgado, donde ocurren las funciones digestivo-absortivas. Las lesiones inflamatorias descritas en la mucosa del colon podrían explicar la colitis descrita en algunos niños infectados por EAEC.

Palabras-clave: *Escherichia coli* agregativa; diarreia persistente; *in vitro*; microscopía.

Introduction

Diarrheal illness still accounts for a substantial portion (20%) of under-five mortality worldwide⁽¹⁾. Persistent

diarrhea has a major impact on morbidity and mortality rates among pediatric populations in developing countries; over 50% of diarrhea-related deaths in these countries are associated with episodes of persistent disease⁽²⁾.

Injury of the small intestine plays a key role in the pathophysiology of persistent diarrhea⁽³⁾, but one must distinguish the enteropathy brought about by persistent bacterial colonization from the post-infectious enteropathy that follows failure or delay in regeneration of the intestinal mucosa⁽⁴⁾. Many studies have focused on characterizing the bowel injury that occurs in these conditions, identifying changes in absorption, secretion, and reabsorption of minerals, carbohydrates, protein, and fat due to chronic enteropathy⁽⁵⁾. The lesions described in children with persistent diarrhea appear to be due to a variety of factors, isolated or acting in tandem. These factors come together and lead to a protracted disease course and delayed mucosal recovery⁽³⁾. The pathogens isolated during persistent diarrhea are not always the same found during the acute stage of the disease, which suggests that secondary infection may play an important role in prolonging diarrheal episodes⁽⁶⁾. Delayed mucosal repair appears to be the key component of the process, and the available evidence suggests that other factors may also perpetuate injury. Enteropathogens cause diarrhea and injury of the intestinal villi and secrete toxins that act on enterocytes. Recovery from the diarrheal illness will not occur until damaged cells have been replaced. Changes that lead to delayed epithelial regeneration thus underlie the pathophysiology of protracted diarrheal illness⁽⁷⁾. On the other hand, these pathogens have been known to colonize the colonic mucosa, producing infectious colitis with bloody diarrhea⁽⁸⁾.

Certain *Escherichia coli* serotypes that display adherence properties in cell culture are of major importance as enteropathogenic agents. By definition, the enteroaggregative *Escherichia coli* (EAEC) group of bacteria comprises *E. coli* serotypes that adhere to cell lines (such as HeLa and HEp-2 cells) *in vitro*, forming bacterial aggregates on the cell surface and on cell-free regions of the cover slip, in clumps resembling piles of stacked bricks (aggregative adherence)⁽⁹⁾. This “pile of bricks” appearance is considered the *sine qua non* of typical aggregative adherence (AA) patterns⁽¹⁰⁾. The pathogenesis of EAEC infection is complex, and EAEC strains are markedly heterogeneous⁽¹¹⁾. Although several studies have stressed the association of these strains with the colonic mucosa, some authors have shown EAEC is capable of colonizing the small intestine⁽¹²⁾. Human and animal studies

have shown that EAEC interacts with the jejunal, ileal, and colonic epithelium^(13,14).

Several virulence factors have been identified in these agents, but the clinical implications of these factors have yet to be fully established⁽¹⁵⁾. Therefore, further research that assesses the interaction of EAEC with the bowel mucosa is required. The present study used light microscopy (LM) to assess the interaction between three EAEC strains, isolated from children with persistent diarrhea, and various regions of the intestinal tract (distal small bowel and large intestine), documenting potential changes in the bowel induced by these interactions in order to define their role in the persistence of diarrheal disease.

Methods

The EAEC samples used in this study (strains 071-1, 101-1, and 171-1) were isolated from stool cultures of children under the age of 6 months with persistent diarrhea (EAEC CASES). Prototype strain 042⁽¹⁰⁾, which has proven cytopathic effects in the bowel regions of interest, was used as a positive control.

EAEC strains were identified on the basis of characteristic patterns of aggregative adherence to HeLa and HEp-2 cells and through hybridization with the AA-specific gene probe pCVD 432, which detects genes associated with the capacity to manifest AA⁽¹⁶⁾. Somatic (O) and flagellar (H) antigen identification was performed by means of standard agglutination tests⁽¹⁷⁾. Nontypeable strains were given the identifier "NT".

The EAEC strains used in this study were the prototype strain 042 (O44:H18) and wild strains 71-1 (ONT:H33), 101-1 (ONT:H10), and 171-1 (ONT:H1).

Human intestine fragments were obtained from pediatric bowel resection surgeries and adult colonoscopies. In both cases, grossly normal ileal and/or colonic mucosal tissues were chosen. Adhesion assays were carried out as described by Knutton *et al*, *em* 1987⁽¹⁸⁾, with some modifications. Biopsy specimens were transported in modified organ culture medium (MOCM), adapted as described by Embaye *et al*⁽¹⁹⁾ and consisting of NCTC-135 medium (Sigma) with 2 mM L-glutamine (Sigma), MTT8 (FLOW) and newborn calf serum. Fragments kept in MOCM were placed in 4% paraformaldehyde for LM processing. EAEC strains were tested on three fragments from each bowel region of interest.

Biopsy fragments were placed, villi side up, on sterile filter paper (AP20; Millipore) on a 35 x 10 mm Petri dish (Corning). The level of MOCM containing 1% D-mannose was

adjusted so that a thin layer of culture medium covered the surface of the biopsy fragments. MOCM-treated fragments were washed and then fixated in 4% paraformaldehyde for 24 hours and dehydrated in a graded ethanol series (from 70%). Samples were then preinfiltrated with a 1:1 mixture of ethanol 100% and glycol methacrylate (GMA HistoResin, LKB) for 12 hours at room temperature, followed by pure HistoResin for 24 hours. Fragments were oriented villi side up with the aid of a stereo microscope and embedded in LKB HistoMolds, at room temperature, with a mixture of HistoResin and GMA. Blocks were then sliced at a thickness of 1.0 μ m in a Sorvall JB-4A ultramicrotome with a glass knife (EMS). Sections were stained with toluidine blue 1% for 4 minutes and then in a fuchsin stat stain (toluidine blue–basic fuchsin) for 30 seconds, and finally viewed under LM at 100, 200, 400, and 1000x magnification.

The study protocol was approved by the UNIFESP-EPM Research Ethics Committee. Written informed consent was obtained from adult patients or from the legal guardians of underage participants.

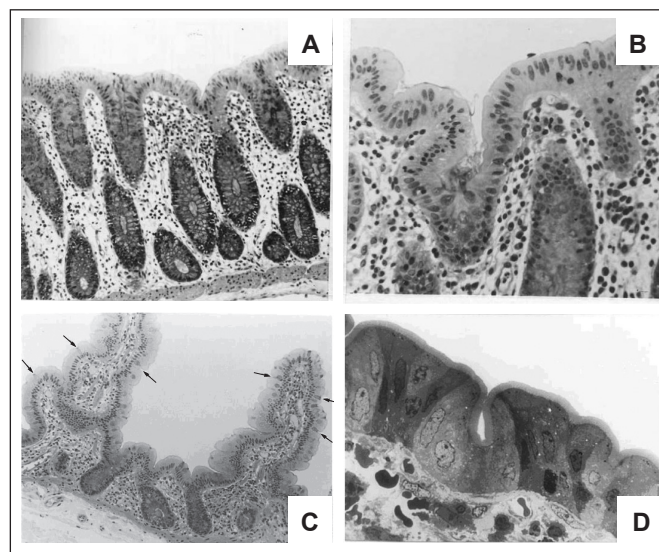


Fig. 1 - Photomicrographs of ileal and colonic fragments (control). A (105x) and B (210x): Normal colonic mucosa, featuring columnar epithelial cells (note nuclei at the basal end) and non-pathologic lymphoplasmacytic infiltration of the lamina propria; C (105x): Normal ileal mucosa, featuring finger-shaped villi, columnar epithelial cells (note nuclei at the basal end), goblet cells throughout the epithelium (\downarrow), and non-pathologic lymphoplasmacytic infiltration of the lamina propria; D (1050x): higher magnification showing intact brush border (microvilli) and columnar epithelial cells (note nuclei at the basal end).

Results

LM analysis of control colon fragments showed normal cell surface structure, with enterocytes in a typical palisade-like arrangement. Goblet cells and the brush border were intact (Figures 1A and 1B). Analysis of colonic mucosa fragments infected with the prototype 042 strain revealed bacteria in the intestinal lumen and in the mucus layer that covers the epithelium (Figure 2). Bacteria were also found in contact with the brush border (Figures 2B and D). Focal areas of epithelial detachment were visible (Figure 2A). In several tissue areas, cytoplasmic vacuolation was visible at the basal portion of enterocytes (Figures 2C and D).

Colonic mucosa fragments infected with the 171-1 strain showed bacterial aggregates in a “pile of bricks” pattern far from the epithelial surface, intermingled with inflammatory cells. A large number of goblet cells with areas of cytolysis, destruction, and extrusion were visible at the epithelium.

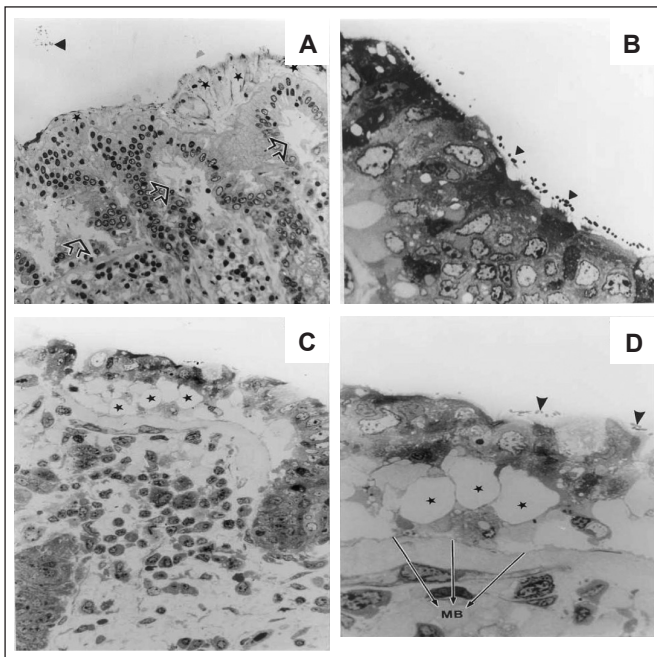


Fig. 2 - Photomicrographs of EAEC-infected colon specimens. A (210x) and B (1050x): Bowel fragment sections showing a thick layer of mucus (*) overlying the epithelial surface and bacterial clumps on the epithelial surface (▶). (⇔) denotes areas of epithelial detachment from the chorion. C (420x) and D (1050x): Semithin sections showing vacuolization of the basal region of enterocytes (*). D: MB denotes normal basement membrane, (*) denotes vacuoles in the basal region of enterocytes, and (▶) denotes bacterial clusters near the epithelial surface.

Sections of colonic mucosa infected with the 71-1 strain showed typical *E. coli* aggregates near the epithelium, which was detached from the chorion in several areas. Cell extrusion was also apparent. The brush border appeared normal in several regions, but microvilli were abnormal at sites of bacterial adhesion.

LM analysis of control ileal fragments (Figures 1C and 1D) showed intact tissue with normal villous architecture, enterocytes in a typical palisade arrangement, and a large number of goblet cells, with no visible abnormalities. The brush border appeared intact. No mucus or cell debris was seen on the epithelial surface of control specimens.

Analysis of ileal mucosa infected with prototype strain 042 showed villi with areas of epithelial detachment, including detachment from the chorion in some sites. Bacterial aggregates were visible near the microvilli on the detached epithelial surface and in areas of tissue destruction.

Analysis of the ileal mucosa infected with strain 171-1 revealed regions of complete epithelial detachment or destruction (Figure 3). Crypt remnants were visible (Figure 3A). Bacteria occurred in typical aggregates and, in some regions, in what appeared to be secondary invasion (Figure 3D).

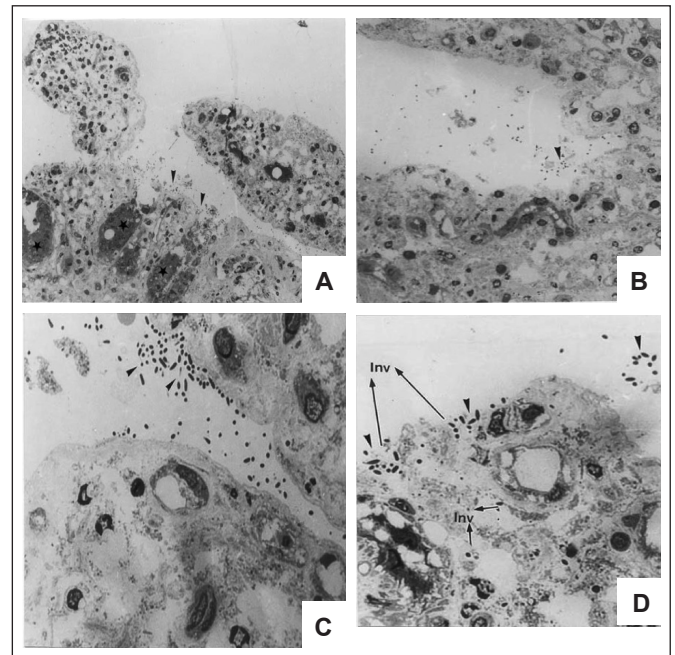


Fig. 3 - Photomicrographs of semithin sections of ileum infected with the 171-1 strain of EAEC. A (210x), B (420x), C (1050x), and D (1056x): Sites of apparent epithelial detachment or destruction, with disaggregated chorion and crypt remnants visible (▶). Bacteria arranged in the typical AA pattern, intermingled with cell debris (▶). D: Tissue invasion secondary to tissue destruction (Inv).

Strain 101-1 induced changes identical to those found with the other analyzed strains (71-1 and 171-1), in both bowel regions.

Discussion

Infection with EAEC strains is a major cause of diarrhea in developing countries⁽²⁰⁾. EAEC was recently identified as the number-one causative agent of diarrheal illness in Brazilian under-fives⁽²¹⁾, and is also commonly implicated in pediatric diarrhea in the United States⁽²²⁾.

Changes in the small intestine have been pinpointed as the determining factor of persistent diarrheal illness⁽³⁾. Evidence suggests that EAEC can colonize the small bowel and colonic mucosa, but this capacity varies from strain to strain^(12,13). *In vivo* analysis of EAEC in animal models⁽²³⁾ and *in vitro* cultures of isolated enterocytes from cell lines such as T84, Caco2, HT29, HeLa, and HEp-2⁽²⁴⁾ or bowel fragments^(12,13) provides a plausible explanation for the finding that infection with this virotype tends to prolong the course of diarrheal illness. However, the mechanisms underlying the cell damage wrought by EAEC have yet to be fully elucidated.

Some studies assessing the interaction between de EAEC strains and the human bowel, using intestinal fragments from both children and adults⁽²⁵⁾, found no difference in the degree of adherence. All three strains assessed in the present study colonized the small and large intestine alike, inducing major cytotoxic changes in both regions. Changes found in the ileal mucosa could account for prolonged diarrheal illness. Several hypotheses have been proposed in an attempt to explain protracted damage to the intestinal mucosa. *In vivo*, malabsorption and the presence of microbial antigens are believed to trigger an immune response that leads to sustained mucosal injury⁽⁷⁾. On the other hand, the lack of defense mechanisms *in vitro* could predispose to the development of cytotoxic changes in these experiments.

In the present study, EAEC formed characteristic, “pile-of-bricks”-like clumps in both bowel regions studied. The affinity of this virotype for mucus-rich areas of the digestive tract was confirmed. Substantial mucus secretion was observed in EAEC-infected fragments of colonic and ileal tissue. These bowel fragments exhibited total or partial destruction of villi, vacuolization of the basal cytoplasm of enterocytes, epithelial detachment, and a complete breakdown of normal tissue structure, including loss of epithelial cells. Some areas featured total destruction of the epithelium, with a complete absence of microvilli. These lesions corroborate the findings

of Vial *et al*, who found areas of substantial tissue destruction in in rat and rabbit bowel loops, suggesting more intense cytotoxic effects in the distal rather than the proximal small intestine⁽²⁶⁾. Hicks *et al* found that EAEC strains interacted with the jejunal, ileal, and colonic mucosa⁽¹³⁾. Similar results were reported by Nataro *et al*, who studied the effects of prototype strain 042 on T84 cells. The authors showed that cells infected with this strain exhibited marked signs of toxicity, particularly in areas to which bacteria were adhered⁽⁵³⁾. In colon specimens, morphological changes were detected that would account for the presence of mucus and blood in the stool of patients with persistent diarrhea due to EAEC infection. LM assessment showed clumps of bacteria arranged in a “pile of bricks” pattern, amid inflammatory and epithelial cells, in samples infected with all analyzed strains (Figures 2A, 2B, and 2C). Near the epithelium, large amounts of goblet cells with areas of cytolysis, cell destruction and extrusion were visible, as were areas in which bacteria appeared closer to abnormal microvilli. Nevertheless, the brush border was intact in substantial portions of each tissue sample. Ileal fragments contaminated with the 171-1 strain showed extensive destruction of the epithelial surface, suggesting cytotoxic action. Strain 171-1 was found to be invasive; infected tissues were completely disaggregated and shorn of their epithelium. With the same duration of incubation (6 hours), prototype strain 042 produced apparently milder, though still significant, changes in ileal tissue. LM showed several areas of epithelial detachment. The lesions found on microscopy might have been due to processing artifacts, but no similar changes were found in control specimens. The clinical manifestations of EAEC diarrhea are believed to vary on a case-by-case basis, depending on several host genetic factors. The genotypic distribution of interleukin-8 (IL-8) in symptomatic and asymptomatic patients is being studied; investigators have suggested that differences in this factor may define population groups with a higher or lower susceptibility to EAEC infection⁽²⁸⁾.

The normal architecture of the brush border was preserved in most of the analyzed tissue. As noted elsewhere in the literature, EAEC strains have greater affinity for mucus-rich regions⁽³⁵⁾. Mucus production in infected fragments from both study regions was higher than in control fragments.

The presence of bacteria within the ileal cells could lead to increased exposure to toxins or other injury-causing substances and, consequently, to malabsorption, favoring penetration by bacterial and dietary antigens. These antigens might then trigger immune responses that would perpetuate mucosal injury and, consequently, persistent diarrhea.

The available data are insufficient to provide a complete understanding of the pathophysiology of EAEC infection, but several hypotheses have been suggested. A three-stage model for the pathogenesis of EAEC has been proposed: 1) bacteria adhere to the intestinal mucosa and/or mucus layer. Fimbriae are most likely the facilitators of this initial colonization event; 2) mucus production increases, creating a biofilm to which bacteria then adhere. The mucus layer could thus be responsible for persistent bacterial colonization and, perhaps, malabsorption; 3) cytotoxins are produced and cause injury of intestinal cells, inducing a local inflammatory response with cytotoxic mucosal injury and intestinal secretion. In addition, malnourished hosts may be at a particular disadvantage in terms of their capacity to recover from injury induced by EAEC, which would predispose this population to protracted diarrheal illness⁽¹⁾.

The gastrointestinal disturbances associated with EAEC infection appear to be due to a complex host–pathogen

interaction, involving factors such as the genetic susceptibility of the host, the heterogeneity of EAEC virulence factors, and the number of bacteria present.

In conclusion, this study examined the interaction between three EAEC strains and human intestinal mucosa samples and confirmed that this virotype is capable of colonizing the small and large bowel. EAEC strains produce changes in the absorptive epithelium of the small intestine, which may lead to persistent diarrheal illness. The inflammatory lesions found in samples of colonic mucosa infected with EAEC may explain the colitis that has been described in some children with EAEC disease⁽³⁰⁾.

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