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Detection of virulence factors in α-haemolytic *Escherichia coli* strains isolated from various clinical materials

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ABSTRACT

In total, 201 α-haemolytic *Escherichia coli* isolates from various clinical materials (urine samples and vaginal and rectal swabs) were examined by PCR for the presence of genes for the virulence factors α-haemolysin (*hly*), cytotoxic necrotising factor type 1 (*cnf1*), P-fimbriae (*pap*), S/F1C-fimbriae (*sfa/foc*), aerobactin (*aer*) and afimbrial adhesin (*afaI*). Among vaginal isolates, 96% were positive for *cnf1*, compared with 80% of urine strains (p 0.02) and 63% of rectal strains (p 0.0001). Similarly, *sfa/foc*-specific DNA sequences were found in 97% of vaginal isolates compared with 75% of rectal strains (p 0.004). The *afa1* and *aer* genes were associated more with rectal α-haemolytic *E. coli* strains than with extra-intestinal isolates. The results suggested that CNF1 and/or S/F1C-fimbriae contribute to colonisation and persistence of α-haemolytic *E. coli* strains in the vaginal environment.

Keywords *afa*, *aer*, *cnf*, *Escherichia coli*, *hly*, *pap*, *sfa/foc*, virulence factors

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INTRODUCTION

Escherichia coli is able to colonise both the intestinal and extra-intestinal environments in humans [1]. A balanced relationship exists between the human immune system and strains of *E. coli*, which normally allows them to occupy the host without causing illness. However, in some specific circumstances (e.g., immediate immune deficiency or because of individual host susceptibility), these apparent commensals may cause diarrhoea or extra-intestinal infections [2]. Newborns, the elderly, cancer patients and patients with transplanted organs are the most endangered groups [3,4].

The ability of *E. coli* strains to cause an illness is influenced by the carriage of virulence factors [5]. For instance, urinary tract infection in non-immunocompromised adult women is often associated with *E. coli* strains producing multiple virulence factors [6]. These include tissue-specific adhesins, toxins, siderophores, etc.

One such virulence factor is α-haemolysin (HlyA), which is associated with both intestinal and extra-intestinal infections [7]. HlyA is produced by most haemolytic *E. coli* strains isolated from clinical material [8]. HlyA is a member of the RTX toxin family, on the basis of a common nonapeptide repeat in the C-terminal part of the molecule [9]. It is known that α-haemolysin acts on a wide range of host cells, such as the erythrocytes of warm-blooded animals and fish, chicken embryo fibroblasts, mouse fibroblasts, monocytes, polymorphonuclear leukocytes and macrophages [10]. *E. coli* strains with increased production of α-haemolysin have also been found to be more serum-resistant than non-haemolytic strains or strains with reduced production of α-haemolysin [11].

It is believed that the persistence of α-haemolytic *E. coli* strains in the host may be a reason for the emergence of intestinal or extra-intestinal infections, and for their recurrence. The faecal flora is a reservoir for more virulent *E. coli* strains,

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but the vaginal environment is also colonised by *E. coli* [12]. Hence, in this study we searched for differences (if any) in the distribution of virulence factors associated with α-haemolysin in *E. coli* isolates from various clinical sites. The distribution of *hly*, *cnf1*, *afa1*, *aer*, *sfa/foc* and *pap* virulence genes was examined in haemolytic *E. coli* strains isolated from vaginal and rectal swabs and urine specimens.

MATERIALS AND METHODS

Clinical isolates of *E. coli*

In total, 201 α-haemolytic *E. coli* strains were collected in this study. Strains were isolated from various clinical specimens by standard bacteriological methods during the period 1993–2002 in our university hospital. Of 201 *E. coli* strains, 71 were isolated from the urine specimens of patients with community-acquired urinary tract infections, of which 24 strains were from patients with pyelonephritis, 23 were from cystitis patients, and 24 were from patients with significant asymptomatic bacteriuria ($> 10^5$ CFU/mL). Vaginal strains comprised 71 genital tract (cervix or vagina) isolates obtained from adult women with either various types of genital tract infections (24 isolates), or pre-cancer or cancer of any part of the genital tract (22), or from healthy pregnant women (25). Rectal strains ($n = 59$) were isolated from the rectal swabs of children aged ≤ 1 year with a diagnosis of gastroenteritis (13 isolates), or with other diagnoses, e.g., respiratory tract infection, sepsis or nutritional deficit (35), or children without any pathological symptoms (11). *E. coli* strains EB28 (*hly*$^+$, *aer*$^+$, *cnf1*$^+$, *sfa*$^+$ and *pap*$^+$), KS52 (*afa*$^+$) and C600 *rif*$^-$ (negative for all detected virulence factors) were used as positive and negative controls in PCR detection.

Detection of α-haemolysin

E. coli strains were tested for the production of a haemolytic phenotype on blood agar plates (Blood Agar Base number 2; Oxoid, Basingstoke, UK) containing defibrinated sheep erythrocytes 5% v/v. Production of haemolysis was read after overnight incubation at 37°C [13].

Bacterial cultures and DNA extraction

E. coli strains were cultivated on blood agar or in Luria–Bertani broth overnight at 37°C. Bacterial cells were disrupted by rapid lysis as described previously [14], and the lysate supernatant was used as a DNA template for PCR assays.

PCR detection of specific gene sequences

Amplification of *cnf1*-specific sequences (543-bp product) was performed as described previously [14], using primers CNF1-A (5′-GAACTTATTAAGGATAGT-3′) and CNF1-B (5′-CATTATTTATAACGCTG-3′) in a total volume of 50 μL containing 1 μL DNA extract, 900 ng each of the *cnf1*-specific primers, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 1 U *Taq* Platinum polymerase (Gibco BRL Life Technologies, Rockville, MD, USA). The published amplification protocol was slightly modified and comprised 94°C for 2 min, followed by 33 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 2 min.

PCR detection of *pap*-specific gene sequences (328-bp product) was performed with primers pap1 (5′-GACG GCTGTACTGCAGGGTGTGGCG-3′) and pap2 (5′- ATATCC TTTCTGCAGGGAT GCAATA), selected from the *papC* gene sequence, 1 μL of DNA extract, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 0.5 U *Taq* Platinum polymerase. PCR was performed at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 2 min [15].

All bacterial strains were also retested by multiplex PCR. A total volume of 50 μL contained 20 pmol each of pap3 (5′-GCA ACAGCAACGCTGGTTGCATCAT-3′) and pap4 (5′-AGAGA GAGCCACTCTTATACGGACA-3′), giving a 328-bp product; sfa1 (5′-CTCCGGAGAACTGGGTGCATCTTAC-3′) and sfa2 (5′-CGGAGGAGTAATTACAAACCTGGCA-3′), giving a 410-bp product; afa1 (5′-GCTGGGCAGCAAACTGATAACTCTC-3′) and afa2 (5′-CATCAAGCTGTTTGTTCGTCCGCCG-3′), giving a 750-bp product; aer1 (5′-TACCGGATTGTCATATGCAGA CCGT-3′) and aer2 (5′-AATATCTTCCTCCAGTCCGGAGA AG-3′), giving a 602-bp product; cnf1 (5′-AAGATGGAGTT TCCTATGCAGGAG-3′) and cnf2 (5′-CATTCAGAGTCCTGCC CTCATTATT-3′), giving a 498-bp product; and 30 pmol of hly1 (5′-AACAAGGATAAGCACTGTTCTGGCT-3′) and hly2 (5′-ACCATATAAGCGGTCATTCCCGTCA-3′), giving a 1177-bp product. In addition, each reaction contained 2.3 mM $MgCl_2$, 0.2 mM each dNTP and 2 U *Taq* Platinum polymerase. PCR was for 30 cycles, each consisting of 95°C for 1 min, 60°C for 30 s (incrementing by 1°C after every five cycles to 65°C) and 72°C for 3 min, with a final extension at 72°C for 7 min (slightly modified) [16].

Following PCR, aliquots (10 μL) of the final reaction mixtures were electrophoresed through agarose 2% w/v gels for 45 min at 80 V. Amplified DNA fragments were visualised by UV fluorescence after staining with ethidium bromide.

Statistics

Statistical analysis was performed by means of the Pearson's χ^2 test. A p value of < 0.05 was considered to be significant.

RESULTS

Detection of virulence factors

The *hly* gene was detected in 200 *E. coli* isolates with a haemolytic phenotype. One strain investigated in this study was phenotypically non-haemolytic, but was positive by PCR for *hly*, *cnf1*, *pap* and *sfa*. In contrast, two haemolytic *E. coli* strains (one vaginal and one rectal isolate) were *hly*-negative, and were excluded from this study. PCR detection of *cnf1* showed a 100% correlation between the results of single PCR and multiplex PCR. Detection of *papC* and *papEF* in multiplex PCRs also showed 100% correlation in the population of 201 α-haemolytic strains.

Table 1. Occurrence of virulence factor genes among α-haemolytic clinical *Escherichia coli* isolates

	Number of positive isolates from each source		
Virulence factor gene	**Urine (*n* = 71)**	**Vaginal (*n* = 71)**	**Rectal (*n* = 59)**
cnf1	57 (80%)	68 (96%)	37 (63%)
pap	63 (89%)	59 (83%)	50 (85%)
sfa	61 (86%)	69 (97%)	44 (75%)
afaI	2 (3%)	0	8 (13.5%)
aer	38 (53%)	35 (49%)	45 (76%)

Table 1 shows the frequency of detection of different virulence factor genes. The *cnf1*, *pap* and *sfa/foc* genes were found to be associated closely with α-haemolytic strains, with overall figures of 86% positive for *pap*, 81% for *cnf1*, and 87% for *sfa/foc*. Aerobactin genes were found less frequently (59%) than *cnfI*, *pap* and *sfa* genes. Interestingly, *aer*-positive *E. coli* strains were detected more frequently among α-haemolytic rectal isolates (76%) than among urine (53%; p 0.01) or vaginal isolates (49%; p 0.003). Only 5% of isolates (two urinary tract isolates and eight rectal isolates) were positive for *afa1.*

Table 2. Statistically significant differences in the occurrence of virulence factors between different groups of *Escherichia coli* isolates

	p Values for compared groups of isolates		
Virulence factor gene	**Rectal vs. urine**	**Rectal vs. vaginal**	**Vaginal vs. urine**
cnf1	0.07	< 0.0001	0.02
pap	NS	NS	NS
sfa	NS	0.0004	0.03
afaI	0.05	0.004	NS
aer	0.01	0.003	NS

NS, not significant.

Table 2 shows the statistically significant differences between the different groups of isolates.

Association of detected virulence factors

Table 3 shows the number of strains with different genotype combinations. Sixteen combinations of the genes were detected in this study. Most combinations were found among both urine and rectal *E. coli* isolates (14 and 13, respectively). There was less variation in the combinations among vaginal isolates, with only five combinations detected (Table 3). The most prevalent combination, found in 69% of the α-haemolytic *E. coli* isolates, was *cnf1*, *pap* and *sfa/foc* (with or without *aer*). This combination was especially common among extra-intestinal isolates, being found in 82% of vaginal and 70% of urine isolates (p 0.0004 and 0.04, respectively, when compared to the frequency among rectal isolates). The *afa* gene was usually associated with *pap* (80%) and *aer* (80%). No *afa*-positive *E. coli* isolates carrying *sfa* and/or *cnf1* were found.

DISCUSSION

As in previous studies [6,17], the *cnf1*, *pap* and *sfa/foc* genes were found frequently (80–90%) with *hly* in a single strain. The results also confirmed previous findings that *afa* gene sequences are generally not found with *sfa* and/or *cnf1*, but are associated positively with *pap* and *aer* (80% for both genes) [6,15].

Statistical analysis revealed an increased occurrence of *cnf1* and *sfa/foc* in *E. coli* isolates from

Table 3. Occurrence of virulence combinations among clinical isolates (VF-virulence factor)

Virulence factor combination					Number of isolates from each source		
cnf1	***pap***	***sfa***	***afaI***	***aer***	**Urine (*n* = 71)**	**Vaginal (*n* = 71)**	**Rectal (*n* = 59)**
+	+	+	−	+	26 (36.6%)	32 (45.1%)	24 (40.7%)
+	+	+	−	−	24 (33.8%)	26 (36.6%)	7 (11.9%)
+	−	+	−	−	2 (2.8%)	10 (14.1%)	4 (6.8%)
+	+	−	−	+	1 (1.4%)	0	1 (1.7%)
+	+	−	−	−	2 (2.8%)	0	1 (1.7%)
+	−	+	−	+	2 (2.8%)	0	0
−	+	+	−	+	4 (5.6%)	1 (1.4%)	7 (11.9%)
−	+	+	−	−	2 (2.8%)	0	1 (1.7%)
−	+	−	−	+	2 (2.8%)	0	3 (5.1%)
−	−	+	−	−	1 (1.4%)	0	0
−	−	+	−	+	0	0	1 (1.7%)
−	−	−	−	+	2 (2.8%)	2 (2.8%)	2 (3.4%)
−	−	−	−	−	1 (1.4%)	0	0
−	+	−	+	+	1 (1.4%)	0	5 (8.5%)
−	−	−	+	+	0	0	2 (3.4%)
−	+	−	+	−	1 (1.4%)	0	1 (1.7%)

vaginal swabs, compared to isolates from urine or rectal swabs. Necrotoxigenic *E. coli* strains in the vagina (i.e., *cnf1*$^+$) have also been reported by other authors using a phenotypic method [18,19]. This finding may be significant for three reasons. First, S-fimbriae are known to comprise a key virulence factor in the pathogenesis of neonatal meningitis caused by *E. coli*. Therefore, persistence of S-fimbriated α-haemolytic *E. coli* strains in the vagina of pregnant women may expose neonates to a higher risk of infection. The risk associated with vaginal carriage of α-haemolytic strains of *E. coli* was also emphasised by Czirok *et al*. [20] in an investigation of virulence factors in *E. coli* isolates from the cerebrospinal fluid of neonates with meningitis. Second, *E. coli* is the most frequent pathogen isolated from bacterial prostatitis patients [21]. Several authors have observed an increased proportion of necrotoxigenic *E. coli* strains from patients with bacterial prostatitis [22,23]. Therefore, vaginal carriage of necrotoxigenic *E. coli* strains could expose sexual partners to a risk of prostatic infection. Third, women often suffer from an enhanced susceptibility to recurrent urinary and genital tract infections in association with uropathogenic *E. coli* strains [24].

Rectal α-haemolytic *E. coli* isolates showed an increased incidence of *aer* (76%; p 0.01 compared to 52% for urine isolates; p 0.003 compared to 49% for vaginal isolates). This finding suggests that aerobactin is advantageous for the survival of α-haemolytic *E. coli* strains in the gut. The precise role of *aer* in intestinal carriage of uropathogenic *E. coli* strains or those associated with diarrhoea in children, remains to be determined. Diffusely adherent (i.e., *afa*-positive) *E. coli* strains have been described in association with more severe forms of urinary tract infection [25]. However, uropathogenic *afa*-positive *E. coli* strains are usually non-haemolytic [6,17,26]. Other authors have reported the frequent occurrence (31%) of *afa*-positive *E. coli* strains in association with diarrhoea [27,28]. Considering the results of the present study, together with those of other authors, it can be concluded that α-haemolytic *afa*-positive *E. coli* strains are probably found more frequently in the intestinal tract, while uropathogenic *afa*-positive *E. coli* strains are usually non-haemolytic.

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