MICROBIAL PATHOGENICITY

Virulence-associated factors in *Escherichia coli* strains isolated from children with urinary tract infections

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Summary. One hundred and sixty-eight strains of *Escherichia coli* were isolated from cases of pyelonephritis (24) and lower urinary tract infections (UTI) (144) from hospitalised and outpatient children up to 2 years old. These strains were investigated for the expression of P fimbriae (PF), mannose-resistant and mannose-sensitive haemagglutination, cell-surface hydrophobicity, serum resistance and the production of α -haemolysin (AH), colicins and aerobactin. PF, AH, aerobactin production and serum resistance were significantly more frequent amongst strains expressing mannose-resistant haemagglutination. PF and AH production was significantly more frequent in pyelonephritogenic strains than in lower UTI strains. Serotypes O6 and O112 were isolated most frequently and plasmids were found in the majority of strains tested.

Introduction

Escherichia coli is one of the most frequent causes of extra-intestinal diseases including urinary tract infections (UTI). In uropathogenic *E. coli* strains, virulence factors include the ability to adhere to uro-epithelial cells,^{1–3} some O and K antigens,^{4,5} and resistance to phagocytosis and to the bactericidal action of normal serum.^{6,7} Other factors known to contribute to the virulence of *E. coli* include the production of α -haemolysin (AH),^{8,9} colicins,^{10,11} aerobactin,^{12,13} cytotoxic necrotising factor^{14,15} and cell-surface hydrophobicity.¹⁶ Adherence to uro-epithelial cells is mediated by fimbrial and non-fimbrial adhesins. Among the former, P, S, M and X fimbriae have been described^{17–19} and these may cause mannose-resistant haemagglutination (MRHA) of human erythrocytes.

In this study, the incidence of virulence factors was investigated in 168 *E. coli* strains isolated from the urine of children with pyelonephritis or lower UTI.

Materials and methods

Bacterial strains

One hundred and sixty-eight strains of *E. coli* were isolated from the urine of patients with either pyelonephritis (24) or lower UTI (144). Most were isolated from hospitalised infants < 2 years old. From each primary isolation plate of MacConkey agar, only one colony was picked for further investigation. Identifi-

cation of *E. coli* was based on biochemical properties. O serotyping by slide agglutination was done first with eight polyvalent (OA, OB, OC, OD, OE, OF, OG, OH) and then with 52 monovalent antisera (OA–O26, O55, O111, O127; OB–O86, O119, O124, O125, O126, O128; OC–O1, O2, O3, O4, O5, O6, O7; OD–O8, O9, O10, O11, O12, O13, O14; OE–O15, O16, O17, O18, O19, O20, O21; OF–O22, O23, O24, O27, O28, O29, O30; OG–Ca18, O32, O50, O75, O143, O144, Ca792; OH–O25, O78, O85, O112, O139, O141, O142) (Imuna, Slovakia).

The diagnosis of acute pyelonephritis and lower UTI (acute cystitis and urethritis) was based on laboratory and clinical investigations. The former was defined by the presence of fever (> 38.5°C), frequency of urination, positive urine culture ($\geq 10^5$ cfu/ml of urine), pyuria (> 10 white blood cells/high power field), elevated erythrocyte sedimentation rate (> 20 mm/h), an increased concentration of C-reactive protein (≥ 20 mg/L) and reduced concentrating capacity (< 600 mOsm/L). Cystitis or urethritis was indicated by a history of frequency of urination, dysuria, suprapubic pain, significant bacteriuria (> 10^5 cfu/ml of urine) but without fever, and with normal renal function.

Haemagglutination test

Bacteria were inoculated into 5 ml of Mueller-Hinton Broth (Difco) and incubated statically at 37°C for 5 days until a pellicle was formed. From the pellicle, bacteria were inoculated on to CFA agar²⁰ containing Casamino acids 1% w/v, yeast extract 0.15% w/v, MgSO₄ 0.005% w/v, MgCl₂ 0.0005% w/v and agar 2% w/v and cultivated at 37°C for 18 h. Haem-

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agglutination was performed in round-bottomed microtitration plates.¹⁵ One drop (100 µl) of bacterial suspension was mixed with one drop of erythrocytes (human type A or guinea-pig; 3% v/v in phosphate-buffered saline, PBS) and one drop of PBS, with or without D-mannose 3% w/v. The plate was left to rotate (15 rpm) for 5 min at laboratory temperature followed by rotation for 5 min at 4°C. Haemagglutination was considered to be mannose-resistant (MRHA) when it occurred in the presence and absence of mannose, and mannose-sensitive (MSHA) when it was inhibited by mannose.

P fimbriae (PF)

PF were detected by a particle agglutination test (PF test, Orion Diagnostica, Espoo, Finland) which was specific for the P adhesin. Briefly, one drop of bacterial suspension was mixed on a plastic slide with one drop of the test solution containing latex particles coated with the α-D-Gal-(1-4)-β-D-Gal P receptor. Latex particles without P receptor served as a control. If PF were expressed by the organism, macroscopic agglutination was apparent, usually within 1 min.³

Cell-surface hydrophobicity

Cell-surface hydrophobicity was measured by the salt aggregation test (SAT) with suspensions (5 × 10⁹ cfu/ml) in 0.2 M phosphate buffer, pH 6.8, of bacteria grown on CFA medium.¹⁵ Suspensions were mixed with ammonium sulphate (Lachema, Czech Republic) solutions at final molar concentrations of 2.0, 1.4, 1.0, 0.4, 0.1, 0.06 and 0.02. Strains were considered to be hydrophobic when they aggregated in ammonium sulphate at concentrations ≤ 1.4 M.

α-Haemolysin production

The method used for determination of α-haemolysin production was a slight modification of those of Smith²¹ and Asnani *et al.*²² Cultures were incubated for 4–6 h at 37°C in alkaline meat extract broth then centrifuged (12000 rpm, 0°C, 20 min) and the supernate was collected. An equal amount of sheep red blood cells (1%, v/v), washed three times in normal saline, and broth supernate were incubated at 37°C for 2 h with intermittent agitation. The suspensions were then centrifuged and the supernates were examined for signs of haemolysis.

Colicin production

Production of colicin was estimated by the method of Šmarda.²³ In this method, bacterial strains were stabbed into nutrient-agar plates which were then incubated for 18 h at 37°C. The agar surface was inverted over a beaker containing about 500 ml of chloroform for 15 min and was then overlaid with 4 ml of soft agar containing a suspension of *E. coli* strain ROW which is sensitive to all colicins. A zone of

inhibition of growth in the overlay surrounding the stab indicated production of a colicin.

Aerobactin production

A slight modification of the chemical method of Csaky²⁴ was used to detect aerobactin production. Briefly, *E. coli* culture supernates (0.5 ml) were mixed with 0.56 M H₂SO₄ and autoclaved for 30 min to generate free hydroxylamine. After neutralisation with 1.5 ml sodium acetate 35% w/v, 0.5 ml of sulphanilic acid 1% w/v and 0.5 ml of iodine 1.35% w/v, both in acetic acid 30% v/v, were added and left to stand for 5 min to allow oxidation of the free hydroxylamine nitrogen to nitrite. Nitrite was then estimated colorimetrically (absorbance at 526 nm) after addition of 0.5 ml of 1-naphthylamine 0.3% w/v in acetic acid 30% v/v, and incubation at room temperature for c. 30 min. The concentration of hydroxylamine nitrogen was estimated from a standard curve constructed with known amounts of hydroxylamine hydrochloride.

Plasmid extraction

Plasmids were extracted by the alkaline lysis method²⁵ and lysates were subjected to agarose 0.7% w/v gel electrophoresis with TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.6). Gels were stained in TAE buffer containing ethidium bromide 0.5 µg/ml for 40 min and destained in distilled water for 10 min before visualisation of plasmid DNA under UV illumination.

Serum bactericidal assay

The assay was done as described previously.²⁶ Briefly, overnight cultures of *E. coli*, grown at 37°C on blood agar, were harvested and the cells were suspended in Hanks's Balanced Salts Solution (HBSS) to 2.5 × 10⁴ cfu/ml. The wells of microtitration plates were used for incubation of bacterial suspensions (0.05 ml) with serum (0.05 ml). Control wells contained 0.05 ml of HBSS instead of serum. The plates were placed on a roller (angle 45°) and rotated for 180 min. Samples (10 µl) were withdrawn after incubation for 180 min at 37°C and spread on blood-agar plates. The plates were incubated for 18 h at 37°C and the viable count was determined. Susceptibility of bacteria to serum bactericidal activity was expressed as the percentage of bacteria surviving after 180 min in relation to the original count of bacteria determined at 0 min in the controls. According to the method of Benge,²⁷ strains were termed serum sensitive if the viable count dropped to 1% of the initial value and resistant if > 90% of organisms survived after 180 min. Strains that gave results between these values were considered to show intermediate sensitivity.

Statistical analyses

The χ² test was used for statistical comparison of groups; values < 0.05 were regarded as significant.

Results

Of the 168 *E. coli* strains investigated, 129 were typable with 52 monovalent O antisera. Serogroups O6 (21 strains) and O112 (17 strains) were detected most frequently. Fifty one (35.4%) of 144 strains from lower urinary tract infections and six (25%) of 24

Table I. Incidence of serotypes in *E. coli* strains from pyelonephritis and lower UTI

Serotype	Number of strains from	
	Lower UTI	Pyelonephritis
O1	2	0
O2	12	0
O3	2	0
O4	4	2
O5	1	0
O6	21	0
O7	3	1
O8	2	1
O9	2	0
O11	2	1
O12	9	3
O13	1	0
O14	3	0
O15	1	0
O16	1	1
O21	5	1
O23	1	0
O25	1	0
O26	1	0
O29	1	0
O32	1	0
O55	2	0
O75	5	1
O86	1	0
O112	14	3
O127	4	0
O141	2	1
O144	1	0
Ca18	5	1
Ca792	1	0
SA*	4	1
NT†	29	5

* Spontaneously agglutinating in saline.

† Non-typable with antisera to 52 O groups.

strains from pyelonephritogenic infections belonged to the "uropathogenic" serotypes O1, O2, O4, O6, O7, O8, O16, O18, O25 and O75. Thirty-nine isolates were not typable, including five strains which showed autoagglutination (table I).

Table II shows the distribution of the various virulence-associated factors, in relation to haemagglutination properties, in all of the 168 isolates. MRHA of human type A erythrocytes was found in 73 (43%), MSHA in 23 (14%), and no haemagglutination (MSHA⁻, MRHA⁻) in 72 (43%) of isolates. Of the 73 strains showing MRHA, expression of PF was found in 82%, production of AH in 80%, cell-surface hydrophobicity in 81%, aerobactin production in 78%, serum resistance in 68% and colicin production in 34%. Of 85 PF⁻ strains, 60 (70%) did not cause haemagglutination compared to 12 (16.7%) non-haemagglutinating strains among 84 PF⁺ *E. coli* ($p < 0.01$). In MSHA strains, expression of PF was found in only 48% ($p < 0.01$, in comparison with the MRHA group), production of AH in 60% ($p < 0.05$), cell-surface hydrophobicity in 83% (not significant—NS; $p > 0.05$), production of aerobactin in 52% ($p < 0.05$), colicins in 39% (NS) and serum resistance in 48% ($p < 0.01$). Furthermore, in MSHA⁻, MRHA⁻ strains, PF were detected in 17% ($p < 0.01$, in comparison with the MRHA group), production of AH in 57% ($p < 0.01$), cell-surface hydrophobicity in 71% (NS), serum resistance in 71% (NS), production of aerobactin in 63% ($p < 0.05$) and colicins in 24% (NS).

Tables III and IV show the distribution of virulence-associated factors in relation to the type of UTI from which the strains were isolated. Of 24 strains isolated from cases of pyelonephritis (table III), 54% were MRHA, 13% were MSHA and 33% MSHA⁻, MRHA⁻. Of 144 lower UTI isolates, 42% were MRHA, 14% MSHA and 44% were MSHA⁻, MRHA⁻ (table IV). Additionally, several isolates of each group showed MSHA of guinea-pig erythrocytes.

Among the pyelonephritogenic strains (table III), expression of PF was found in 71%, AH in 92%,

Table II. Virulence-associated factors in 168 *E. coli* strains in relation to haemagglutination of human type A erythrocytes

Virulence factor	Number (%) of strains showing haemagglutination		
	MRHA (73)	MSHA (23)	MRHA ⁻ , MSHA ⁻ (72)
PF	60 (82.2)	11 (47.8)**	12 (16.7)**
Hydrophobicity	59 (80.8)	19 (82.6)	51 (70.8)
α -Haemolysin	60 (80.2)	14 (60.0)*	41 (57.0)**
Colicins	25 (34.2)	9 (39.1)	17 (24.0)
Aerobactin production	57 (78.1)	12 (52.2)*	45 (62.5)*
Serum resistance	50 (68.5)	11 (47.8)**	51 (70.8) ^{NS}

MRHA, strains causing mannose-resistant haemagglutination; MSHA, strains causing mannose-sensitive haemagglutination; MRHA⁻, MSHA⁻, strains not causing haemagglutination.

* Significant, $p < 0.05$; ** highly significant, $p < 0.01$; χ^2 test used for comparison with MRHA isolates.

Table III. Virulence-associated factors in 24 *E. coli* from pyelonephritis

Haemagglutination of human erythrocyte	Number (%) of strains	Number (%) of strains expressing							
		haemagglutination of guinea-pig erythrocytes		PF	hydrophobicity	AH	colicin	aerobactin	serum resistance
		MSHA	MRHA						
MRHA	13 (54)	6	0	13	12	12	3	9	6
MSHA	3 (13)	1	0	2	3	3	1	2	1
MRHA ⁻ , MSHA ⁻	8 (33)	4	0	2	7	7	1	7	7
Total	24	11 (46)	0	17 (71)	22 (92)	22 (92)	5 (21)	18 (75)	14 (58)

See footnote to table II.

Table IV. Virulence-associated factors in 144 *E. coli* strains from lower UTI

Haemagglutination of human erythrocyte	Number (%) of strains	Number (%) of strains expressing							
		haemagglutination of guinea-pig erythrocytes		PF	hydrophobicity	AH	colicin	aerobactin	serum resistance
		MSHA	MRHA						
MRHA	60 (42)	31	4	44	46	48	22	48	44
MSHA	20 (14)	16	0	9	16	11	8	10	10
MRHA ⁻ , MSHA ⁻	64 (44)	38	7	13	45	34	16	38	44
Total	144	85 (59)	11 (8)	66 (46)	107 (74)	93 (65)	46 (32)	96 (67)	98 (68)

See footnote to table II.

Figure. Plasmid patterns of *E. coli* strains from UTI. Lanes 1 and 9: *Hind*III digest of λ bacteriophage used as a mol.-wt marker; 2–8, plasmid profiles of eight *E. coli* strains from lower UTI; 10–16, plasmid profiles of eight pyelonephritogenic *E. coli* strains.

colicins in 21%, aerobactin production in 75%, cell-surface hydrophobicity in 92% and serum resistance in 58% of the strains. When compared to these strains, PF and AH were found significantly less frequently in isolates from lower UTI (table IV), in 46% of isolates ($p < 0.05$) and in 65% of isolates ($p < 0.01$), respectively. No significant differences were found in the production of colicins (32% of lower UTI isolates) and aerobactin (67%) or in the expression of cell-surface hydrophobicity (74%) and serum resistance (68%) when lower UTI strains were compared to the pyelonephritogenic strains.

Plasmid DNA was found in 61 (69%) of 89 strains tested, including all ampicillin-resistant isolates. The majority of the strains carried one to three plasmids of 23.1, 2.1 and < 2.0 kb (figure). There was no marked

difference in plasmid DNA content between pyelonephritogenic and lower UTI isolates.

Discussion

E. coli strains isolated from urinary tract infections were investigated for their serotype, possession of seven virulence-associated factors and, in selected isolates, for plasmid DNA. On the basis of agglutination of human erythrocytes, isolates were divided into three groups: MRHA, MSHA and MSHA⁻, MRHA⁻. The expression of PF and production of AH and aerobactin were significantly more common in MRHA strains than in the other two compared groups. A significant difference in serum resistance

was found only when MSHA and MSHA⁻, MRHA⁻ strains were compared (48% versus 71%, $p < 0.05$). The highest percentage of serum-resistant strains was found among MSHA⁻, MRHA⁻ strains. To our knowledge, only Jacobson *et al.*²⁸ have looked for a correlation between PF or type I fimbriae and resistance to the bactericidal action of serum but none was found. Differences in the expression of cell-surface hydrophobicity and aerobactin production were found between the groups in the present work but these were not significant. As in the work of Hull *et al.*,²⁹ expression of PF and AH production were common in our collection of strains and particularly frequent in bacteria possessing MRHA.^{20,30}

PF were more frequently present in pyelonephritogenic strains than in lower UTI strains. This finding is in agreement with those of others.³¹⁻³³

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