ADHERENCE OF ENTEROAGGREGATIVE *Escherichia coli* TO THE ILEAL AND COLONIC MUCOSA: an in vitro study utilizing the scanning electron microscopy

Jacy Alves Braga de **ANDRADE**[1], Edna **FREYMÜLLER**[2] and Ulysses **FAGUNDES-NETO**[1]

ABSTRACT - ***Context*** - Enteroaggregative *Escherichia coli* strains have been associated with persistent diarrhea in several developing countries. In vivo procedures with animal models, in vitro assays with cellular lines and in vitro organ culture with intestinal fragments have been utilized to study these bacteria and their pathogenicity. ***Objective*** - The present experimental research assessed the pathogenic interactions of three enteroaggregative *Escherichia coli* strains, using the in vitro organ culture, in order to show the adherence to different regions of both, the ileal and the colonic mucosa and demonstrate possible mechanisms that could have the participation in the prolongation of diarrheiogenic process. ***Methods*** - This study used intestinal fragments from terminal ileum and colon that were excised from pediatric patients undergoing intestinal surgeries and from adult patients that underwent to colonoscopic procedures. Each strain was tested with three intestinal fragments for each region. Tissue was fixed for scanning electron microscopic analysis. ***Results*** - These bacteria colonized ileal and colonic mucosa in the typical stacked-brick configuration in the ileum and colon. In both regions, the strains were seen over a great amount of mucus and sometimes over the intact epithelium. In some regions, there is a probable evidence of effacement of the microvilli. It was possible to see adhered to the intestinal surface, bacteria fimbrial structures that could be responsible for the adherence process. ***Conclusion*** - In order to cause diarrhea, enteroaggregative *Escherichia coli* strains adhere to the intestinal mucosa, create a mucoid biofilm on the small bowel surface that could justify the digestive-absorptive abnormalities and consequently, prolonging the diarrhea.

HEADINGS - *Escherichia coli*. Diarrhea. Microscopy, electron, scanning. Organ culture techniques.

INTRODUCTION

Diarrheal disorders in childhood account for a large proportion (20%) of children deaths with an estimated 2.2 million deaths yearly[9]. Persistent diarrhea has high impact on infantile morbidity and mortality rates in developing countries and more than 50% of the deaths due to diarrhea are associated to persistent episodes[23, 35, 53].

Small intestinal mucosa injury has been incriminated as a central mechanism in the persistence of diarrhea[6, 19]. However it is important to discriminate between the persistence of the infection that leads to an enteropathy and a post infectious enteropathy that fails to heal[31]. Persistent diarrhea is often manifested by a chronic enteropathy, with impaired mucosal healing and diminished digestive and absorptive capacity[6, 19, 33]. The majority of the studies have focused on the characterization of injury, identifying changes in the digestive-absorptive process, secretory and reabsorptive capacities for minerals, carbohydrates, protein and fats[6, 7]. The small bowel lesions described in infants with persistent diarrhea may be caused by several noxious factors, acting separately or in an associated pattern, namely nutritional deficiencies, direct action of some enteropathogenic agents on the enterocyte, milk or food intolerance as well as prior to antibiotic therapy[19, 26, 34, 55].

Several studies have demonstrated different degrees of alterations in the small bowel mucosa[19, 20, 47, 48, 49] due to persistent diarrhea. Deficient intestinal repair is regarded as a key component of abnormal mucosal morphology[8]. However the exact factors underlying ineffective repair processes and continuing injury are poorly understood. The end result of mucosal derangement is malabsorption of luminal nutrients and increased permeability of the gut to dietary and microbial antigens[6].

Division of Pediatric Gastroenterology and the Center of Electron Microscopy of the Universidade Federal de São Paulo - Escola Paulista de Medicina (UNIFESP-EPM), São Paulo, SP, Brazil
[1] Division of Pediatric Gastroenterology, UNIFESP-EPM; [2] Center of Electron Microscopy of the UNIFESP-EPM, São Paulo, SP, Brazil.
Correspondence: Prof. Ulysses Fagundes-Neto - Avenida Conselheiro Rodrigues Alves, 1239 - Vila Mariana - 04014-012 - São Paulo, SP, Brasil. E-mail: ufagundesneto@uol.com.br

Enteric pathogens commonly cause illness by damaging the epithelial intestinal cells and/or secreting toxins, which bind to the enterocytes.

Some *Escherichia coli* serotypes presenting diverse adherence properties in tissue culture assays have been reported as important enteropathogens. One of these types of enteropathogenic properties called enteroaggregative is characterized by the ability of some *Escherichia coli* strains (EAEC) to produce an aggregative pattern of adherence (AA) in cultured HEp-2 cell monolayers[37]. AA is identified by the presence of prominent bacterial autoagglutination on the cells' surfaces as well as on the glass coverslip free from cells. The main characteristic of AA, however, is the layering of bacteria, best described as a stacked-brick configuration.

The pathogenesis of EAEC is complex and EAEC strains are very heterogeneous[16]. Human and animal studies indicate that these bacteria are able to bind to jejunal, ileal and colonic epithelium[5, 15, 28, 29, 30, 42, 44, 57, 58].

After epidemiologic evidences of association between EAEC infection and diarrhea[40], efforts have been made to explain the interaction of these agents with the intestinal mucosa. Numerous putative virulence factors have been identified, but the clinical implication of these factors remains unclear[4, 18, 36, 44]. In order to cause diarrhea, EAEC needs to adhere to the intestinal epithelial cells, form a mucoid biofilm and induce toxic effects on the small bowel. More investigations on the interactions of EAEC with the intestinal mucosa are desirable.

The present study was designed to investigate the interaction of three EAEC strains, isolated from infants with persistent diarrhea with ileal and colonic mucosa utilizing the scanning electron microscopy.

METHODS

Bacterial strains and culture conditions

EAEC strains: 171-1(ONT: H1), 101-1(ONT: H1) and 71-1(ONT: H33), previously isolated from stool specimens of hospitalized infants with persistent diarrhea in São Paulo, Brazil, were selected for this study. These strains were characterized as EAEC based on the production of the AA pattern in HEp-2 cells adhesion assay[37] and EAEC probe hybridization[4].

As a positive control, the prototype strain 042 (O44: H18)[39] was used to infect the ileal and colonic fragments.

As a negative control, non-infected intestinal fragments were used.

Bacterial strains stored at room temperature in nutrient agar were routinely grown on tryptic soy broth, in a period of 18 hours at 37°C.

In vitro organ culture adhesion assay

Intestinal fragments were obtained from terminal ileum and colon that were excised from pediatric patients that underwent abdominal surgeries and from adult patients that underwent colonoscopic procedure. In both cases, macroscopically normal tissues were selected. The adhesion assays were performed as described by Knutton et al.[32] with some modifications, as follows: intestinal fragments obtained by biopsies were transported to the laboratory, after being placed in modified organ culture medium (MOCM) adapted from that described by Embaye et al.[17] consisting of NCTC-135 (SIGMA) with 2 mm of L-glutamin (SIGMA), MTT8 (FLOW) and new born calf serum. Some fragments were immediately fixed to microscopic evaluation (uncultured controls). Fragments were fixed in modified Karnovsky (mk) for analysis by scanning electron microscopy (SEM). EAEC strains were tested with three intestinal fragments of each intestinal region. Three biopsy samples were placed with their villi tip in the upper position on sterile filters (AP 20; Millipore) in a plastic Petri dish (35 × 10 mm; Corning). The level of MOCM containing 1.0% D-mannose was adjusted to cover the biopsy specimens with a thin film of medium by capillary action. For adhesion studies, 60 µL of a bacterial broth culture (overnight cultures at 37°C) was added. Fragments of biopsy specimens were incubated with tissue culture medium without the addition of bacteria as negative controls.

Petri dishes were then incubated at 37°C in a 95% O_2-5% CO_2 atmosphere, for 2 hours. Then, biopsy samples were washed 4 times with sterile phosphate buffered saline and incubated with 2 mL of MOCM containing 1.0% D-mannose for additional 2, 4 and 6 hours at 37°C. The culture medium was changed completely every 2 hours to maintain pH and nutrient levels, without reinoculation with bacterial culture. After these incubation periods, biopsy samples were washed and prepared for SEM.

Tissue processing

For SEM, the intestinal fragments were fixed in mk and washed in 0.1M cacodylate solution and then dehydrated. After, biopsies were dried in a CPD 030 critical point dryer. Subsequently specimens were attached to 0.5 - inch aluminum stubs and coated with silver colloid (Silver Print). Later, they received a thin layer of gold (sputtering method) to become conductors. The observations were performed in SEM (JEOL JSM – 5300) in 10 Kv.

Ethical Considerations

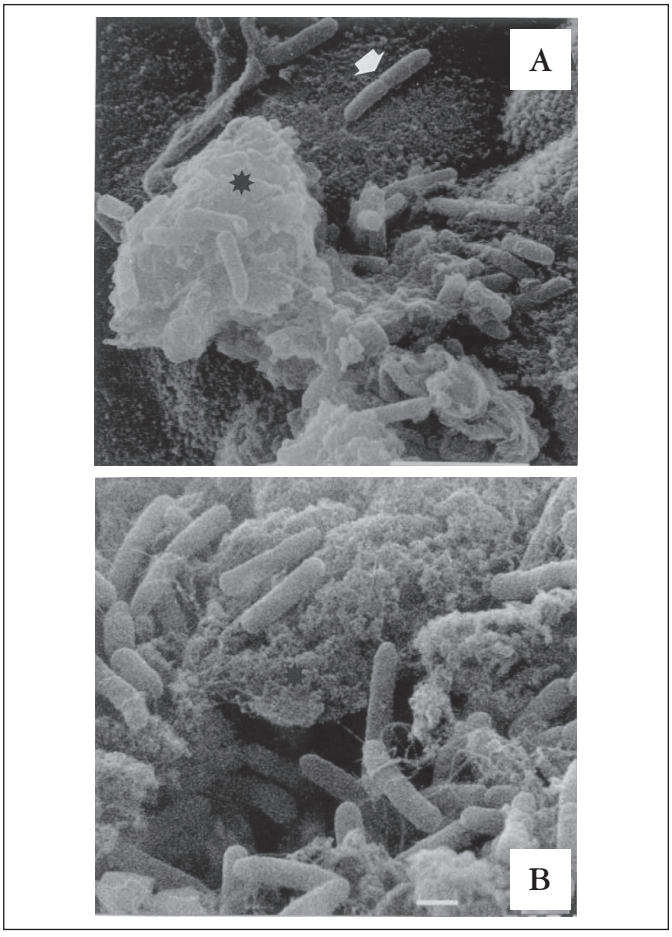
This study protocol was approved by the Universidade Federal de São Paulo — Escola Paulista de Medicina (UNIFESP-EPM) Ethical Commitee for Human Experimentation. Informed written consent was obtained from adults and parents of each participating infant.

RESULTS

Adhesion to ileal fragments

Ileal fragments that were excised from children and from adults showed the same pattern of lesions. Analysis by SEM of the intestinal fragments infected with 171-1 strains revealed bacterial aggregates, with a stacked brick pattern similar to that seen on HEp-2 cells[39] adhering to a layer of mucus overlying the mucosa and inducing effacement of the microvilli (Figures 1A and 1B). In some specimens of tissue the bacterial aggregates were also seen adhering directly to the epithelium (Figure 2). Non-characterized fimbrial structures

on bacterial surface were observed, apparently mediating bacteria-bacteria and bacteria-cell interactions (Figures 3A and 3B). Negative controls showed apparently intact microvilli and lack of adherent bacteria on the mucosal surface (Figure 4). The different strains showed the same pattern of adherence as well as the 042 strain (positive control).



FIGURES 1A. (5200X; bar- 1 µm) and 1**B.** (7500X; bar- 1 µm). Scanning electronic microscopy of enteroaggregative *Escherichia coli* infected ileal mucosa. Bacterial aggregates in a stacked brick pattern adhering to a layer of mucus overlying the mucosa are seen (*). In some areas, there is a probable evidence of effacement of the microvilli

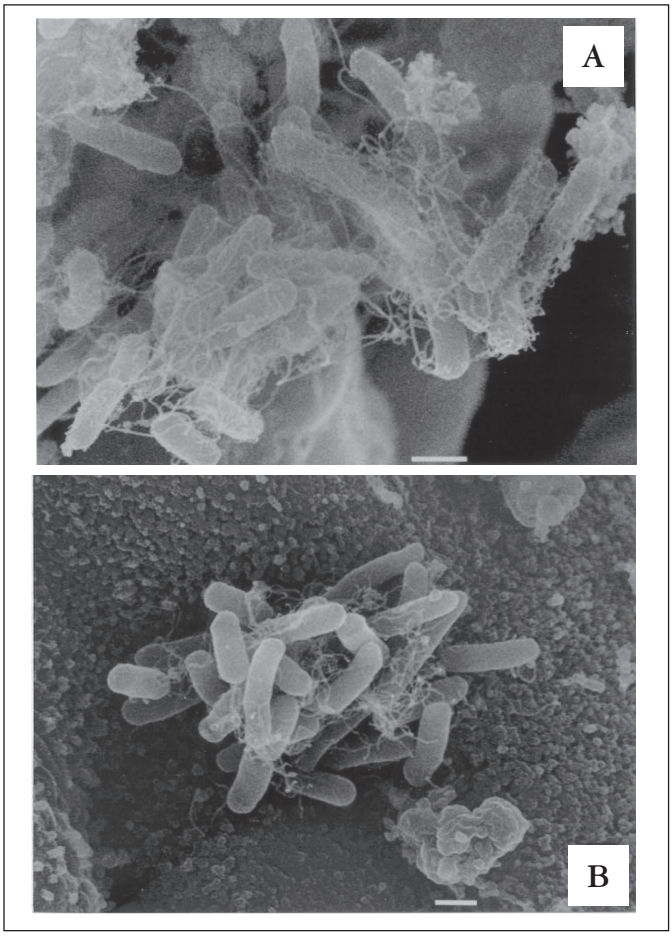


FIGURE 3. Scanning electronic microscopy of enteroaggregative *Escherichia coli* strains overlying the mucus **(A)** and directly over the epithelium **(B).** It is possible to observe fimbrial structures over the epithelial surface (A – 10000X; bar- 1 µm) e (B-7000X; bar- 1 µm)

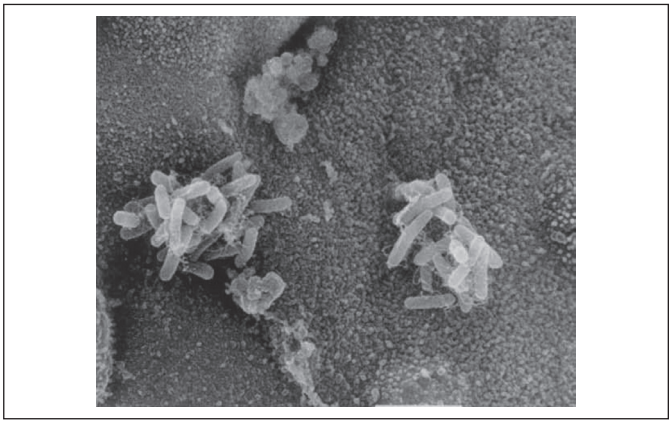


FIGURE 2. Scanning electronic microscopy of enteroaggregative *Escherichia coli* infected ileal mucosa. Bacterial aggregates in a stacked - brick pattern with a probable evidence of effacement of the microvilli are seen. (3800X; bar- 5 µm)

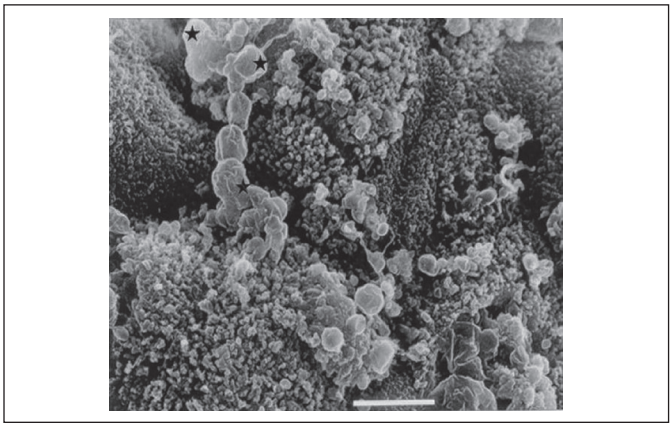


FIGURE 4. Scanning electronic microscopy of intact ileal mucosa with mucus over the preserved brush border (★- mucus layer). (3600X; bar - 5 µm)

Adhesion to colonic fragments

Colonic fragments that were excised from children and from adults showed the same pattern of lesions. In the infected fragments of the colonic mucosa bacteria were seen over a large amount of mucus and in some other areas, bacteria were seen in contact directly with the intact mucosa (Figures 5A, 5B and 5C). Analysis of negative controls showed intact epithelium (Figure 6). The positive control showed the same aspects that were observed with the wild strains.

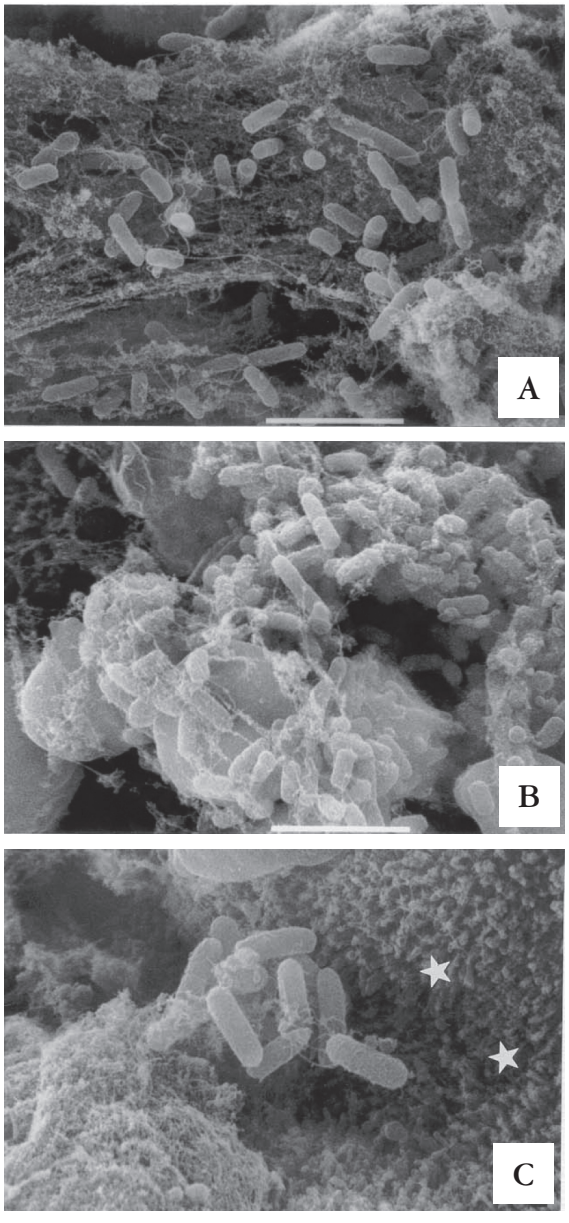


FIGURE 5. (Scanning electronic microscopy) of enteroaggregative *Escherichia coli* (EAEC) in stacked - brick pattern on infected colonic mucosa. EAEC strains over a thick mucus layer (A/B/C) and directly over the intact epithelium (C). Intact brush border is shown (★). A (5400X, bar - 5 μm) / B (5400X, bar - 5 μm) / C (7000X, bar - 5 μm)

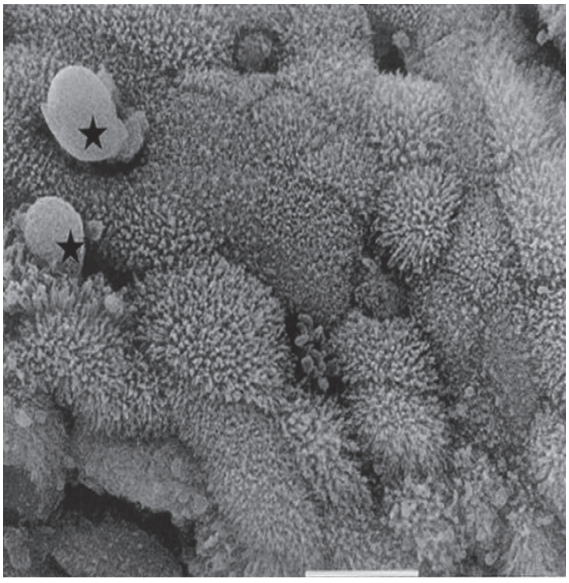


FIGURE 6. Scanning electronic microscopy of intact colonic mucosa with mucus (★) over the preserved brush border (2000X; bar - 10 μm)

DISCUSSION

EAEC infection represents an important cause of diarrhea in developing countries[2, 3, 8, 12, 21, 22, 25, 41, 45]. A Brazilian study identified EAEC infection as the most common cause of bacterial diarrhea in infants[2]. In United States, EAEC has also been reported as a common pathogen in children with diarrhea[24].

The analysis of EAEC strains in several models, such as in vivo assays in animal models[50, 51, 52] and in vitro organ culture (IVOC) with isolated enterocytes[42] or cellular lines like T84, Caco 2, HT29, HeLa e Hep-2[1, 11, 27, 54] and with intestinal fragments[33] have shown that a plausible explanation for the persistent nature of EAEC disease involves intestinal mucosal damage. However, the mechanism of this mucosal damage is not fully understood.

There are evidences that EAEC can colonize both, the small bowel and the colonic mucosa, but there may be variations among the different EAEC strains[28, 29, 30, 57, 58].

Hicks et al.[30] examined the interaction between EAEC and the human intestine using the IVOC model utilizing intestinal mucosa biopsies obtained from infants with diarrhea. They used two prototype strains (17-2 and JM221) that adhered to jejunal, ileal and colonic mucosa. Their wild type strains showed a variation in adhesion location: two adhered to all intestinal levels, one adhered to jejunum and ileum, another adhered to the ileum only and the last one adhered to both. Most bacteria were associated with the mucus layer above the intestinal mucosa and few of them were found in close association with the mucosal surface. EAEC adhered in large groups which obscured the view of the underlying epithelial surface. In the present study the three wild strains adhered to both, the ileal and colonic mucosa inducing the same pattern

of mucosal injury. We have shown that wild EAEC strains adhered to both evaluated regions over a thick mucus layer and sometimes directly to the mucosa. In some regions, there is a probable evidence of effacement of the microvilli (Figure 1).

In all cases in which EAEC strains adhered to the mucosa in significant numbers, they did so in aggregates with a stacked brick pattern similar to that seen on HEp-2 cells in culture.

Hicks et al.[30] suggested that these bacteria must penetrate the mucus layer to reach the epithelium. The IVOC model allows a direct access to the mucosa, and it indicates that this large production of mucus that was observed in the infected fragments by EAEC strains could be an inflammatory response to infection, since that this excessive amount of mucus production was not observed in control fragments. Wanke et al.[56] demonstrated this great affinity of these bacteria to the biofilm that covers the epithelium suggesting that this adherence to the mucus seems to be an important role in the bacterial colonization.

Several aspects of EAEC mucosal adherence have been elucidated. It was described the related aggregative adherence fimbriae I, II and III (AAF/I, AAF/II and AAF III), which are encoded on ≈ 60 mda virulence plasmids called pAA[5, 14, 15, 38]. Other investigators have described in outer membrane of proteins that may contribute to adherence as well[13, 15]. Thus, the full understanding mechanism of EAEC adhesins has yet to be defined. The production of mucus was confirmed by Sheikh et al.[46] that suggested that the EAEC biofilm is distinct from the biofilms described previously for non-pathogenic *E. coli* and has features that distinguish it from well-characterized biofilms formed by other bacteria. They used an in vitro model of EAEC biofilm formation and found that the defining property of EAEC, aggregative adherence, appears to represent the early stages of biofilm formation in rich growth medium. This mucus layer has unique aspects and is dependent on the AAF or functionally similar structures. Further analysis of this system may yield other important observations concerning adherence and colonization by EAEC.

In the present study, it was observed increased mucus discharge in both evaluated regions that were infected by these strains.

The fimbrial structures that were shown binding bacteria – bacteria and bacteria – epithelium could be involved with other virulence factors that must be better studied. Immunohistochemical analyses would be necessary for a better understanding of these interactions.

In conclusion, this study, which examined the interaction of three EAEC strains with human intestinal tissues, has confirmed that EAEC colonizes both the small bowel and the colonic mucosa. EAEC infection may lead to damage to the absorptive epithelium of the small bowel, resulting in perpetuation of diarrhea. In the colonic mucosa the inflammatory lesions described in the present study could explain the colitis that had been reported in children suffering from EAEC infection[10, 43].

Andrade JAB, Freymüller E, Fagundes-Neto U. Aderência da *Escherichia coli* enteroagregativa a mucosas ileal e colônica: estudo in vitro utilizando microscopia eletrônica de varredura. Arq Gastroenterol. 2011;48(3):199-204.

RESUMO - ***Contexto*** - Cepas de *Escherichia coli* enteroagregativa têm sido associadas à diarreia persistente em vários países em desenvolvimento. Procedimentos in vivo com modelos animais, cultura de órgão in vitro com fragmentos intestinais e ensaios in vitro com linhas celulares têm sido utilizados para estudar essas bactérias e a sua patogenicidade. ***Objetivo*** - A presente investigação experimental avaliou as interações patogênicas de três cepas de *Escherichia coli* enteroagregativa, usando cultura de órgão in vitro, para mostrar a aderência a diferentes regiões do intestino: íleo e cólons e demonstrar possíveis mecanismos que poderiam ter participação na perpetuação do processo diarréico. ***Métodos*** - Este estudo usou fragmentos de íleo terminal e cólon que foram retirados de pacientes pediátricos submetidos a cirurgias intestinais e de pacientes adultos que foram submetidos a colonoscopias. Cada cepa foi testada com três fragmentos intestinais para cada região. O tecido foi fixado para análise sob microscopia eletrônica de varredura. ***Resultados*** - Estas bactérias colonizaram mucosa ileal e colônica na configuração típica de pilhas de tijolos. Em ambas as regiões, as bactérias foram vistas sobre grande quantidade de muco e, às vezes, sobre o epitélio intacto. Em algumas áreas, há evidência de provável achatamento de vilosidades. Foi possível ver sobre a superfície intestinal, estruturas fimbriais bacterianas que poderiam estar relacionadas ao processo de adesão. ***Conclusões*** - Para causar diarreia, cepas de *Escherichia coli* enteroagregativa aderem à mucosa intestinal e criam um biofilme de muco sobre a superfície do intestino delgado, o que poderia justificar as anormalidades digestivo-absortivas e, por conseguinte, prolongar a diarreia.

DESCRITORES - *Escherichia coli*. Diarreia. Microscopia eletrônica de varredura. Técnicas de cultura de órgãos.

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Received 22/11/2010.
Accepted 19/1/2011.