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Biological and Genetic Characteristics of *Escherichia coli* Strains Isolated from Children with Neurogenic Bladder

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Abstract

Backgrounds: The urine of the patients with neurogenic bladder frequently contains bacteria, and *Escherichia coli* are among the most frequent bladders colonizers.

Objectives: The aim of the present study was to examine the virulence properties of *E. coli* isolated from bacteriuria symptomatic and asymptomatic of children with neurogenic bladders.

Methods: In this study some virulence properties were examined such hemagglutinins, hemolysins, cytotoxins and adhesins in 50 strains of *Escherichia coli* isolated.

Results and Discussion: The production of hemolysin was observed in 36% of the *E. coli* examined but none produced cytotoxins or enterotoxin. Thirty four (72%) isolates of the *E. coli* showed mannose-sensitive hemagglutinating activity with guinea-pig eythrocytes and 12%, showed mannose-resistant hemagglutinating property. No one isolates were possible to fit into adherence patterns known on HeLa cells. However, these isolates adhered on kidney epithelial cells such as Vero, MDBK, MDCK and HTB-9. Only 60% of the 50 *E. coli* isolates could be grouped with O antisera, and the serogroups O86 and O6 predominanted. No significant difference was observed in frequency of resistance to antimicrobial agents and serum resistance among *E. coli* analysed. Only 10 strains carried sequences related to the three adhesin-encoding operon families investigated. 18% of isolates carried the *hly* operon and *cnf* genotypes. Altogether, 62 % of the *E. coli* strains were encapsulated; these were more common in asymptomatic bacteriuria group (72.7%) than in symptomatic bacteriuria (41%). The *usp* was detected in 24.2% *E. coli* isolated from asymptomatic bacteriuria and 3.6% in the symptomatic group.

Conclusion: These results suggest that strains without the specific virulence factors of UPEC may be able to cause infection in the urinary tract in patients with neurogenic bladder with reduced resistance to infection.

Keywords: *Escherichia coli*; Virulence; Neurogenic bladder; Adhesins; Hemolysin.

1. Introduction

Urinary tract infection (UTI) is a frequent medical complication during the rehabilitation of many spinal-cord injureed individuals. The urine of the patients with neurogenic bladder frequently contains bacteria, and *Escherichia coli* are among the most frequent bladders colonizers [1]. *Escherichia coli*, wich causes over 80% of cases of uncomplicated urinary tract infection (UTI), may carry a number of virulence factors of relevance in urinary tract infections. However, relatively avirulent strains of *Escherichia coli* often cause complicated infections in immunocompromised patients because these bacteria may cause a symptomatic bacteiruria (SB) or an asymptomatic bacteriuria (AB) [2].

The subset of *E. coli* causes uncomplicated cystitis and acute pyelonephrites is distinct from commensal *E. coli* strains that comprise most of the *E. coli* populating the lower colon of humans [3]. The severity of cystitis depends on the virulence of the infecting bacteria and the susceptibility of the host. Children who will develop recurrent UTI can be identified out of the larger populations who have first infections [4].

Although numerous studies on UTI have been published, limited data is available on the urinary tract pathogens involved and the properties of the urinary *Escherichia coli* strains in specific groups' patients, so the aim of the present study was to examine the virulence properties of *E. coli* isolated from bacteriuria symptomatic and asymptomatic of children with neurogenic bladders.

2. Material and Methods

2.1 Patient Characteristics and *Escherichia coli*

Fifty *Escherichia coli* were isolated from children's urine obtained by vesical sonda from 33 children with neurogenic bladder on intermitent catheterization and acquired at least one colonization of the urinary tract at Departament of Nephrology, Faculdade de Medicina de Santa Casa, São Paulo, SP, Brazil. Nine patients (1 male and 8 females) developed 17 symptomatic UTI (urinary tract infection). Twenty-four patients (4 males and 20 females) were colonized 33 times with *E. coli* but remained asymptomatic bacteriuria. Symptomatic UTI was definied as a fever of at least 38.5°C plus pyuria (>10 leukocytes per high-power field).

2.2. Antimicrobial Resistance

The disk diffusion method was used to determine antimicrobial susceptibility. Antimicrobials tested included amikacin, ampicilin, cefazolin, ceftriaxone, cefuroxime, cephalotin, ciprofloxacin, gentamicin, nitrofurantoin, norfloxacin, tetracycline, tobramycin and trimethoprim-sulfamethoxazole (Oxoid, Basingstoke, United Kingdom) [5].

2.3. Serum Resistance Assay

Sensitivity to the bactericidal effect of human normal serum was tested according to the procedure of Pelkonem & Finne [6]. Overnight bacterial culture in Luria Broth (LB) was diluted 1:10 (10^9bacteria/ml) in fresh LB medium and incubated at 37°C for 90 min on a rotary shaker (150rpm). Cultures were centrifuged at 4°C 15.000 x g for 15 min, and ressuspended in phosphate-buffered saline (PBS) pH 7.4 (10^9 bacteria/ml). 100µL of bacterial suspension and 100µL of PBS were pipetted into the wells. 100 µL serum (36% final concentration) was added into the wells and incubated at 37°C. Absorbance at 630 nm was measured at 0, 30, 60, 90, 120 and 180 min. Before each measurement, the plates were shaken althought bacterial sedimentation was not expected to influence the absorbance. The strains were defined resistant, intermediate or sensitive as presented by Taylor [7].

2.4. Detection of Type 1 and P Fimbriae by Hemagglutination Assay

The type 1 fimbriae was determinated by agglutinating guinea pig red blood cell without D-mannose as described by Evans et al. [8] and P fimbriae was detected as described by Gander et al. [9] using the human erythrocytes expressing a P^1 antigen (kindly provided by Hemocentro, Faculdade Ciências Médicas, UNICAMP, Campinas, São Paulo, Brazil).

2.5. Hemolysin Production

Hemolysin production was tested on Muller-Hinton agar plates with defibrinated sheep, ox, horse, guinea pig, chicken, rabbit and A, B and O human bloods. The hemolytic activity was also analyzed on agar plates containing 5% washed bloods in phosphate-buffered saline (PBS 5mM, pH 7.4). Hemolysis was read after overnight incubation at 37°C.

2.6. Detection of Cytotoxic Activity

Escherichia coli were cultivated in 5mL of Tryptone Soy Broth (TSB), pH 7.5, in shaker (150 rpm) at 37°C. 20μL containing 2.5μg of mitomycin C (Sigma, USA) solution was added to cultures 5h after inoculation. After 20h incubation, cultures were centrifuged (6000 x *g* /15 min/ 4°C). The culture supernatants obtained were filtered through 0.22μm membranes (Millipore, USA) and assayed on Vero cells monolayers (2×10^5 cells mL^{-1}) [10], grown in Eagle minimal essential medium (EMEM, Seromed, Biochrom KG, Berlin) with 10% fetal bovine serum (FBS, Sigma, St Louis, MO).

Cultures were incubated at 37°C in 5% CO_2 atmosphere and the morphological changes in cells were examined using an inverted microscope (Nikon Instruments, Japan) after 24 and 48h of inoculation.

2.7. Bacterial Adhesion to Cultured Cells

Adhesion and invasion assays of *E. coli* isolated from patients with symptomatic (BS) and asymptomatic bacteriuria (BA) were performed on kidney cells such as Vero (African green monkey), MDBK (bovine), MDCK (dog) and human bladder cell (HTB-9) and HeLa cell (human cervix, as a cell standard) cultured in 24 wells plates, as described by Scaletsky et al. [11].

2.8. Serotyping

The determination of the O antigens was carried out by the method described by Guinée et al. with all available O (O1 to O181) antisera. The O antisera were produced in the Laboratorio de Referencia de *E. coli* (Lugo, Spain [http://www.lugo.usc.es/ecoli]).

2.9. Detection of the pap, afa, K Antigens, MR Adhesins, Aerobactin, CNF, Hemolysin and Uropathogenic–specif Protein (USP) Sequences by PCR

DNA to be amplified was released from whole cells by boiling. Bacteria were harvested from TSA agar, suspended in 200μL of sterile water, incubated at 100°C for 10 min and centrifuged. The supernatant was used in the PCR reaction as described by Blanco et al. [12]. Base sequences, locations and predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table 1.

3. Results

3.1 Hemolysin and Detection of Cytotoxic Activity

The hemolysin was produced in 18 (36%) of the 50 strains of E. coli and the most sensitive erythrocytes was horse followed by sheep and A, B and O human erythrocytes. None of the strains produced cytotoxins (VT and CNF) or heat-labile enterotoxin (LT), because we did not detect alterations in morphology of Vero cells (Table 2).

Table 1: Base sequences, predicted sizes of amplified products, and location of the oligonucleotide primers used in PCR.

Location	Oligonucleotide sequence (5'-3')	Size amplicons	Reference
Cn*f1*	CNF1:GAACTTATTAAGGATAGT CNF2: CATTATTTATAACGCTG	533	Blanco et al., [12]
HlyA	1:AACAAGGATAAGCACTGTTCTGCTT 2:ACCATATAAGCGGTCATTCCCGTCA	1.177	Yamamoto et al., [6]
*Pap*C	1:GACGGCTGTACTGCAGGGTGTGGCG 2:ATATCCTTTCTGCAGGGATGCAATA	328	Daigle et al., [4]
Afa	1:GCTGGGCAGCAAACTGATAACTCTC 2:CATCAAGCTGTTTGTTCGTCCGCCG	750	Daigle et al., [4]
Sfa	1:CTCCGGAGAACTGGGTGCATCTTAC 2:CGGAGGAGTAATTACAAACCTGGCA	410	Daigle et al., [4]
*Pap*E/F	E:GCAACAGCAACGCTGGTTGCATCAT F:AGAGAGAGCCACTCTTATACGGACA	336	Picket et al., [5]
*Pap*G Classe I	PG1:CAACCTGCTCTCAATCTTTACTG PG2:CATGGCTGGTTGTTCCTAACAT	692	Karkkainen et al., [14]
*Pap*G Classe II	PG1:GGAATGTGGTGATTACTCAAAGG PG2:TCCAGAGACTGTGCAGAAGGAC	562	Karkkainen et al., [14]
*Pap*G Classe III	PG1:CATGGCTGGTTGTTCCTAACAT PG2:TCCAGAGACTGTGCAGAAGGAC	421	Karkkainen et al., [14]
USP	Usp1:ATGCTACTGTTTCCGGGTAGTGTGT Usp2:CATCATGTAGTCGGGGCGTAACAAT	1000	Nakano et al., [22]

Table 2: Frequency of virulence-associated phenotypes in *Escherichia coli* isolated from children with neurogenic bladder

Frequency of phenotype (%)				
Patient status	MRHA[a]	MSHA[b]	Hly[c]	Cyt[d]
B.symptomatic (17)	2/17 (11.7)	12/17 (70.5)	2/17 (11.7)	0
B.asymptomatic(33)	4/33 (12.1)	24/33 (32.7)	18/33 (54.5)	0
Total (50)	6/50 (12.0)	36/50 (72.0)	20/50 (40.0)	0

3.2 Expression of Type 1 and P Fimbriae

Thirty four (72%) of the *E. coli* exanined showed hemagglutinating activity with guinea pig erythrocytes, in the absence of D-mannose. 06 (12%) of strains showed mannose-resistant hemagglutinating property (MRHA) with human erythrocytes possessing P^1 antigen (Table 2).

3.3 Bacterial Adhesion

The adherence tests carried out in the present study demonstrated that all 50 *E. coli* strains showed adhesive capacity in presence or absence of D-mannose (data not shown), but adherence patterns such localized, diffused and aggregative were not observed. The Hly producing *E. coli* were excluded from the adherence assay because these strains induced detachment and lyis of clture cells within 30 min of incubation (data not shown).

3.4 Serotyping

Only 30 (60%) of the 50 *E. coli* isolates could be grouped with O antisera used, 15 (30%) were group O indeterminate and 05 (10%) of the isolates were rough. The serogroups O86 and O6 predominanted (11 strains – seven O86 and four O6) and together comprising 36% of the grouped with O antisera *Escherichia coli* examined- (08 related to symptomatic bacteriuria and 03 from bacteriuria asymptomatic). However**,** 19 of the grouped with O antisera *Escherichia coli* examined (64% of 30 strains) presented a wide range of O antigens and O:K serotypes, mainly of intestinal *E. coli*, in each patient group showed that there was no indication of a clone or clones characteristics of any of these groups.

3.5 Antimicrobial and Serum Resistance

No significant difference was observed in frequency of resistance to one or more antimicrobial agents and frequency of serum resistance between *E. coli* isolates from isolates of symptomatic and asymptomatic bacteriuria (data not shown).

3.6. PCR

Out of 50 uropathogenic *E. coli* strains tested by PCR (Table 3), only 20% (10) carried sequences related to the three adhesin-encoding operon families (*pap, sfa, afa*) investigated. It was found that 16% (9) of the strains exhibited the *pap* genotypes, (simultaneous presence of pap GII- GIII was detected in 06 strains), 4 (8%) the *sfa* genotypes and 3 (6%) the *afa* genotype. A total of 2 (4%) of the strains (Table 3) carried both *pap* and *sfa* operons. The simultaneous presence of *pap-afa-sfa* operons was not detected. The *pap* operon was detected more in isolates from asymptomatic bacteriuria (AB) and the *afa* operon in symptomatic bacteriuria (SB) and no difference was detected for *sfa* operon in each group tested (Table 3).

Nine of isolates carried the *hly* operon and this was more detected (21%) in patients with asymptomatic bacteriuria 7 and 2 (11%) in SB group (Table 3). The *cnf* genotypes were detected in 9 of the *E. coli* isolates (29% in SB and 12% in AB) (Table 3). Altogether 62% (31) of the *E. coli* strains were encapsulated (Table 3); these capsulated strains were more common in asymptomatic bacteriruria group (72.7%) than in symptomatic bacteriruria group (41%) (Table 3). The *usp* was detected in 18% (9) of the *E. coli* isolates and the frequency was higher in group of AB (24.2%) than the SB (3.6%) (Table 3).

4. Discussion

The main purpose of this study was to determine and identify the virulence properties of *Escherichia coli* isolated from the patients with bladder disease.

The type 1 fimbriae, P fimbriae, S fimbriae and afimbrial adhesin have been identified as main specific virulence factors of uropathogenic *E. coli* (UPEC) [13]. These factors play an important role in adhesion, but not all the UPEC isolates have the specific virulence factors that are detectable by phenotypic diagnostic methods or genotypic methods. Carriage of G adhesin genes and expression of MR Adhesins were found to be the most important virulence factors for discriminating between immunocompromised and immunocompetent patients [14]. However, the lower presentation of *pap*, *sfa* and *afa* in our isolates when compared with previously published studies is unsual and its significance in uropathogenesis is not known currently.

E. coli adhesin genes (*pap, sfa, afa*) are the factors studied largely for the adherence trait in UPEC. P fimbriae are highly prevalent (77%) [15] among uroseptic *E. coli* strains, in addition type 1 piliated *E. coli* is reportedly capable of invading the bladder epithelial cells, and replicating intracellularly, resulting in the formation of a large collection of intracellular bacteria [16, 17]. On other hand, 25-40% of urinary isolates of *E. coli* causing UTI shows no adherence to uroepithelial cells [18], however in our study all of the *E. coli* isolates were able to adhere to uroepithelial cells. The absence of adherence pattern in UPEC could explain such controversial findings proposed that adherence may not be an essential step for bacteria with shorter doubling time in case of cystitis [19]. Since all isolates, including the ones without any recognized adhesins can adhere to the cells even in the presence of D-manose in the culture medium, it is clear that not only P, S fimbriae and afimbrial adhesin but also type 1 fimbriae are not required for adherence or colonization of bladder and kidney epithelial cells [13].

Hemolysin is a cytolytic enzyme secreted by many *E. coli* related with extraintestinal infections. Our results suggest that hemolysin may not contribute to clinical symptoms of UTI with neurogenic bladder. This result is consistent with Schlager et al. [20] that serum resistance, antimicrobial resistance, hemolysin, type 1 fimbriae and serotype associated with pyelonephritis were not overly represented in the UTI clones. The frequencies of these properties tested among *E. coli* from symptomatic and asymptomatic bacteriruria were not significantly different. None strains showed cytotoxic activities such as CNF, VT or LT on Vero cells in vitro, besides 9 isolates carry out CNF gene detected by PCR (Table 3).

The most common serogroups in this study were O86 and O2, and these two serogroups have been reported to account for a part of O-groupable in *E. coli* related to UTI from different parts of the world, but the majority of grouped with O antisera uropathogenic *E. coli* examined in our study presented a wide range of O antigens in each patient group and there was no indication of a clone or clones characteristic (data not shown).

Recently, was described a protein named USP (uropathogenic specific protein) in *Escherichia coli* related to UTI [21, 22], this protein was detected 79.8%, and 93.8, and 24% in *E. coli* isolated from cystitis, pyelonefritis and healthy feces, respectively. Our results, the frequency of the USP were 1 (5%) in *E. coli* isolated from symptomatic bacteriuria and 8 (24%) from asymptomatic bacteriuria (Table 3). Moreover, the serogroups of *E. coli* isolates examined in the present study the protein USP was detected on O1, O4, O6, O22, O18, O25 serogroups related to uropathogenic *E. coli* as described by Kurazono et al. [21], however the majority of these isolates were in asymptomatic bacteriuria group (Table 3).

In conclusion, we have determined that *E. coli* isolated from patients with symptomatic UTI undergoing intermittent catherization may be less virulent than those in the asymptomatic bacteriuria group, as indicate by a lower incidence of the virulence factors (carriage of G adhesin genes, expression of MR adhesins and hemolysin production). These results suggest that strains without the specific virulence factors of UPEC may be able to cause infection in the urinary tract in patients with neurogenic bladder with reduced resistance to infection. So we may conclude that others factors as changes in host may be more important than virulence factors in cause of symptomatic infection of the neurogenic bladder.

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