SUSCEPTIBILITY OF NEWBORN MICE TO INTESTINAL COLONIZATION BY ENTEROPATHOGENIC *Escherichia coli* ISOLATED FROM MILK OF BOVINE WITH MASTITIS

(SENSIBILIDADE DE CAMUNDONGOS RECÉM-NASCIDO À COLONIZAÇÃO INTESTINAL POR ESCHERICHIA COLI ISOLADAS DE LEITE DE BOVINOS COM MASTITE)

(SENSIBILIDAD DE RATONES RECIÉN NACIDOS A LA COLONIZACIÓN INTESTINAL POR ESCHERICHIA AISLADAS DE LECHE DE BOVINOS COM MASTITIS)

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SUMMARY

The aim of this study was to improve the understanding of the mouse model for colonization by enteropathogenic *Escherichia coli* (EPEC). An inoculum of 10^8 CFU of three different types of *E. coli* strains: (i) a EPEC strain 3111-90 isolated from human infantile diarrhea (*eae*+, EAF +); (ii) the EPEC-like strains 537-1 and 263 (*eae*+ EAF +) isolated from milk of bovine with mastitis; (iii) the strains 304-3 and 988-2 without the intimin gene (*eae*-, EAF +) isolated from milk of bovine with mastitis was independently administered to three twenty-days-old male albino Swiss mice. The epithelial cells from the intestinal mucosa of mice colonized with the strains 263 and 988-2, examined by light microscopy showed a mild damage in the mucosa with breaks in the superficial stratum, irregularly shaped epithelial cells and an infiltration of inflammatory cells.

KEYWORDS: *E. coli*. EPEC. Experimental mouse model. Histopathological lesions.

RESUMO

O objetivo deste estudo foi melhorar o entendimento do modelo de colonização intestinal em camundongo por *Escherichia coli* enteropatogênica (EPEC). Um inóculo de 10^8 Unidades Formadoras de Colônia (UFC) de três linhagens diferentes de *E. coli*: (i) uma linhagem EPEC 3111-90 isolada de diarréia humana infantil (*eae*+, EAF +); (ii) as linhagens EPEC-like 537-1 e 263 (*eae*+ EAF +) isoladas de leite de bovinos com mastite; (iii) as linhagens 304-3 e 988-2 sem a presença do gene da intimina (*eae*-, EAF +) isoladas de leite de bovino com mastite, foram independentemente administradas a três camundongos Suíço albino, macho, de vinte e oito dias de idade. As células epiteliais da mucosa intestinal dos camundongos colonizadas com as linhagens 263 e 988-2, examinadas por microscopia de luz, mostraram lesões suaves na mucosa com a quebra da camada celular superficial da mucosa, aparecimento de células epiteliais com contornos irregulares e infiltração de células inflamatórias.

PALAVRAS–CHAVE*: E. coli*. EPEC. Modelo experimental em camundongo. Lesões histopatológicas.

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RESUMEN

El objetivo de este estudio fue mejorar el entendimiento del modelo de colonización intestinal en ratones por Escherichia coli enteropatógena (EPEC). Un inóculo de 108 Unidades Formadoras de Colonias (UFC) de tres linajes diferentes de E. coli: (i) un linaje EPC 3111-90 aislado de diarrea humana infantil (eae+, EAF +); (ii) los linajes EPC-like 537-1 y 263 (eae+ EAF +) aislados de leche de vacas com mastitis; (iii) los linajes 304-3 y 988-2 sin la presencia del gen de la intimina (eae-, EAF +), aislados de leche de vacas con mastitis, fueron independientemente administrados a tres ratones Suizos Albinos, machos, de veintiocho días de edad. Las células epiteliales de la mucosa intestinal de los ratones colonizadas con los linajes 263 y 988-2, examinados por microscopia de luz, mostraron lesiones leves en la mucosa con ruptura de la capa celular superficial de la mucosa, aparecimiento de células epiteliales con contornos irregulares e infiltración de células inflamatorias.

PALABRAS-CLAVE: E. coli, EPEC, modelo experimental en ratones, lesiones histopatológicas.

RESUMEN

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INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of infantile diarrhea in developing countries. In industrialized countries, the frequency of these organisms has decreased, but they continue to be an important cause of diarrhea (NATARO & KAPER, 1998). The pivotal mechanism of EPEC pathogenesis is a lesion called attaching and effacing (A/E), characterized by an intimate attachment of the bacterium to the host cell, the effacement of enterocyte microvilli and formation of pedestal-like structures (KNUTTON et al., 1989). The genetic determinants for the production of A/E lesions are located on the locus of enterocyte effacement (LEE) (McDANIEL et al., 1995), a pathogenicity island that contains the gene encoding intimin, a 94-KDa outer membrane protein encoded by the *eae* gene, which is responsible for the intimate adherence of bacteria to enterocyte membranes (NATARO & KAPER, 1998). The strains presenting this kind of lesion have been regrouped under the name of attaching/effacing *E. coli* (AEEC); Adu-Bobie et al. (1998) identified five variants of the *eae* gene classified as α, β, γ, δ and ε.

Many EPEC strains produce a characteristic adherence pattern, called localized adherence, in tissue culture cells (SCALETSKY et al., 1984). This phenomenon is associated with the presence of a large EPEC adherence factor (EAF) plasmid, which carries the so-called EAF sequence (NATARO & KAPER, 1998). The EAF plasmid is not essential for the formation of A/E lesions, although its presence enhances the efficiency of the process (NATARO & KAPER, 1998).

Although EPEC is a frequent cause of disease in humans, it has been difficult to establish experimental infections by these organisms in animals. The most successful results have so far been obtained by feeding cultures to mice treated with antibacterial drugs prior to the test in order to suppress their indigenous intestinal flora (ASBBURNER & MUSHIN, 1962, SHIMIZU et al., 2003). However, this treatment eliminates the competitive balance between the animal's indigenous flora and pathogenic bacteria. The present study describes a experimental model for intestine colonization by *E. coli* using newborn mice.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Five EPEC strains were used: strain 1, a standard EPEC strain CDC 3111-90 *eae*+, EAF+- human origin;

strain 2, an isolate 537-1 of EPEC-like strain *eae*+, EAF+; strain 3, an isolate of 263 EPEC-like strain *eae*+, EAF+; strain 4, an isolate 304-3 *eae*-, EAF+; strain 5, an isolate 988-2 *eae*-, EAF+, all isolates had been obtained from bovine mastitic milk in another work (CORREA & MARIN, 2002), and had been kept in the Laboratory of Genetics, FORP/USP. Luria broth (L broth) and Luria agar (L agar) were used for routine culturing of bacteria and when necessary McConkey agar (Difco Laboratories, Detroit, Mich, USA) as also used. Bacteria were grown in L broth at 37°C for 16 h, with shaking. Viable bacteria were counted in cells plated on agar media.

ANIMAL AND INFECTION

Twenty-days-old male albino "Swiss" mice were infected with a suspension of 10^8 UFC of *E. coli* cells/mL. One mL of the suspension was administrated orally using a gastric catheter; 100μL of the suspension were injected intraperitonially. Afterwards only water was offered during the day of the treatment to the mice. Five hours following the administration of the bacterial suspension, the animals were submitted to euthanasia by diethyl ether. Three animals were used for each bacterial strain. The control group was inoculated with 0.8% sodium chloride (wt/v).

HISTOLOGICAL EXAMINATION

Animal's intestines were fixed in 10% buffered neutral Formalin and processed by standard procedures. Sections of paraffin-embedded intestinal tissues were stained with hematoxylin and eosin and examined by light microscopy.

RESULTS AND DISCUSSION

Tissue intestinal specimens from mice used as controls, when colonized with standard EPEC strain or colonized with 537-1 and 304-3 strains appeared normal under light microscopy (figure 1- A, B, C, D respectively); in contrast, the epithelial cells from the intestinal mucosa of mice colonized with strains 263 and 988-2 (figure 2 - E, F respectively) presented a superficial stratum showing breaks and epithelial cells of an irregular shape; it was also possible to see inflammatory cell infiltration at the site.

Freter et al (1983) reported that wild-type *E. coli* strains from human and K-12 strain appeared to pass through the mouse intestine without multiplication or death and no evidence of colonization. It is also difficult to explain the somewhat puzzling finding that *E. coli* of a given host species, can not be easily implanted into the gut flora of other individuals of the same host species (VAN DER WAAIJ et al., 1971).

Mushin & Dubos (1965) reported that high inoculums of over 9 x 10^8 cells/mL should be employed for experimental mouse infection, and that irrespective of the infective dose used, *E. coli* O26: K60 from infantile diarrhea became established in 13 days old or younger mice. In contrast, the percentage of colonization in 18 days old mice was related to the dose used. Infection rates were even lower in 24-days-old animals; in this case, the number of *E. coli* tended to decrease rapidly in the gastrointestinal tract within 48 hours following infection. In the present study we used a 10^8 -cells/mL solution as an infection dose; however, we experienced difficulty in the handling of less than 20-days old mice; this could have a bearing on our results.

Tzipori et al (1989) reported that the most virulent EPEC strains they found were those causing attaching-effacing lesions (A/E) in the proximal small intestine as well as in the remaining gastrointestinal tract. A/E lesions due to EPEC isolates that caused little or no diarrhea were primarily restricted to the large intestine and the distal portion of the small intestine. It is worth recalling that the bovine *E. coli* isolates used in this work were not isolated from feces of diarrheic cattle but came from milk of mastitis-bearing cows and may express an entirely different set of genes involved in the production of tissue adherence.

A/E histopathology has been characterized in EPEC and Shiga toxigenic *E. coli* (STEC) pathogens (KNUTTON, 1996, KAPER et al., 1998); lesions have been seen in tissue culture cells, animal models and samples from human infected with EPEC (NATARO & KAPER, 1998), but not clearly so in murine infection models (WADOLKOWSKI et al., 1990). An explanation of this problem could be the competition of the indigenous *E. coli* mouse flora for the same points of attachment (FRETER et al., 1983, SHIMIZU et al., 2003); therefore a way to explore mouse intestine colonization by pathogenic *E. coli* could be the use of antibacterial drugs to suppress the indigenous intestinal flora (MUSHIN & DUBOS, 1965). In this case however, competition between host and invading bacteria would be eliminated, an effect that could mask the results of colonization.

In the present study, the strain 988-2 (*eae*-, EAF+) showed the ability to colonize and to damage the epithelial cells, agreeing with the results of Nakagawa et al (2002) and Best et al (2006) which reported that the intimin gene (*eae*) was not necessary to the colonization of the intestine, suggesting the existence of other mechanisms that do not require the intimin gene.

In the mouse model employed in this study, the intestinal mucosa was found to be mildly damaged by some of the *E. coli* strains used; however, the results obtained were not conclusive, further research on this model will be necessary to improve our methodology to provide a better understanding of the behavior of the *E. coli* bovine strain in the mouse intestine.

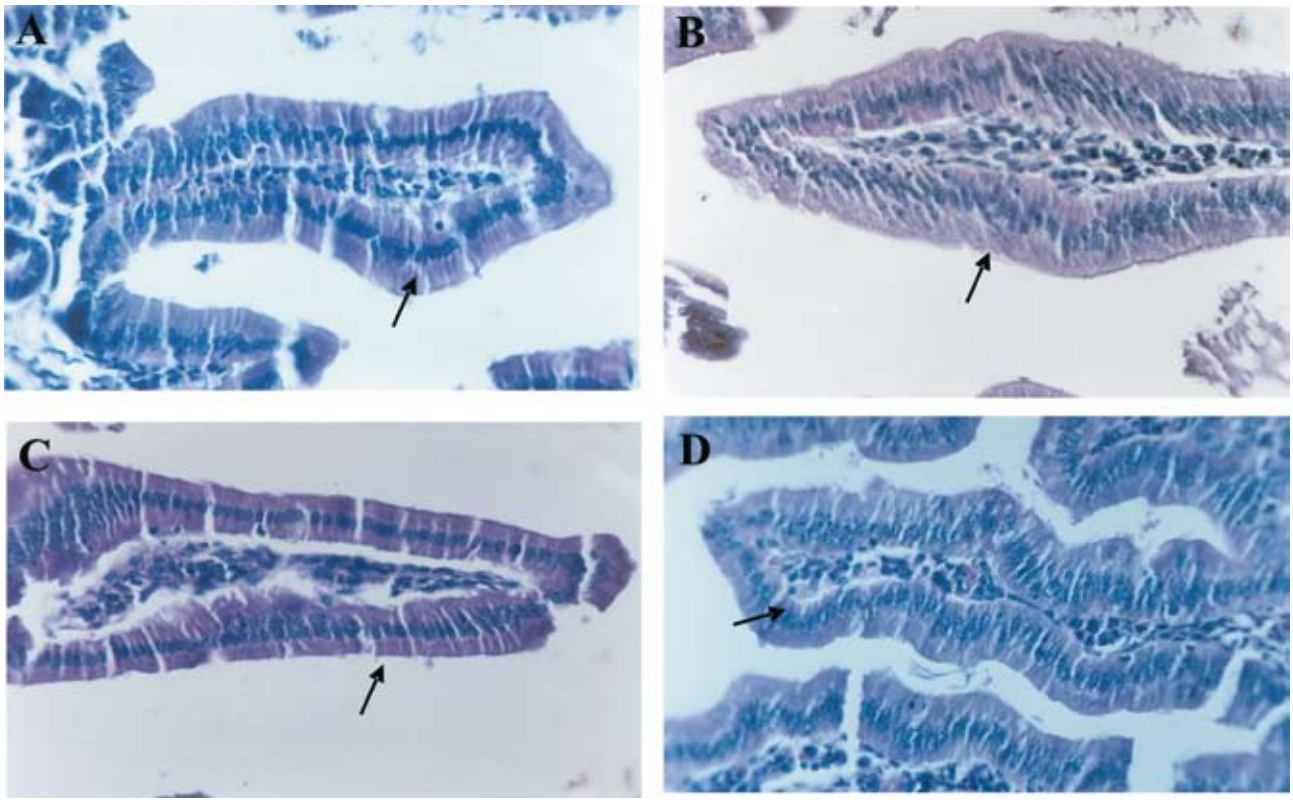

FIGURE 1- Histological examination by hematoxylin-eosin staining of the small intestine of mouse. A- control, untreated normal mouse; B- mouse infected with EPEC standard strain 3111-90; C- mouse infected with isolate 537-1; D- mouse infected with isolate 304-3. Tissues were histologically normal, with epithelial cells of regular shape, and no signs of inflammatory infiltration.

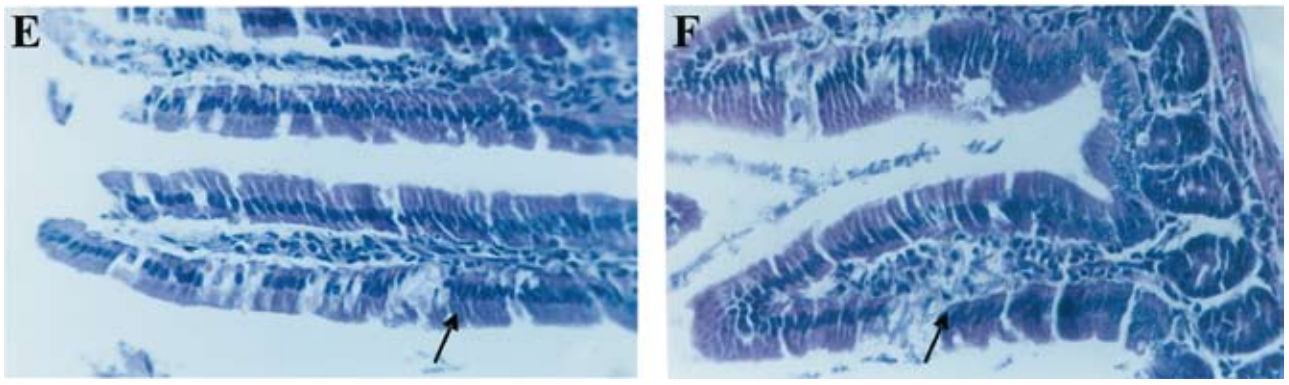

FIGURE 2 - Histological examination by hematoxylin-eosin staining of the small intestine of mouse. E- mouse infected with isolate 263; F- mouse infected with isolate 988-2. The tissues showed abnormalities, with superficial stratum breaks, epithelial cells of irregular shape and signals of inflammatory infiltration.

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REFERENCES

ADU-BOBIE, J., FRANKEL, G., BAIN, C., GONÇALVES, A.G., TRABULSI, L., DOUCE, G., KNUTTON, S., DOUGAN, G. Detection of intimins α, β, γ and δ four intimin derivatives expressed by attaching and effacing microbial pathogens. **Journal of Clinical**

Microbiology, v.36, p.662-8, 1998.

ASBBURNER, F. M., MUSHIN, R. Experimental intestinal coliform infections in mice. **Journal of Hygiene**, v.60, p.175-82, 1962.

BEST, A., LA RAGIONE, R. M., CLIFFORD, D., COOLEY, W. A., SAYERS, A. R., WOODWARD, M. J. A comparison of Shiga-toxin negative *Escherichia coli* O157 aflagellate and intimin deficient mutants in porcine in vitro and in vivo models of infection. **Veterinary Microbiology**, v.113, p.63-72, 2006.

CORREA, M. G. P., MARIN, J. M. O-serogroups, *eae* gene and EAF plasmid in *Escherichia coli* isolates from cases of bovine mastitis in Brazil. **Veterinary Microbiology**, v.85, p.125-32, 2002.

FRETER, R., BRICKNER, H., FEKETE, J., VICKERMAN, M. M., CAREY, K. E. Survival and implantation of Escherichia coli in the intestinal tract. **Infection and Immunity**, v.39, p.686-703, 1983.

KAPER, J. B., GANSHEROFF, I. J., WACHTEL, M. R., O'BRIEN, A. D. Intimin-mediated adherence of Shiga toxin-producing *Escherichia coli* and attaching-and-effacing pathogens. **Escherichia coli O157:H7 and other Shiga toxin-producing E. coli strains**. Washington, DC: ASM, 1998. p.148-56.

KNUTTON, S. Attaching and effacing *Escherichia coli*. **Escherichia coli in domestic animals and humans**. Wallingford, UK: CAB International, 1996. p.567-91.

KNUTTON, S., BALDWIN, T., WILLIAMS, P. H., McNEISH, A. S. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. **Infection and Immunity**, v.57, p.1290-8, 1989.

McDANIEL, T. K., JARVIS, K. G., DONNENBERG, M. S., KAPER, J. B. A genetic lesion of enterocyte effacement conserved among diverse enterobacterial pathogens. **Procedings of National Academy of Science of U.S.A**, v.92, p.1664-8, 1995.

MUSHIN, R., DUBOS, R. Colonization of the mouse intestine with *Escherichia coli.* **The Journal of Experimental Medicine**, v.122, p.745-57, 1965.

NAKAGAWA, I., NAKATA, M., YAMAMURA, T., WAKISAKA, S., KAWABATA, S., HAMADA, S. Infection and pathogenesis of a murine strain of *Escherichia coli* with genetically introduced Shiga toxin type I operon in conventional mice. **Microbial Pathogenesis**, v.33, p.63-72, 2002.

NATARO, J. P., KAPER, J. B. Diarrheogenic *Escherichia coli*. **Clinical Microbiology Review**, v.11, p.142-201, 1998.

SCALETSKY, I. C. A., SILVA, M. L. M., TRABULSI, L. R. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. **Infection and Immunity**, v.45, p.534-6, 1984.

SHIMIZU, K., ASAHARA, T., NOMOTO, K., TANAKA, R., HAMABATA, T., OZAWA, A., TAKEDA, Y. Development of a lethal Shiga toxin-producing *Escherichia coli*-infection mousemodel using multiple mitomycin C treatment. **Microbial Pathogenesis**, v.35, p.1-9, 2003.

TZIPORI, S., GIBSON, R., MONTANARO, J. Nature and distribution of mucosal lesions associated with enteropathogenic and enterohemorrhagic *Escherichia coli* in piglets and the role of plasmid-mediated factors. **Infection and Immunity**, v.57, p.1142-50, 1989.

VAN DER WAAIJ, D., BERGHUIS-DE VRIES, J. M., LEKKERKERT, J. E. C. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. **Journal of Hygiene**, v.69, p.405-511, 1971.

WALDOSKOWSKI, E. A., BURRIS, J. A., O'BRIEN, A. D. Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157: H7. **Infection and Immunity**, v.58, p.2438-45, 1990.