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EXPRESSION OF AGGREGATIVE ADHERENCE TO HELA CELLS BY *ESCHERICHIA COLI* STRAINS ISOLATED FROM SICK HORSES

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ABSTRACT

The virulence attributes of 56 *Escherichia coli* strains isolated from sick horses (secretions of uterine cervices; gastrointestinal and lung fragments of necropsy; diarrheic feces, and tracheal washings) was examined by determining their adherence pattern to HeLa cells and searching for the presence of virulence genes of the various *E. coli* pathotypes. Two non-adherent strains presented *astA*, which encodes the enteroaggregative *E. coli* heat-stable toxin. Twenty-seven strains (48.2%) adhered to HeLa cells, 21 (77.8%) of which presented the aggregative adherence pattern (AA) that characterize the Enteroaggregative *E. coli* pathotype (EAEC). Nine of the strains presenting AA were isolated from secretions of uterine cervix, including one carrying virulence genes of the EAEC pathotype (*aggR, aap*, *irp2*, and *pic)*. This is the first description of the AA phenotype amongst *E. coli* strains from sick horses. Such strains should be further evaluated regarding their potential role in the pathogenesis of diverse equine diseases and as reservoirs of human infections.

Key words: horse, *Escherichia coli*, aggregative adherence, virulence

INTRODUCTION

Although commensal *Escherichia coli* inhabits the intestinal lumen of humans and animals, certain *E. coli* strains carrying specific combinations of virulence genes may cause diarrhea, urinary tract infections or sepsis/ meningitis (22,32). According with their set of virulence genes and the signs and symptoms generated in the host, diarrheagenic *E. coli* strains are classified in distinct pathotypes named enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC) or enterohemorrhagic *E. coli* (EHEC), diffuselly adherent *E. coli* (DAEC), and enteroaggregative *E. coli* (EAEC) (28). In addition, *E. coli* strains causing human extra-intestinal infections are collectively known as Extra-intestinal *E. coli* (ExPEC) (32).

Colonization is a fundamental step in the establishment of most bacterial diseases and depends on the ability of bacteria to adhere to host surfaces. Adhesiveness can be examined in HeLa and HEp-2 cell lines *in vitro*, where *E. coli* strains may present at least 3 distinct adherence patterns: localized (LA), aggregative (AA), and diffuse (DA) adherence (28). These patterns distinguish strains belonging to the EPEC, EAEC and DAEC pathotypes, respectively. Furthermore, some *E. coli* strains have been reported to express a LA-like (LAL) pattern of adherence or to present a non-characteristic (NC) pattern with few bacteria attaching to the cells surfaces (13,22).

In various animal species, *E. coli* strains sharing one or more characteristics with human pathogenic *E. coli* have been reported (3,5,6,16,25,27,30). In horses, *E. coli* is one of the most common organisms in feces and blood of septic foals (19,20,23) and is frequently detected in samples from endometritis and other important equine diseases (17,18,20,26,31). Holland *et al.* (19) showed that *E. coli* strains from foal diarrheic feces harbored the *stx1 and stx2* genes (associated with the STEC/EHEC

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pathotypes) and *eae*, encoding the adhesive protein intimin (associated with both EPEC and STEC/EHEC). These findings, in addition to the demonstration of a characteristic ultra-structural damage to the brush border of equine epithelial cultured cells by an EPEC strain isolated from a diarrheic child (1), suggest that certain *E. coli* strains may be potential equine pathogens. However, little is known about their potential virulence attributes. In the present study we sought to examine the virulence potential of a collection of *E. coli* strains detected among various clinical samples from sick adult horses.

MATERIALS AND METHODS

Origin of the strains studied

Fifty-six strains, previously isolated and identified as *E. coli* according to Ewing (12), were selected randomly (one strain per sample) from a collection of *E. coli* strains detected among various clinical samples from sick adult horses infected by one single bacterial type (monobacterial infections) in the Microbiology Laboratory of Veterinarian Hospital of São Paulo Jockey Club (JCSP) in São Paulo city, Brazil. Fifteen strains, derived from sick mares of breeding farms, were formerly collected with swabs from uterine cervices secretions. Fragments of gastrointestinal tract (23 strains) and lungs (2 strains) were obtained from horses that died of diverse diseases. The remaining strains were isolated from clinical specimens from Thoroughbred ill horses and comprised feces (13 strains) from diarrheic horses and tracheal washings (3 strains) from horses with pneumonia.

Determination of the pattern of adherence

Adherence assays were performed in HeLa cells using the 3 h and 6 h assays (8). HeLa cells at 60% confluence were cultivated in 24-well tissue culture plates containing Minimal Essential Media (MEM) supplemented with 5% of fetal calf serum. After two washes with PBS, 1.0 ml of fresh media (MEM supplemented with 2% D-mannose) was dispensed on the cell monolayers. *E. coli* strains were grown overnight in Luria broth without shaking, and diluted 1:50 in the media contained in the microplates. After an incubation period of 3 h at 37ºC, monolayers were washed 10 times with PBS, fresh media was added to the wells, and an additional incubation period of 3 h proceeded (6 h assay). After 5 washes with PBS, the preparations were fixed with methanol, stained with May Grünwald-Giemsa, and examined blindly by light microscopy. Strains were considered adherent when more than 10% of cells per field presented bacteria (8). Laboratory *E. coli* strain HB101 was used as a non-adherent control while strains E2348/69 (28), JPN15 (28), C1845 (4), and 0431-4 (13) were used as controls of LA, LAL, DA, and AA, respectively.

Search for virulence genes of diarrheagenic *E. coli*

All *E. coli* strains were submitted to colony hybridization assays as previously described (36), using cloned or amplified genetic probe sequences for ETEC (LT-I, ST-Ih, and ST-Ip probes), EIEC (invasiveness plasmid probe), EPEC (*eae*, *bfpA*, and EAF probes), STEC/EHEC (*stx* and *eae* probes), EAEC (EAEC plasmid sequence), DAEC (*daaC* probe), and ExPEC (α-hemolysin probe) as well as for Cytolethal distending toxin (*cdt* probe), Cytotoxic necrotizing factor (*cnf* probe) and Enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) (*ast*A probe).

To further characterize the *E. coli* strains that expressed AA in HeLa cells identified in this study, the presence of putative EAEC virulence genes was tested by PCR amplifications. The primers and conditions used were described previously (10). DNA extracts of prototype strains 042 (*aafC*, *aggR*, *aap*, *shf*, *irp2*, *pet*, and *pic* probes) and 17-2 (*aggC* probes) were used as positive controls.

RESULTS

Among the 56 strains tested, 27 (48.2%) adhered to HeLa cells; 21 (77.8%) of these 27 strains presented AA (AA+) and 6 (22.2%), presented a non-characteristic pattern (NC+). Table 1 presents the frequency of adherent strains among the different clinical samples analyzed as well as their adherence patterns and distribution. Adherent strains were most frequently observed among *E. coli* strains isolated from secretions of uterine cervix (60.0%) and gastrointestinal tract fragments (56.5%), and were also detected in lungs (50.0%) and feces (30.8%). Most of the 21 AA+ strains were isolated from secretions of uterine cervix (9 strains, 42.8%) and gastrointestinal tract necropsy fragments (8 strains, 38.1%) while the NC+ strains were most frequent in gastrointestinal necropsy fragments (83.3%) (Table 1).

None of the strains carried *cdt*, *cnf* or the virulence DNA sequences associated with EPEC, STEC/EHEC, EIEC, ETEC, DAEC or ExPEC. Two non-adherent strains (from feces) reacted with *astA*.

The EAEC putative virulence genes described so far are located on a high molecular weight plasmid and comprise the *agg* and *aaf* operons (involved in the biogenesis of aggregative adherence fimbriae) (2, 28), *aggR* (encoding the transcriptional activator AggR) (29), *aap* (formerly *aspU*, encoding dispersin) (34), *shf* (encoding a cryptic secreted protein, Shf) (9), and *pet* (encoding the Plasmid encoded toxin, Pet) (11). Chromosomal virulence genes have also been described in some EAEC strains, which comprise *pic*, encoding a Protein involved in colonization (Pic), and *irp2*, encoding the iron-repressible high-molecular-weight protein 2 (Irp2) involved in Yersiniabactin expression (9). Only 5 of the 21 AA+ strains carried any of the genes searched for: one AA+ strain (from uterine cervix) reacted with the EAEC probe and four strains (one from uterine cervix and 3 from gastrointestinal fragments) carried *irp*2. The strain from uterine cervix that had the EAEC probe sequence also carried *aggR*, *aap*, *pic*, and *irp2*.

Table 1. Adherence properties in HeLa cells of 56 *Escherichia coli* strains isolated from various equine clinical samples.

Clinical samples	Nº of strains tested	Nº (%) of adherent strains	Adherence pattern[a] N°/%[b]
Gastrointestinal Tract (necropsy)	23	13 (56.5)	AA 8 / 38.1[c] NC 5 / 83.3
Uterine cervix	15	9 (60.0)	AA 9 / 42.8[cd]
Feces	13	4 (30.8)	AA 3 / 14.3 NC 1 / 16.7
Tracheal washing	3	0	-
Lungs (necropsy)	2	1 (50.0)	AA 1/ 4.8
Total	56	27 (48.2)	AA 21 / 77.8 NC 6 / 22.2

[a] AA, aggregative adherence; NC, non-characteristic adherence; [b] Number of adherent strains and % of total AA (n=21) and NC (n=6) adherent strains; [c] Three gastrointestinal and one uterine cervix strains carried *irp* 2; [d] One strain reacted with the EAEC, *pic*, *aap*, *aggR*, and *irp*2 probe sequences.

DISCUSSION

E. coli is one of the most commonly isolated organisms from tissues and blood of septic foals but their virulence potential is fairly unknown. Therefore, the present study was conducted to elucidate the nature of the virulence determinants encoded by *E. coli* strains isolated from various clinical samples of sick equines using an approach already used by our group in studies of infant diarrhea (36). None of the gene sequences tested, which were designed to detect 5 of the 6 human diarrheagenic *E. coli* pathotypes (ETEC, EIEC, EPEC, STEC/EHEC, and DAEC) were found among the strains tested. Two non-adherent *E. coli* strains (from feces) reacted with *astA*, but as many *E. coli* isolates from other diarrheagenic pathotypes as well as commensal strains were shown to carry *astA*, this finding should not be emphasized until the role this toxin plays in diarrheal diseases be confirmed (24,33).

Among the 56 *E. coli* tested, we found 21 strains expressing the AA pattern. Most AA+ strains were isolated from secretions of uterine cervix (42.8%) and from Gastrointestinal fragments (38.1%). One of these AA+ strain (from uterine cervix) probably carried the EAEC plasmid, as indicated by the fact that it reacted with the EAEC probe and carried the *aggR*, *aap*, *irp2*, and *pic* genes. Thus, of those AA+ *E. coli*, only one was shown to harbor all those factors generally associated with clinically significant human EAEC. EAEC strains are considered human emerging diarrheagenic pathogens, but it is commonly accepted that it is a heterogeneous group comprising pathogenic and non-pathogenic strains that share the common AA phenotype (22). Recently, Kaper *et al.* (22) suggested that the term "typical EAEC" should be used for strains carrying *aggR* and a group of regulated genes, and "atypical EAEC" for the AA+ strains lacking these genes. According to this proposal, most of the AA+ strains found in the equine specimens should be regarded as atypical EAEC. However, although most authors consider only typical EAEC as pathogens, there are reports on outbreaks of diarrheal infections (7,21) caused by atypical EAEC (EAEC probe negative strains). As discussed earlier, although only one strain was identified as typical EAEC, atypical EAEC strains were also obtained from animals with no other known pathogens (monobacterial infections), which were associated with clear clinical symptoms of infection, and responded very well to antibiotic therapy. Therefore, we considered these strains as potential pathogens. However, since many of these strains were isolated from extra-intestinal specimens (and not from diarrheic feces) it might not be appropriate to designate them as "enteroaggregative" *E. coli*.

AA+ *E. coli* has been previously found in feces of monkeys and dogs (6,27), but to our knowledge, the present study is the first description of such strains in horses. Moreover, as far as we know, the presence of AA+ *E. coli* strains in host regions outside the intestinal tract has been reported only in human urinary tract infections (14,15). Interestingly, although AA+ strains have been detected in feces and necropsy intestinal fragments, in the present study they were isolated mainly from secretions of uterine cervices and from specimens collected from intestines (feces and necropsy fragments) and lung fragments. One explanation for finding EAEC in extra-intestinal sites could be that in horses, fecal contamination can spread to unusual sites, possibly due to the contiguity of the cervix to the anus and the easy fecal contact with the respiratory tract by their housing characteristics. Thus, since this survey used a strain collection that is likely to hold pathogenic, non-pathogenic commensals and possible cross-contaminating isolates, the significance of this finding is unclear. Recently, Uber *et al.* (35) characterized a collection of EAEC strains of human and animal specimens in order to investigate a possible role of animals as reservoir for EAEC human infections. In their work they included some of the EAEC strains used in the present study. Their results demonstrated that EAEC strains of human and animal sources are serologically and genotypically distinct, suggesting that the animal strains may not represent potential reservoirs for human infections. However, those data may not be conclusive due to the small number of equine strains studied. Besides, due to the proximity between human and animals, a possible contamination of people who work with infected animals cannot be dismissed. Further studies analyzing a larger

collection of strains from sick and healthy horses (matched controls) should be performed to better assess the association of AA+ *E. coli* strains with different equine diseases and as a reservoir of human infections.

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RESUMO

Expressão de aderência agregativa em células HeLa por amostras de *E. coli* isoladas de eqüinos doentes

Características de virulência de 56 amostras de *Escherichia coli* isoladas de eqüinos doentes (secreção de colo uterino, fragmentos de necrópsia do trato gastrointestinal e de pulmões, fezes diarréicas e lavado traqueal) foram examinadas para determinar o padrão de aderência em células HeLa e pesquisar a presença de genes de virulência de vários patotipos de *E. coli*. Duas amostras não aderentes apresentaram *astA*, gene que codifica a toxina termo-estável de *E. coli* enteroagregativa. Das vinte e sete amostras (48,2%) que aderiram a células HeLa, 21 (77,8%) apresentaram o padrão de aderência agregativa (AA) que caracteriza o patotipo de *E. coli* Enteroagregativa (EAEC). Nove destas amostras que apresentaram AA foram isoladas de secreção de colo uterino, incluindo uma que apresentava genes de virulência de patotipos de EAEC (*aggR, aap, irp2* e *pic*). Esta é a primeira descrição do fenótipo AA em amostras de cavalos doentes. Estas amostras deverão ser melhor avaliadas em relação a sua potencial função na patogênese de diferentes doenças eqüinas, bem como à possibilidade destes animais representarem um reservatório de infecções humanas causadas por esta bactéria.

Palavras chave: eqüino, *Escherichia coli*, adesão agregativa, virulência

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