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BIOLOGICAL AND GENETIC CHARACTERISTICS OF UROPATHOGENIC *Escherichia coli* STRAINS

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SUMMARY

The aim of the present study was to determine biological characteristics such as expression of fimbriae, Congo red binding, production of hemolysin and aerobactin, adhesion to HeLa and uroepithelial cells and invasion of HeLa cells by *Escherichia coli* isolates obtained from patients showing clinical signs of urinary tract infection (UTI). Also, the presence of genes (*apa, afa, spa*) for fimbria expression and cytotoxic necrotizing factors (CNF1, CNF2) was assayed using specific primers in PCR. The data obtained were compared with the clonal relationships obtained by analysis of multilocus enzyme electrophoresis (MLEE), restriction fragment length polymorphism (RFLP) of the rDNA (ribotyping) and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR). All isolates but one presented a combination of at least two of the characteristics studied, a fact suggesting the presence of pathogenicity islands (PAIs). Diffuse adherence type to HeLa cells was observed to occur in most of the strains, but adhesion to uroepithelial cells seems to be a more reliable test to verify pathogenicity. Although four strains seemed to be able to invade HeLa cells when assayed by light microscopy, electron microscopy studies demonstrated that these strains were not invasive. MLEE, RFLP and ERIC-PCR were able to group the isolates differently into main clusters that were not correlated with the presence of pathogenic traits.

KEYWORDS: *Escherichia coli;* Clonal relationship; Urinary infection; Virulence traits; Genetic analysis.

INTRODUCTION

Escherichia coli is one of the major causes of human infectious diseases and is by far the most common cause of urinary tract infection (UTI). The biological characteristics of uropathogenic *E. coli* strains (UPEC) include hemolysin and aerobactin production, expression of P fimbriae, serum resistance, cytotoxic necrotizing factor (CNF), and capsule production. These strains belong to a small number of O serogroups[10,13,14,17,24,25,27,32,40]. The genes responsible for expression of these characteristics are normally clustered in DNA regions denominated pathogenic islands (PAIs)[9,20,37].

Biochemical and molecular techniques such as multilocus enzyme electrophoresis (MLEE), restriction fragment length polymorphism (RFLP) of rDNA (ribotyping) and DNA profile obtained after the polymerase chain reaction with ERIC primers specific for enterobacterial repetitive intergenic consensus (ERIC-PCR) have been used to identify and characterize distinct bacterial populations and to study the clonal relationships among subgroups inside these populations[1,23,28,30,38,41,47,48,49].

In the present study we analyzed urinary tract *E. coli* isolates to obtain possible evidence of a correlation between biological characteristics that could represent pathogenicity traits of these strains and the clonal relationships as assayed by MLE, RFLP of rDNA (ribotyping) and ERIC-PCR.

MATERIAL AND METHODS

Bacterial strains and media: Thirteen wild-type uropathogenic *E. coli* strains were isolated from patients with urinary tract infection (UTI) at the School of Medicine of the University of Campinas, (UNICAMP), Campinas, Brazil. CFA[15], LB and LA media[39] were used routinely for bacterial growth. All strains were stored at –70 °C in LB medium plus 15% glycerol to avoid plasmid losses. Strain ORN115[33] was used as a standard for expression of type 1 fimbriae. Strain LG1522 was used for production of aerobactin.

Hemagglutination and expression of type 1 and P fimbriae: The expression of type 1 fimbriae, or D-mannose-resistant fimbria types by the bacterial strains was determined by agglutinating human and guinea pig red blood cells in the presence or in the absence of D-mannose, as described by EVANS *et al*.[16]. Type P fimbria expression was determined using a commercial P-fimbria-specific agglutination test as described by BLANCO *et al*.[4].

Hemolysin production: Production of hemolysin was assayed by growing the different strains in LB medium overnight (37 °C) and dropping 50 μL of this culture on a Petri dish containing sheep blood agar. The culture was incubated at 37 °C overnight and hemolysin production was verified by the presence of a clear hemolytic halo around the colony.

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Detection of cytotoxic necrotizing factors (CNF) by PCR: Primers CNFA (5'CTGGACTCGAGGTGGTGG3') and CNFB (5'CTGCTG TCAACCACAGCC3') were first used to detect the CNF1 or CNF2 genes, primers CNF1-A (5'GAACTTATTAAGGATAGT3') and CNF1-B (5'CATTATTTATAACGCTG3') were used to detect CNF1, and primers CNF2-A (5'AATCTAATTAAAGAGAAC3') and CNF2-B (5'CATGC TTTGTATATCTA3') were used to detect CNF2. PCR was carried out as described by BLANCO *et al.*[8].

Aerobactin production: Production of aerobactin by the isolated strains was assayed by growing strains in LB medium containing 200 μM of α-α-dipyridyl at 37 °C for 24 h without shaking. The growth was spun for 3 min (12,000 g), the supernatants were filtered through a nitrocellulose membrane (0.22 μm) and aliquots of 50 μL were added to orifices made in LA medium previously seeded with strain LG 1522[11]. The Petri dishes were kept at 37 °C for 48 h and the production of aerobactin was visualized by the growth of strain LG 1522 around the orifices.

Congo red binding: Congo red binding was assayed as described by BERKHOFF & VINAL[3]. Briefly, strains were grown in LB medium (37 °C, 24h) and seeded onto CR agar (trypticase soy agar supplemented with 0.03% Congo red dye and 0.15% bile salts) and the cultures were incubated for 24 h (37 °C). Congo-red-positive *E. coli* isolates were identified by the appearance of red colonies.

Adherence to uroepithelial cells: The adherence capacity of the different bacterial isolates to uroepithelial cells was assayed as described by SVANBORG-EDEN[44] and SVANBORG-EDEN *et al.*[45]. Briefly, squamous and transitional epithelial cells from the urine sediment of one human female donor without a known previous history of urinary tract infection were suspended in PBS. Bacteria (10^8 cells) were added to 10^5 epithelial cells in PBS with D-mannose diluted to a final concentration of 0.5% in a volume of 1.0 mL. After incubation for 60 min at 37 °C, unattached bacteria were eliminated by repeated washing with PBS and the cells fixed and Gram stained. The number of bacteria attached was counted by directed light microscopy. Adherence was defined as the mean number of bacteria attached to 40 epithelial cells. Strain ORN 115 was used as the type 1 fimbria positive control.

HeLa cell adherence and invasion assays: The adherence of the different isolates to HeLa cells was determined as described by CRAVIOTO *et al.*[12], with infection periods of 3 and 6 h. Strains that produced cell lysis within these periods were assayed by adherence for periods of 10 minutes to 3 hours separated by 10 minute intervals. Invasion assay was performed by observation of internalized bacterial cells by light microscopy and confirmed by electron microscopy as described by JOUVE *et al.*[26].

Detection of *pap*, *sfa* and *afa* sequences by PCR: Primers pap1 (5'GACGGCTGTACTGCAGGGTGTGGCG3'), pap2 (5'ATATC CTTTCTGCAGGGATGCAATA3'), sfa1(5'CTCCGGAGAACTGGG TGCATCTTAC 3'), sfa2(5'CGGAGGAGTAATTACAAACCTG GCA3'), afa1(5'GCTGGGCAGCAAACTGATAACTCTC 3'), and afa 2 (5'CATCAAGCTGTTTGTTCGTCCGCCG 3') described by BLANCO *et al.*[7] were used to detect the *pap*, *sfa* and *afa* sequences, respectively, under the conditions described by the authors.

Preparation of enzyme extracts and electrophoretic enzyme typing: *Escherichia coli* isolates were grown overnight at 37 °C in 50 ml of LB medium and pelleted by centrifugation (8,000 g – 2 min) at 4 °C. The sediment obtained was suspended in 2 mL of 10 mM Tris-1 mM EDTA-0.5 mM NADP, pH 6.8, and the cells were lysed with a Brown-sonic sonicator with three sonication pulses of 20 seconds, each followed by at least 1 min of ice bath cooling. Each sample was centrifuged in a 1.5 ml microcentrifuge tube for 20 min at 12000 g (4 °C). The supernatant was filtered through 0.2 μm pore sterile filters and 200 μl aliquots were stored frozen. Electrophoretic analysis of enzymes and subsequent staining procedures were performed as described by SELANDER *et al.*[41]. The following enzymes were assayed: adenylate kinase (ADK; EC 2.7.4.3), isocitrate dehydrogenase (IDH; EC 1.1.1.42), alpha esterase (EST; EC 3.1.1.1), phosphoglucose isomerase (PGI; EC 5.3.1.9), hexokinase (HEX; EC 2.7.1.1), malate dehydrogenase (MDH; EC 1.1.1.37), and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49).

Ribotyping (RT) analyses: Genomic bacterial DNA was extracted as described by VAN SOOLINGEN *et al.*[46] and resuspended in TE buffer plus 10 mg/mL RNAse. DNA (4 μg) was digested with 50 U of *Eco*RI or *Hind*III as specified by the manufacturer (Life Technologies) and analyzed by electrophoresis using 0.7% submersed agarose gels in TE buffer as described by SAMBROOK *et al.*[39]. One Kb DNA standard (Life Technologies) was used as migration reference in each gel. Size-separated restriction fragments were transferred to a 0.45 μm nitrocellulose membrane (Pharmacia) which was processed for southern blotting as described by SAMBROOK *et al.*[39]. The *Sal*I fragment of plasmid pUC 18 containing *Streptomyces (lividans) violaceoruber* TK21 *rrnB*[51] was used as a probe. The probe was randomly labeled with [α-^{32}P]dCTP. Hybridization was performed at 45 °C (high stringency) as recommended by SAMBROOK *et al.*[39].

ERIC-PCR analysis: Genomic bacterial DNA was extracted as described for ribotyping analysis. DNA (2 μL) was amplified by PCR (30 cycles of 30 seconds at 90 °C, 1 min at 52 °C, 8 min at 72 °C followed by a final extension cycle of 16 min at 72 °C) using the sequences ERIC 1 (5'ATGTAAGCTCCTGGGGATTCAC3') and ERIC 2 (5'AAGTAA GTGACTGGGCTGAGCG3') as specific primers for the enterobacterial repetitive intergenic consensus[47]. DNA (10 μL) from each reaction was run on 1.2% submersed agarose gel. The DNA profiles were recorded using black and white films after ethidium bromide staining of the gel. Each reaction was performed twice to ensure the accuracy of the reaction and reactions yielding different results were double-run again.

Data analysis: Isoenzymes obtained by multilocus enzyme electrophoresis and rDNA and ERIC-PCR patterns were recorded and the presence of a band was coded as 1 and the absence of a band as 0 in a data matrix and analyzed by the POPGENE software, version 1.31[50]. Dendrograms of dissimilarity were constructed for each case.

RESULTS

Several biological characteristics of 13 *Escherichia coli* strains isolated from patients showing clinical signs of urinary tract infection were studied. These biological characteristics (Table 1) included *in vitro* absorption of Congo red dye, expression of type 1 and type P fimbriae; search for *pap*, *sfa*, *afa* and *cnf* genes by PCR amplification using specific

Table 1
Uropathogenic strains and biological characteristics studied

Strains	Adhesion HeLa								Fimbr. Expr.		
	WM	M	Uro	Cyt	Hemo	AE	CRB	INV	Type1	P	PCR amp.
01	DA	DA	13	-	+	-	+	-	+	+	pap, sfa
02	DA	DA	16	-	-	+	+	-	-	+	pap
03	DA	DA	62	-	+	-	+	-	+	+	pap, sfa, afa
04	-	-	19	-	-	+	+	+	-	+	pap, afa
05	DA	DA	25	-	+	-	+	-	+	-	pap
06	-	-	20	-	-	-	-	-	-	+	pap
07	DA	-	8	-	+	-	+	-	+	-	sfa, cnf1
08	EA	EA	45	+	+	-	+	-	+	-	pap, sfa, cnf1
09	DA	DA	18	-	-	-	+	-	+	+	pap
10	DA	DA	17	-	+	+	+	+	+	ND	sfa
11	-	-	08	-	+	-	+	+	+	-	sfa
12	LA	LA	15	-	-	-	+	-	+	-	pap
13	DA	DA	63	+	+	+	+	+	-	-	pap, afa

Uro = adhesion to uroepithelial cells (mean observed in 40 cells); Adhesion to HeLa cells: M = with mannose; WM = without mannose; Cyt = cytotoxin production; Hemo = hemolysin production; AE = aerobactin expression; CRB = Congo red binding; INV = invasion of HeLa cells; Fimbr. Expr. = fimbrial expression; DA = diffuse adherence; LA = localized adherence; EA = enteroaggregative adherence; ND = not determined.

primers; production of hemolysin and aerobactin; capacity of adherence to uroepithelial cells and adherence to and invasion of HeLa cells cultured *in vitro* (Fig. 1) in the presence and absence of the sugar D-mannose. These same strains were also compared by gel electrophoresis of isozymes (ADK, IDH, α-EST, PGI, HEX, MDH, G6PDH), restriction fragment length polymorphism (RFLP) of ribosomal DNA, and ERIC-PCR (Fig. 2-7).

The results showed that most of the strains (92.3%) were able to absorb Congo red dye. Eight (61.53%) strains, when grown on blood agar medium, produced hemolysin.

Of four aerobactin-producing isolates (30.7%), two were able to express hemolysin and had the ability of absorbing Congo red dye. The other two aerobactin-producing isolates were able to bind Congo red dye but did not produce hemolysin.

Bacterial adherence to and colonization of the urinary tract by uropathogenic *E. coli* strains are mediated by the expression of several types of fimbrial and nonfrimbrial adhesins[19,21,22,24,52]. Under our experimental conditions, when using anti-P fimbria serum and red blood cell agglutination, nine strains (69.2%) were able to express type 1 fimbriae. Three (23%) of these (01, 03, 09) also expressed type P fimbriae and another three (02, 04, 06) expressed type P but not type 1 fimbriae. Amplification by PCR using specific primers for the *pap*, *sfa* and *afa* genes indicated that strains 02, 05, 06, 09 and 12 had *pap*-related sequences only (38.46%) and strains 10 and 11 had *sfa*-related sequences only (15.38%); strain 01 had the *pap* and *sfa* genes (7.69%); strains 04 and 13 had the *pap* and *afa* genes (15.38%), and strain 03 had the *pap*, *sfa* and *afa* genes (7.69%).

The PCR amplification tests for detection of *cnf*–related sequences also demonstrated that two strains (07, 08) had **CNF1**-related DNA sequences. **CNF2** related sequences were not found in any of the strains studied.

The adherence and invasion tests carried out in the present study with HeLa cells (Fig. 1) demonstrated that three isolates (04, 06, 11) were unable to adhere (Fig. 1b), one (12) showed localized adherence (Fig. 1d), one (08) presented enteroaggregative adherence (Fig. 1e), and eight (61.53%) showed diffuse adherence (Fig. 1c) similar to that described by GERMANI *et al*.[18] also in uropathogenic *E. coli* strains. Although *afa*-related sequences were present in three of these strains (03, 04, 13), and four (04, 10, 11, 13) seemed to be able to invade this cell type as assayed by light microscopy (Fig. 1f), the invasion capacity was not confirmed by electron microscopy, indicating that these strains are noninvasive.

Strains 08 and 13 when tested for adhesion capacity showed cytotoxic activity, which was higher in strain 08. The adhesion analysis of these strains was possible because they exhibited adhesion capacity over periods of time (10 and 20 minutes) shorter than those used as standards and prior to the appearance of the cytotoxic effect.

Adherence of *E. coli* isolates to uroepithelial cells is used to differentiate between uropathogenic and fecal strains[44,45]. These authors suggest that uropathogenic strains may present a mean of 20 bacteria/cell or more, while fecal strains may present a mean of about 7 bacteria/cell. Our adherence assays (HeLa and uroepithelial cells) showed that 76.9% of the isolates presented adherence to HeLa cells and the mean number of bacteria adhered to uroepithelial cells ranged from 8 to 63. Isolates 04 and 06, that were negative for adherence to HeLa cells, showed a mean number of bacteria/cells very close to that needed for a strain to be considered uropathogenic. Both of them produced P fimbriae but isolate 06 had no capacity for expression of any other biological characteristic, while strain 04 produced aerobactin and absorption of Congo red dye. On the other hand, isolates 07 (DA with D-mannose)

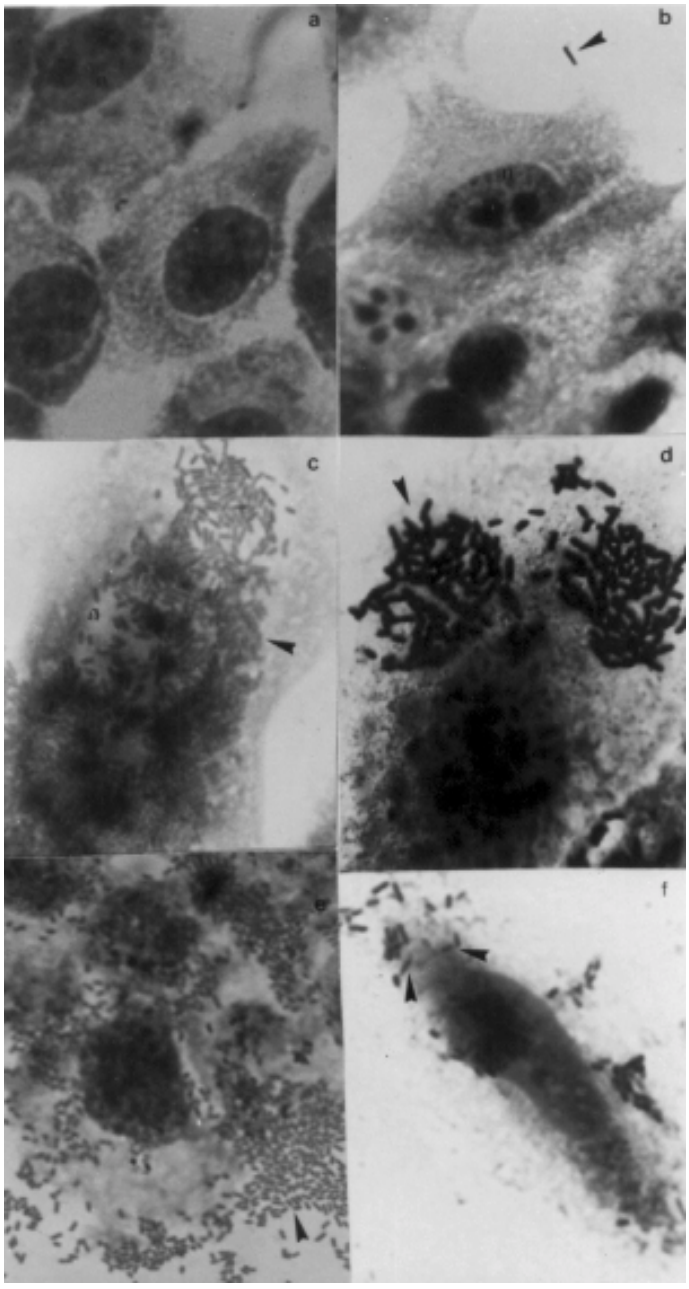

Fig. 1 - Patterns of adherence to HeLa cells shown by urinary *Escherichia coli* isolates: **(a)** HeLa cells cultivated in the absence of bacterial cells; **(b)** Arrows point to negative adhesion bacteria; **(c)** diffuse adherence; **(d)** localized adherence; **(e)** enteroaggregative adherence; **(f)** invasion-positive bacteria. Magnification, 1000X.

and 11 (adhesion negative) presented a low mean frequency of bacterial cell adherence to uroepithelial cells. These same isolates showed biological characteristics normally considered to be pathogenic traits and produced type 1 fimbriae, but not type P fimbriae. All three isolates (03, 08, 13) showing the highest adherence to uroepithelial cells had diffuse adhesion and expressed hemolysin and absorption of Congo red dye. Isolate 13 did not express type P fimbriae but expressed aerobactin.

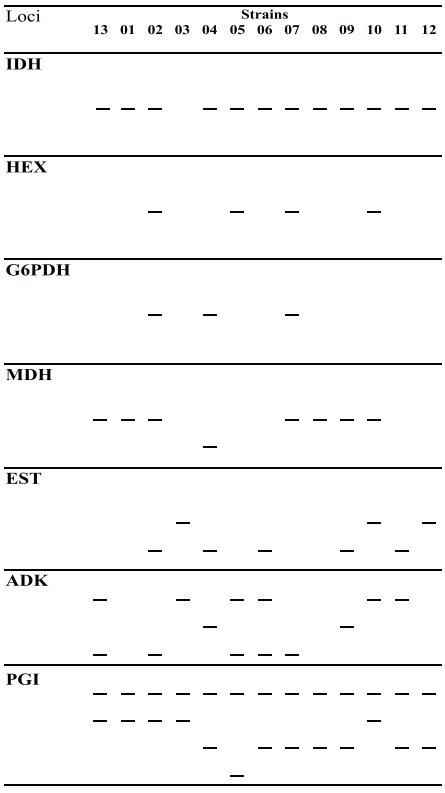

Fig. 2 - Multilocus enzyme electrophoresis (MLEE) profile of the *E. coli* strains studied in the present investigation. (**IDH**): isocitrate dehydrogenase; (**HEX**): hexokinase; (**G6PDH**): glucose-6-phosphate dehydrogenase; (**MDH**): Malate dehydrogenase; (**EST**): α-esterase; (**ADK**): adenylate kinase; (**PGI**): phosphoglucose isomerase. Bars indicate the presence of the enzyme.

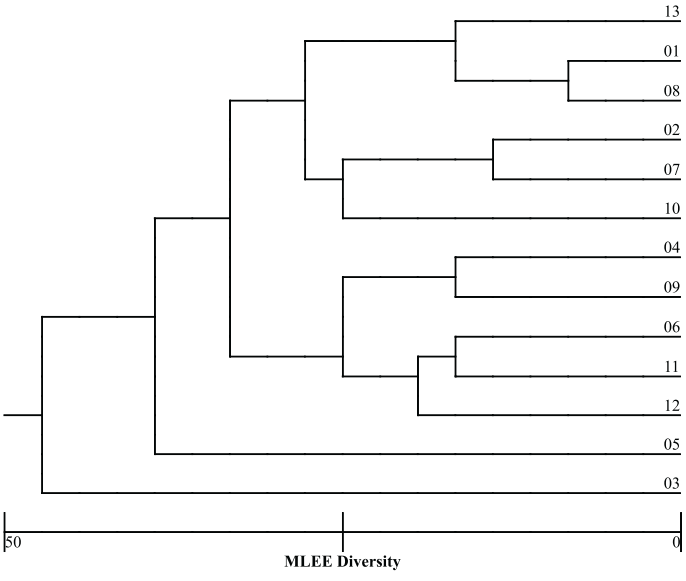

Fig. 3 - Genetic distance of urinary *Escherichia coli* isolates based on multilocus enzyme electrophoresis.

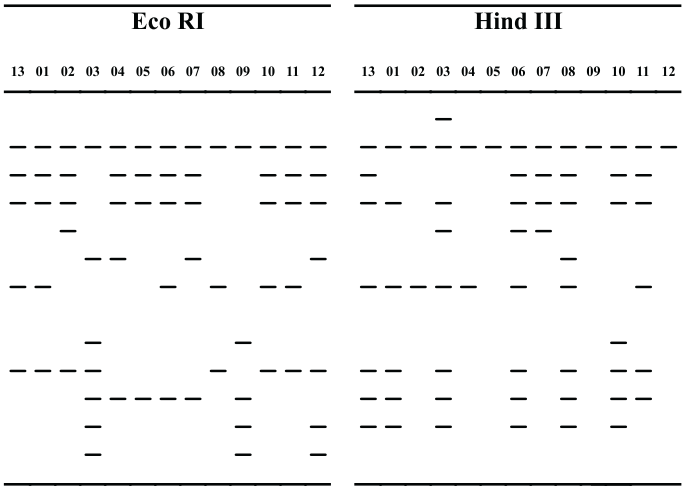

Fig. 4 - Ribotyping profiles of the urinary *E. coli* strains studied in the present investigation.

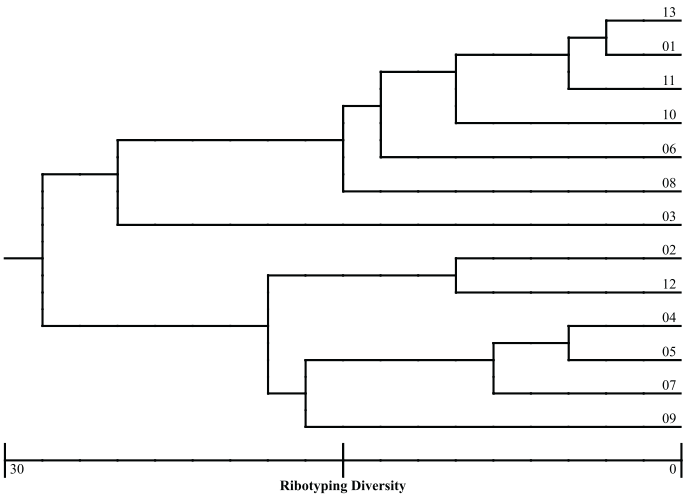

Fig. 5 - Genetic distance of urinary *Escherichia coli* isolates based on rybotype analyses.

Although multilocus enzyme electrophoresis, ribotyping and ERIC-PCR were able to discriminate between the different isolates (Fig. 2, 3, 4, 5, 6, 7), separating them into pattern clusters (thirteen), the results were not the same, i.e., the genetic distances were different for each isolate depending on the method used.

DISCUSSION

The majority of the strains were able to absorb Congo red dye, a characteristic associated with pathogenicity in *Shigella flexneri*[29,31], *Yersinia pestis*[43], *Neisseria meningitidis*, *Vibrio cholerae*[34], and avian septicemic *Escherichia coli* strains[3], suggesting that this characteristic could be used as a marker for pathogenicity in UPEC strains. To our knowledge, this is the first report on absorption of this dye by UPEC.

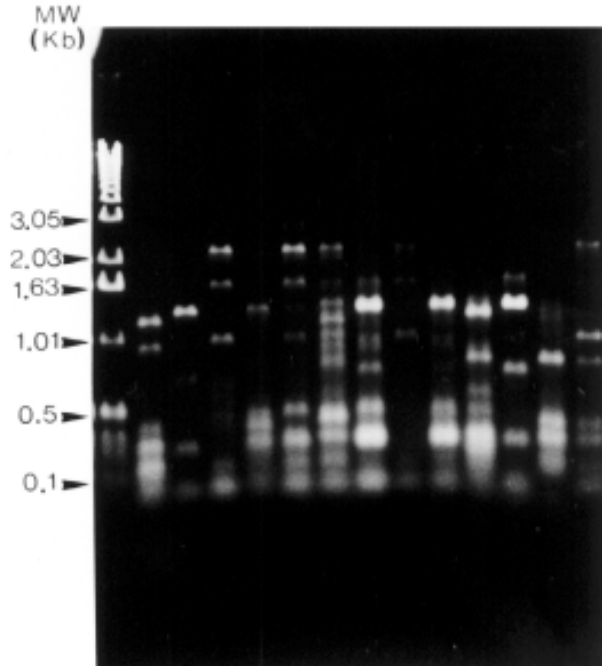

Fig. 6 - Agarose gel electrophoresis (1.2%) of ERIC-PCR profiles of the different urinary *E. coli* strains studied in the present investigation.

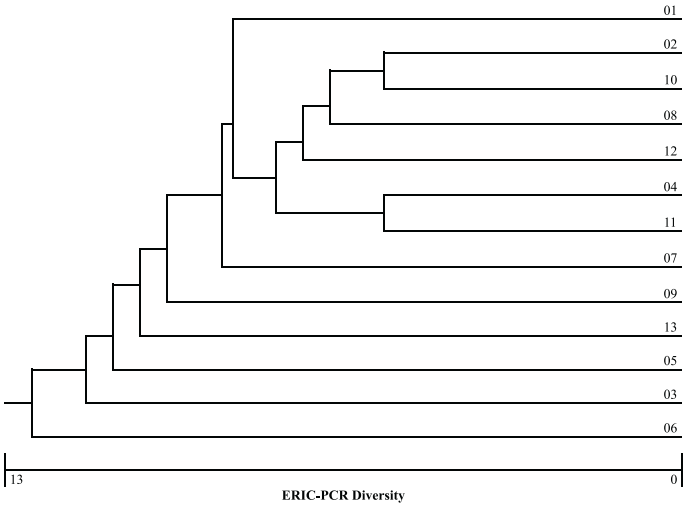

Fig. 7 - Genetic distance of urinary *Escherichia coli* isolates based on ERIC-PCR analysis.

A considerably high number of strains produced hemolysin, a characteristic normally described as being an important trait, although hemolysin production alone does not always equate with virulence, but may be a decisive factor in the virulence of many nephropathogenic strains, as demonstrated by BLANCO *et al.*[5,6]. ARRIAGA-ALBA *et al.*[2] recently described a lower frequency of hemolysin-producing strains than that found by us. This could be explained by differences in the pathogenic status of strains because the cited studies analyzed UPEC isolated from symptomatic

and asymptomatic infections as well. In this case, asymptomatic infections probably are caused by low pathogenic UPEC strains. Two strains were able to show cytotoxic effect in the monocell layer assay, indicating the production of a still unknown cytotoxin.

The expression of several types of fimbrial and nonfrimbrial adhesins is a common characteristic among UPEC strains[18,20,21,23,42,49]. A survey of the Pap, Afa and Sfa adhesins yielded results similar to those obtained by BLANCO *et al*.[7]. Only *afa*-related sequences were not found, indicating that this sequence alone is uncommon among uropathogenic bacterial strains. However, in contrast to the cited authors, we found three strains having *afa*-related sequences associated either with *pap* or with *pap* and *sfa* sequences. Our results also demonstrate a high correlation (100%) between α-haemolysin and one or more of the *pap*, *sfa* and *afa* operons, a fact suggesting the presence of pathogenicity islands in these strains.

The different capacities of strains to adhere to uroepithelial cells suggest the existence of different adhesin types with different adhesion capacities. In fact we found a direct correlation between adhesion capacity and number of adhesin genes. Some discrepancies observed between adhesion assays using HeLa or uroepithelial cells led us to believe that there is no correlation between these adhesion tests. We think that although the uroepithelial cells are more difficult to obtain they will yield more reliable results concerning the pathogenicity of urinary tract infection induced by *E. coli*. For instance, SHRIKHANDE *et al*.[42] used this approach to characterize UPEC strains isolated in India.

One intriguing fact was that, independently of the method used to assess the clonal relationships among strains, strain 03 (*pap*, *sfa*, *afa*) seems to have a longer genetic distance from all the strains (MLEE) either from a group containing a larger number (ERIC-PCR) or a smaller number of strains (ribotyping). This fact led us to propose that, if pathogenicity islands are present in these strains they may have undergone an evolutionary process to adapt to their human hosts, either keeping genes and having a uropathogenic behavior or losing genes and having a non-uropathogenic behavior. This idea is supported by the fact that all the strains with only one type of operon (*pap* or *sfa*) had the smallest mean number of bacteria/uroepithelial cell and the presence of more than one fimbrial operon increased the mean number of bacteria adhered to this type of cell in two out of four strains. In this scenario, UPEC strains of low virulence are still able to cause disease in susceptible or immune-compromised hosts. Indeed, PICARD *et al*.[35] found a direct correlation between the number of pathogenic traits exhibited by UPEC strains and virulence assayed in an animal model. These same authors and others[36] also suggested that UPEC strains, despite the existence of some grade of genetic diversity, are of clonal origin.

RESUMO

Características biológicas e estrutura clonal em amostras uropatogênicas de *Escherichia coli*

O objetivo deste trabalho foi estudar características biológicas tais como a expressão de fímbrias e adesinas, capacidade de absorção do corante Vermelho Congo, produção de hemolisina e aerobactina, adesão e invasão a células HeLa e adesão a células do epitélio urinário em amostras de *Eschericia coli* isoladas de pacientes com sinais clínicos de infecção do trato urinário (UTI). A presença dos genes responsáveis pela expressão de fímbrias (*apa, afa e spa*) e das Citotoxinas Necrotizantes CNF1, CNF2 foi avaliada por PCR. Esses dados foram comparados com a estrutura clonal das amostras obtidas por análises de isoenzimas (MLEE), Ribotipagem (RFLP) e ERIC-PCR. Com uma única exceção, os isolados apresentaram combinação de ao menos duas das características estudadas, fato que sugere a existência de Ilhas de Patogenicidade (PAIs). A maioria das amostras apresentaram um padrão difuso de aderência a células HeLa. Os resultados indicam que a capacidade de adesão a células epiteliais do sistema urinário poderia ser um teste mais específico e correlacionado à patogenicidade. Embora os estudos com microscopia óptica indicassem que quatro linhagens pudessem ser invasivas, dados de microscopia eletrônica não confirmaram tais achados. As técnicas de MLEE, Ribotipagem e ERIC-PCR separaram os isolados em diferentes grupos principais mas estes não foram correlacionados à patogenicidade.

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