

Virulence factors and biofilm production among *Escherichia coli* strains causing bacteraemia of urinary tract origin

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The aim of the present study was to gain an insight into the role of virulence determinants and biofilm production in bacteraemia of urinary tract origin. For this purpose 105 *Escherichia coli* isolates from patients with bacteraemia of urinary tract origin, isolated at the Institute of Microbiology and Immunology, University of Ljubljana, Slovenia, were investigated. A total of 88 strains (84 %) were isolated from immunocompromised patients and 17 (16 %) from non-immunocompromised patients. The prevalence of virulence factor (VF)-encoding genes and associations with phylogenetic background, antibiotic resistance, biofilm production and patient status were analysed by PCR and bioassay. Biofilm was produced by 55 (53 %) of the strains. No combination of VFs was highly associated with biofilm production. Of the tested VF-encoding genes, *usp*, *papC* and the adhesin-encoding *sfa/foc* were significantly more prevalent among strains from non-immunocompromised patients. Our results indicate that the uropathogenic specific protein (USP) may be, as judged by predominance and associations of the *usp* gene, an important VF contributing significantly to bacteraemia of urinary tract origin.

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INTRODUCTION

Pathogenic *Escherichia coli* strains are a common cause of extraintestinal infections such as urinary tract infections, neonatal meningitis, as well as bacteraemia (Stamm & Hooton, 1993; Ronald, 2002). Strains that cause extraintestinal infections harbour virulence factors (VFs) that enhance the ability to cause systemic infection (Maslow *et al.*, 1995; Johnson *et al.*, 1998; Johnson & Stell, 2000; Ruiz *et al.*, 2002). While an increase in the occurrence of *E. coli* bacteraemia and urosepsis has been reported in recent years (McBean & Rajamani, 2001), relatively few studies have investigated the characteristics of *E. coli* strains causing bacteraemia. Furthermore, it is possible that the virulence genotypes and phylogenetic background of *E. coli* differs in different geographical regions. While some previous studies have shown that bacteraemia in adults is caused by *E. coli* strains predominantly from phylogroups B2 followed by D, A and B1 (Johnson *et al.*, 2002), Martínez *et al.* (2006) demonstrated a predominance of

strains of the D phylogroup. The authors postulated that the relative predominance of different phylogroups may be subject to geographical variations. In addition, fully pathogenic group D strains, such as those of serotype O15:K52:H1, in certain geographical areas are endemic and exhibit an increasing involvement in bloodstream infections (Martínez *et al.*, 2006). Therefore, the characteristics of isolates from various regions need to be assessed. The aim of the present study was to gain further insight into the role of virulence determinants and biofilm production in bacteraemia. For this purpose the prevalence and associations of VFs, with regard to patient status, phylogenetic background, antibiotic resistance and biofilm formation, among *E. coli* strains causing urinary bacteraemia from Slovenia were investigated. In addition, several previous investigations have shown that quinolone- and fluoroquinolone-resistant uropathogenic *E. coli* (UPEC) strains exhibit reduced virulence and invade immunocompromised patients, while susceptible strains are virulent and affect non-immunocompromised patients (Horcajada *et al.*, 2005; Moreno *et al.*, 2005). To gain more insight into the basis of this relationship we analysed the VFs, antibiotic resistances and phylogenetic group of the strains from Slovenia.

Abbreviations: MDR, multidrug resistance; MRHA, mannose-resistant haemagglutination; MSHA, mannose-sensitive haemagglutination; MV, multivirulence; UPEC, uropathogenic *Escherichia coli*; USP, uropathogenic specific protein; VF, virulence factor.

METHODS

Bacterial infections, isolates and media. A total of 105 *E. coli* isolates from patients with bacteraemia of urinary tract origin, isolated at the Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana, from patients admitted to various departments of the University Medical Center in Ljubljana, from 2000 and 2001, were studied. The large majority of the infections, 81 (77%), were community acquired, and the majority of the patients, 88 (84%), were immunocompromised, and had one or more underlying diseases, such as chronic urinary tract disease (83 patients), diabetes mellitus, malignant, rheumatologic or neurological disease, and/or had undergone immunosuppressive treatment (20 patients), had a urinary catheter or nephrostomy (15 patients), had undergone recent surgery (13 patients) or invasive diagnostic procedures (18 patients). There were 36 males (34%) and 69 women (66%) (data not shown). Only one isolate per patient was analysed. The strains were freshly cultivated onto nutrient agar (SIFIN) and Luria–Bertani agar (LB plates).

Oligonucleotide primers and PCR for phylogenetic group analysis and detection of virulence genes. The phylogenetic grouping of the studied isolates was determined by multiplex PCR (Clermont *et al.*, 2000). The primers and PCR conditions used to amplify adhesins, *papC* (encoding pilus associated with pyelonephritis), *sfa/focDE* (adhesin-encoding operons: the central region of the *sfa/foc* operon, encoding S fimbriae and F1C fimbriae), *afa* (encoding afimbrial adhesin I) (Le Bouguenec *et al.*, 1992), *papGIII*, *fimH* (encoding type 1 fimbrial adhesin) (Usein *et al.*, 2001); siderophores *fyuA* (encoding yersiniabactin) (Johnson & Stell, 2000), *iucD* (encoding aerobactin), *iroN* (encoding salmochelin) (Johnson *et al.*, 2000); toxins *hlyA* (encoding α -haemolysin) (Yamamoto *et al.*, 1995), *cnf1* (encoding cytotoxic necrotizing factor 1) (Ambrožič *et al.*, 2001), *usp* [encoding uropathogenic specific protein (USP)] (Nakano *et al.*, 2001), and capsules K1, K5 (Nowrouzian *et al.*, 2001), were the same as described in the references cited.

Dot blot hybridization experiments were performed with the DIG DNA labelling and detection kit (Roche) to confirm the PCR assays. Appropriate positive and negative controls were performed.

Biofilm production. The capacity to form biofilms was assayed in microtitre plates essentially as described by O'Toole & Kolter (1998) and Vieira *et al.* (2004) with slight modification. Briefly, cells were initially grown for 24 h in 7 ml M9 minimal medium at 37 °C with shaking at 120 r.p.m. Subsequently, 100 μ l overnight culture was added to 96-well polystyrene microtitre plates and incubated for 24 h without shaking at 37 °C. Unattached bacterial cells were then removed from the culture medium, and the biofilm was stained with 0.2% (w/v) crystal violet for 15 min (this dye stains the cells but not the polystyrene). The excess crystal violet dye was washed out, and this was followed by washing the samples three times with bidistilled water. To release the dye, 200 μ l 96% ethanol was added to the wells. Subsequently, 125 μ l sample was transferred to another well, and the absorbance was measured at 595 nm to estimate the amount of biofilm formed. The experiments were performed in triplicate.

Antibiotic susceptibility testing. Antimicrobial susceptibility testing to the antimicrobial agents ampicillin, ciprofloxacin and trimethoprim/sulfamethoxazole was performed by the disc diffusion method, as well as by MIC determination using microtitre plates (CLSI, 2007).

Haemagglutination assays and haemolysis. Haemagglutination tests to determine mannose-sensitive haemagglutination (MSHA) and mannose-resistant haemagglutination (MRHA) were performed in duplicate by the microtitre method as described by Izumi *et al.*

(2005). Production of α -haemolysin was tested on 5% sheep blood agar. *E. coli* strains were inoculated onto blood agar plates, incubated overnight at 37 °C and haemolysis was detected by the presence of a zone of complete lysis of the erythrocytes around the colony.

Statistical analysis. The significance of the results was established using the Fisher's exact test and the level of significance was set at a *P* value <0.05.

RESULTS AND DISCUSSION

E. coli is the leading cause of Gram-negative bloodstream infections; however, data are relatively scarce regarding strain characteristics, particularly from various geographical settings. In the present study, molecular, as well as biological, assays were employed to investigate the phylogenetic background, virulence-associated characteristics and biofilm production of 105 *E. coli* strains causing bacteraemia of urinary tract origin. The majority, 81 (77%), of the strains examined here were from community acquired urinary bacteraemia. In addition, 88 (84%) patients were immunocompromised.

E. coli strains can be assigned to one of the four main phylogenetic groups: A, B1, D1 and B2 (Herzer *et al.*, 1990). Extraintestinal pathogenic strains belong mainly to phylogenetic groups B2 and to a lesser extent D (Picard *et al.*, 1999). Altogether 54 (51%) of the studied isolates belonged to group B2, 21 (20%) to group D, 16 (15%) to group A and 14 (13%) to the B1 group.

As iron is limiting in the bloodstream, iron acquisition systems are important VFs. Of the studied siderophore-encoding genes, *iucD* was found in as many as 91% of the tested strains, followed by *fyuA* found in 74% strains and *iroN* in 56% strains. At least one of the tested siderophores was present in 104 (99%) of the tested isolates. Our results showed an unexpectedly high prevalence of aerobactin encoding sequences (91%) among the investigated strains, compared to 79% reported by Houdouin *et al.* (2006), and 78% reported by Moreno *et al.* (2005), while all other studies reported lower percentages (Johnson *et al.*, 2005; Soto *et al.*, 2007). Such differences might be due to divergences in geographical variation or differences in association with host characteristics. Among the studied adhesins *fimH* was present in 95% of the studied isolates, followed by *papC* in 55% (*papGIII* 14%), *sfa/foc* 24% and *afa* in 3% of the isolates. Of the toxin-encoding genes *usp* was present in 34% of isolates, while *hlyA* and *cnf1* were detected in 26 and 12%, respectively, of the studied strains. Haemolysis and haemagglutination were employed to confirm production of VFs. A good correlation was found between the presence of *hlyA* and haemolysis, since 26% of the tested strains harboured *hlyA* sequences and 27% exhibited haemolytic activity. Other studies have revealed that MSHA is associated with the presence of type 1 fimbriae (Salit & Gotschlich, 1977; Duguid *et al.*, 1979). While *fimH* was detected in 95% of the investigated strains, only 85% exhibited MSHA. This discrepancy

might be due to phase variation (Hultgren *et al.*, 1985). On the other hand MRHA activity can be mediated by P fimbriae, X, FIC and DR fimbriae (Johnson, 1991). Of the 63 strains exhibiting MRHA, 58 harboured *papC*, among these 20 also harboured *sfa/foc*, 4 strains harboured only *sfa/foc* and 1 only *afa*. Capsule K1- and K5-encoding genes were present in 26 and 11% of the studied strains, respectively. Prevalences of the investigated VF-encoding genes were somewhat lower than reported by some other studies of *E. coli* strains causing bacteraemia, most probably because the majority of the strains were from immunocompromised patients.

Biofilms are microbial communities of organisms adherent to each other and/or a target surface. Biofilm formation protects bacteria from hydrodynamic flow conditions, for example in the urinary tract, and against phagocytosis and host defence mechanisms, as well as antibiotics (Hanna *et al.*, 2003). More than 50% of all bacterial infections reported involve biofilm formation (Costerton *et al.*, 1999). A cascade of several precisely, tightly regulated events are required for proper biofilm formation. A majority of the investigated strains in our study (56%) were *in vitro* positive for biofilm production. The prevalence of biofilm production was thus higher than reported in another study: 17% for faecal strains, 43% for strains isolated from patients with cystitis, 40% for pyelonephritis and 42% for bacteraemic *E. coli* strains (Soto *et al.*, 2007). On the other hand, the same authors reported a high, 63%, prevalence of biofilm formation among strains from patients with prostatitis. In addition, the latter study showed that haemolysin and type 1 fimbriae expression were significantly associated with biofilm production. Type 1 fimbriae, which promote adhesion to host epithelial cells, have been found to be important in the initial steps of biofilm formation (Prüss *et al.*, 2006). Among the strains investigated in our study biofilm production was not statistically associated with any virulence determinant or combination of virulence determinants (data not shown). Biofilm formation thus was not statistically associated with the multivirulent B2 phylogenetic group but was associated with phylogroups B1 and D (for group B1/D vs non-phylogroup B1/D, $P=0.007$) (data not shown).

Prior studies have tried to determine the minimal requirement for bacterial passage into the bloodstream. It was demonstrated that the chromosomal determinants for P fimbriae (Johnson *et al.*, 1988), aerobactin and haemolysin are conserved in antibiotic susceptible UPEC strains that invade non-immunocompromised patients. Two additional studies indicated that *papC*, *fyuA* and *aer* (*iucD*) (Johnson & Stell, 2000; Bingen-Bidois *et al.*, 2002) represent the minimal prerequisite for bacterial passage and infection of the bloodstream. A recent investigation (Horcajada *et al.*, 2005) indicated that the *papG* allele II is a critical trait for reaching the kidney, while *cnf1* and *sfa/focDE* are critical for bacteraemia of urinary tract origin. In addition, previous investigations have indicated that a greater complement of VFs is needed for invasion of a non-

immunocompromised host than for an immunocompromised one (Maslow *et al.*, 1993; Johnson, 1994; Johnson *et al.*, 1988; Otto *et al.*, 2001). Among the tested VFs in our study only *usp*, *papC* and the adhesin-encoding *sfa/foc* exhibited a statistically significant higher prevalence among strains from non-immunocompromised patients (Table 1). Among the investigated strains those from non-immunocompromised patients exhibited a statistically significant higher prevalence of multivirulence (MV) ($MV \geq 3$ and $MV \geq 5$) than strains from immunocompromised patients (i.e. more non-immunocompromised patients harboured ≥ 3 and ≥ 5 of the tested virulence traits).

Additionally, it should be noted that strains from phylogenetic group A and B1 were almost exclusively isolated from immunocompromised patients, as was also reported by Moreno *et al.* (2005), while Bingen-Bidois *et al.* (2002) found no association between phylogenetic group and patient status. Strains from phylogenetic group B2 were slightly more common among non-immunocompro-

Table 1. Host characteristics in relation to phylogenetic group, biofilm production, VFs and antibiotic resistance

The virulence score (VF score) was calculated for each isolate as the sum of all virulence-associated genes detected (*papC*, *sfa/foc*, *afa*, *hlyA*, *cnf1*, *usp*, *fyuA*, *iroN*, *iucD*, K1, K5), the sum of all the VF scores of the isolates was then calculated, and finally this sum was divided by the number of isolates to give the mean VF score.

	Associated host characteristic [no. of isolates (%)]		P value
	Non-immuno- compromised (n=17)	Immuno- compromised (n=88)	
Phylogenetic group			
A	1 (6)	15 (17)	NS
B1	1 (6)	13 (15)	NS
B2	11 (65)	43 (49)	NS
D	4 (24)	17 (19)	NS
Biofilm	12 (71)	43 (49)	NS
VF*			
<i>usp</i>	10 (59)	26 (30)	0.027
<i>papC</i>	16 (94)	42 (48)	<0.001
<i>sfa/foc</i>	8 (47)	17 (19)	0.026
<i>cnf1</i>	4 (24)	9 (10)	NS
Mean VF score	5.4	3.9	
MV (≥ 3)	17 (100)	58 (66)	0.003
MV (≥ 5)	12 (71)	35 (40)	0.031
MDR	1 (6)	21 (24)	NS
Cip ^r	0 (0)	12 (14)	NS
Tp/Sxt ^r	1 (6)	21 (24)	NS
Ap ^r	1 (6)	37 (42)	0.005

NS, Not significant.

*Distribution of other VFs does not differ significantly among immunocompromised/non-immunocompromised patients.

mised patients. Similarly, biofilm-producing strains were also found more frequently in this patient group. However, none of the differences was statistically significant (Table 1).

Analysis of the correlation between antibiotic resistance and patient status revealed that only resistance to ampicillin was statistically associated with immunocompromised status. Nevertheless, resistant strains, as well as multidrug resistance (MDR) strains, were more often, albeit not significantly, isolated from immunocompromised patients, which is not surprising as such patients are more often treated with antibiotics (Table 1).

Several previous investigations have shown that ciprofloxacin resistance and MDR are associated with a shift towards less virulent strains and non-B2 phylogenetic groups (Houdouin *et al.*, 2006; Moreno *et al.*, 2005; Horcajada *et al.*, 2005; Johnson *et al.*, 2005). Thus, as expected, susceptible strains from this study exhibited a higher VF score. In addition, our results showed that sensitivity to ciprofloxacin was significantly associated with *papC*, *hlyA*, *usp* and *fyuA*, but not with *cnf1*, and not with the aerobactin iron uptake system (Table 2) as determined in a recent study (Piatti *et al.*, 2008). The association

between sensitivity to ciprofloxacin and MV (Table 2) was also highly significant. A slightly higher, albeit not statistically significant, capacity to form biofilms was determined among *Cip^r* isolates, 75 % (12), compared to 49 % (46) among *Cip^s* isolates. No correlation between biofilm production and resistance/sensitivity to trimethoprim/sulfamethoxazole or ampicillin was observed (data not shown).

The underlying basis for the association of susceptibility to antibiotics and virulence is still unclear. It is possible that a low presence of certain VFs precedes resistance (Johnson *et al.*, 2005; Moreno *et al.*, 2005) or that low presence of these VFs is followed by acquisition of resistances (Piatti *et al.*, 2008; Vila *et al.*, 2002; Horcajada *et al.*, 2005). The coexistence of both mechanisms is also plausible (Houdouin *et al.*, 2006; Drews *et al.*, 2005). A recent study indicated that a specific chromosomal background, only partially corresponding to the phylogenetic background could precede mutation to antibiotic resistance (Drews *et al.*, 2005).

The most relevant finding of our study is that *usp*, USP-encoding sequences, even though detected at a relatively low prevalence among the investigated strains, exhibited a

Table 2. MDR and ciprofloxacin resistance in correlation with VFs

The virulence score (VF score) was calculated for each isolate as the sum of all virulence-associated genes detected (*papC*, *sfal*, *foc*, *afa*, *hlyA*, *cnf1*, *usp*, *fyuA*, *iroN*, *iucD*, K1, K5), the sum of all the VF scores of the isolates was then calculated, and finally this sum was divided by the number of isolates to give the mean VF score. MDR was determined as resistance to antibiotics belonging to at least two different classes of antimicrobials (penicillins, antifolates and quinolones/fluoroquinolones).

VF	MDR prevalence [no. (%)]		P value	Cip ^r prevalence [no. (%)]		P value
	MDR (n=22)	Non-MDR (n=83)		Cip ^r (n=12)	Cip ^s (n=93)	
Adhesins						
<i>papC</i>	8 (36)	50 (60)	NS	1 (8)	57 (61)	0.001
<i>papGIII</i>	0 (0)	15 (18)	0.037	0 (0)	15 (16)	NS
<i>sfal</i> / <i>foc</i>	0 (0)	25 (30)	0.002	0 (0)	25 (27)	NS
<i>afa</i>	2 (9)	1 (1)	NS	0 (0)	3 (3)	NS
<i>fimH</i>	20 (91)	80 (96)	NS	10 (83)	90 (97)	NS
Toxins						
<i>hlyA</i>	3 (14)	24 (29)	NS	0 (0)	27 (29)	0.034
<i>cnf1</i>	0 (0)	13 (16)	NS	0 (0)	13 (14)	NS
<i>usp</i>	1 (5)	35 (42)	<0.001	0 (0)	36 (39)	0.007
Siderophores						
<i>fyuA</i>	11 (50)	67 (81)	0.006	2 (17)	76 (82)	<0.001
<i>iroN</i>	12 (55)	47 (57)	NS	8 (67)	51 (55)	NS
<i>iucD</i>	19 (86)	76 (92)	NS	10 (83)	85 (91)	NS
K1	2 (9)	25 (30)	NS	1 (8)	26 (28)	NS
K5	3 (14)	8 (10)	NS	1 (8)	10 (11)	NS
Mean VF score – mean	2.8	4.5		1.9	4.5	
MV (≥3)	11 (50)	64 (77)	0.017	3 (25)	72 (77)	<0.001
Non-MV (≤2)	11 (50)	19 (23)		9 (75)	21 (23)	
MV (≥5)	4 (18)	44 (53)	0.039	1 (8)	47 (51)	0.006
Non-MV (≤4)	18 (82)	39 (47)		11 (92)	46 (49)	

NS, Not significant.

statistically significant association with bacteraemia of urinary tract origin among non-immunocompromised patients. Previous studies have shown USP to be a VF more frequently found in UPEC than in faecal strains: 79.4 % from cystitis, 93.4 % from pyelonephritis and 88.8 % from prostatitis compared to only 24 % from faecal strains from healthy individuals (Yamamoto *et al.*, 2001; Kanamaru *et al.*, 2003). *usp* sequences were also found to be highly associated with phylogenetic group B2 (Kanamaru *et al.*, 2006). Furthermore, Parret & De Mot (2002) reported that USP itself could be a bacteriocin acting as an endonuclease, since the *usp* gene showed high homology to genes encoding DNase-type pyocins and colicins. Recently, in a mouse model, it has been shown that *usp*, as well as *malX*, a pathogenicity-island marker, *pap* (encoding P fimbriae) and *fyu* (encoding the yersiniabactin system), were found to be most closely associated with killer strains (Johnson *et al.*, 2006). The prevalence of the studied isolates harbouring *papC*, *hly*, *cnf-1* and *aer* was comparable to that reported by other studies. However, those authors did not test for the prevalence of *usp* and association with patient status.

In conclusion, our study indicates, on the basis of the distribution of the *usp* gene, that USP may be a significant VF with an essential role in contributing to bacteraemia of urinary tract origin in patients with a normal status. USP-encoding sequences were frequently associated with highly virulent strains belonging to phylogroup B2 and with infections in patients with normal, non-immunocompromised status.

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