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SHORT COMMUNICATION

Features of uropathogenic *Escherichia coli* able to invade a prostate cell line

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SUMMARY

RWPE-1 normal prostate cells were tested as an experimental model for adhesion/invasion assays by genotypically and phenotypically characterized community uropathogenic strains of *Escherichia coli* (UPEC), a frequent cause of urinary tract infections (UTIs) and significant etiologic agent also in bacterial prostatitis. Adhesive ability and strong biofilm production was significantly associated with the bacterial invasive phenotype. Invasive strains derived mainly from male and pediatric patients. This study suggests that such a cell model could usefully integrate other available methods of urovirulence analysis, to deepen knowledge on the bacterial interaction with host cells.

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Urinary tract infections (UTIs) are one of the most common infectious diseases with high rates of morbidity both in the hospital setting or in the community. UTIs classification should consider variables such as gender, state of health and symptomatology of the urinary tract of each patient (Grabe *et al*., 2013) in conjunction with the type of pathogen. Uropathogenic *Escherichia coli* (UPEC) are the microorganisms most frequently involved in community-acquired UTIs and in bacterial prostatitis that may occur as a result of ascending urethral infection by reflux of infected urine into prostatic ducts (Lipsky *et al*., 2010). *E. coli* strains are categorized into one of four phylogenetic groups A, B1, B2 and D: uropathogens belong mainly to phylogenetic group B2 and in a lesser extent to group D, while commensal strains fall predominantly into groups A and B1 (Clermont *et al*., 2000). UPEC strains represent a genetically heterogeneous bacterial group with a multiplicity of virulence factors (VFs) that cooperate in successful colonization of the urinary tract leading to acute or recurrent and persistent infections. Several pili types, such as type 1, pyelonephritis-associated pilus (Pap) and S pili, the Dr adhesins and others, contribute to infections in the urinary tract. VFs in UPEC include also toxins such as α-hemolysin, cytotoxic necrotizing factor 1 and iron acquisition systems (Wiles *et al*., 2008, Garcia *et al*., 2013, Flores-Mireles *et al*., 2015). Their expression enables the pathogen to adhere to the uroepithelial cells and spread from low urinary tract up to bladder, kidneys, as well as genital tract in males, resulting in ascending infections. Several studies have shown that UPEC, until recently considered extracellular bacteria, can invade epithelial cells of the urinary tract both *in vitro* and *in vivo* (Mulvey, 2002, Dhakal *et al*., 2008). The ability to invade the urothelium as well as the development of intracellular bacterial communities, large biofilm-like inclusions, correlate with prolonged persistence of UPEC in the host (Flores-Mireles *et al*., 2015). The majority of studies focused on bladder cells but the ability of UPEC strains to adhere, invade, replicate and persist in the normal prostate epithelial cell line RWPE-1 has been also demonstrated (Rudick *et al*., 2011). In order to extend the knowledge on the mechanisms linked to uropathogenic *E. coli* infection process, in the present study, 58 urinary *E. coli* strains were tested for adhesiveness to and invasion ability in the normal human prostate cell line, RWPE-1. Strains from a laboratory collection were isolated in 2006 from community patients diagnosed with UTI by general practitioners (Longhi *et al*., 2012). The cohort of patients included 25 men and 33 women, average age 58 (17-86 years), and 7 children aged between 3 months and 9 years. The possible relationship between antibiotic resistance, virulence factors and *in vitro* biofilm formation was also evaluated. Antibiotic susceptibility was evaluated by Kirby-Bauer disc diffusion method according to the CLSI guidelines (CLSI, 2005) and re-checked whenever necessary by broth dilution test. Bacterial biofilm production was determined by crystal violet assays after incubation for 48 h at room temperature and classified as reported by Stepanovic *et al*. (2004). Non-neoplastic, immortalized human prostatic epithelial RWPE-1 cells (ATCC, Rockwell, MD), maintained in complete keratinocyte serum-free medium, were incubated in medium lacking antibiotics for at least 12 h prior to experiments. RWPE-1 cells, infected with bacteria

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grown in Luria Broth (LB) at 37°C for 48 h under static conditions (multiplicity of infection of about 10 cfu/cell), were centrifuged twice at 500 x *g* for 2.5 min to promote bacterial adherence to the cell monolayer and incubated for 2 h at 37°C in 5% CO_2 as reported by Thumbikat *et al*. (2009) and Rudick *et al*. (2011). To measure bacterial adherence, after extensively washing with PBS, cells were lysed with 0.1% cold Triton X-100 and plated onto Tripticase Soy Agar (TSA). Bacterial adhesion was defined as the percentage of attached bacteria compared with the initial inoculum. Bacterial invasion was measured using a gentamicin protection assay: after 2 h of infection, RWPE-1 cells were incubated for 60 min with 100 µg/ml gentamicin (concentration at which all strains were susceptible), followed by washing, lysing, and bacterial plating as above described. Invasion was normalized to the number of adherent bacteria (number of cfu invasive/adherent x 100); the strains were considered invasive when the mean invasion level was equal or superior to 0.1% of the adhesion values. *E.coli* MG1655 and *E.coli* EIEC HN280 strains were utilized as negative and positive controls respectively. The phylogenetic group was determined by PCR assays (Clermont *et al*., 2000). Multiplex PCR for some genes associated with virulence in *E. coli* (PAI, *pap*A, *fim*H, *kps*MT III, *pap*EF, *ibe*A, *fyu*A, *sfa/foc*DE, *iut*A, *pap*G allele III, *K1*, *hly*A, *rfc*, *nfa*E, *pap*G allele I, *kps*MTII, *pap*C, *gaf*D, *cva*C, *foc*G, *tra*T, *pap*G allele II, *pap*G alleles II and III, *afa/dra*BC, *cnf*1, *sfa*S, *K5*), was performed as described by Johnson and Stell, (2000). Random amplified polymorphic DNA (RAPD) reactions were performed using two arbitrary primers: 3 - [5'-d[GTAGACCCGT]-3'] and 4 - [5'-d[AAGAGCCCGT]-3']. PCR was performed in triplicate as follows: 1 cycle at 95°C for 5 min, followed by 45 cycles at 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min (Mastercycler pro, Eppendorf). Results were analyzed using Total Lab TL120 Trace version 2006 (Nonlinear Dynamics) setting position tolerance at 1.5%. The Dice coefficient of similarity was calculated, and the unweighted pair group method with arithmetic averages (UPGMA) was used to generate cluster XLstat 7.5 (Addinsoft). The similarity percentage cut-off to distinguish between clonally distinct groups was set at 95%. In order to compare VFs score between clus-

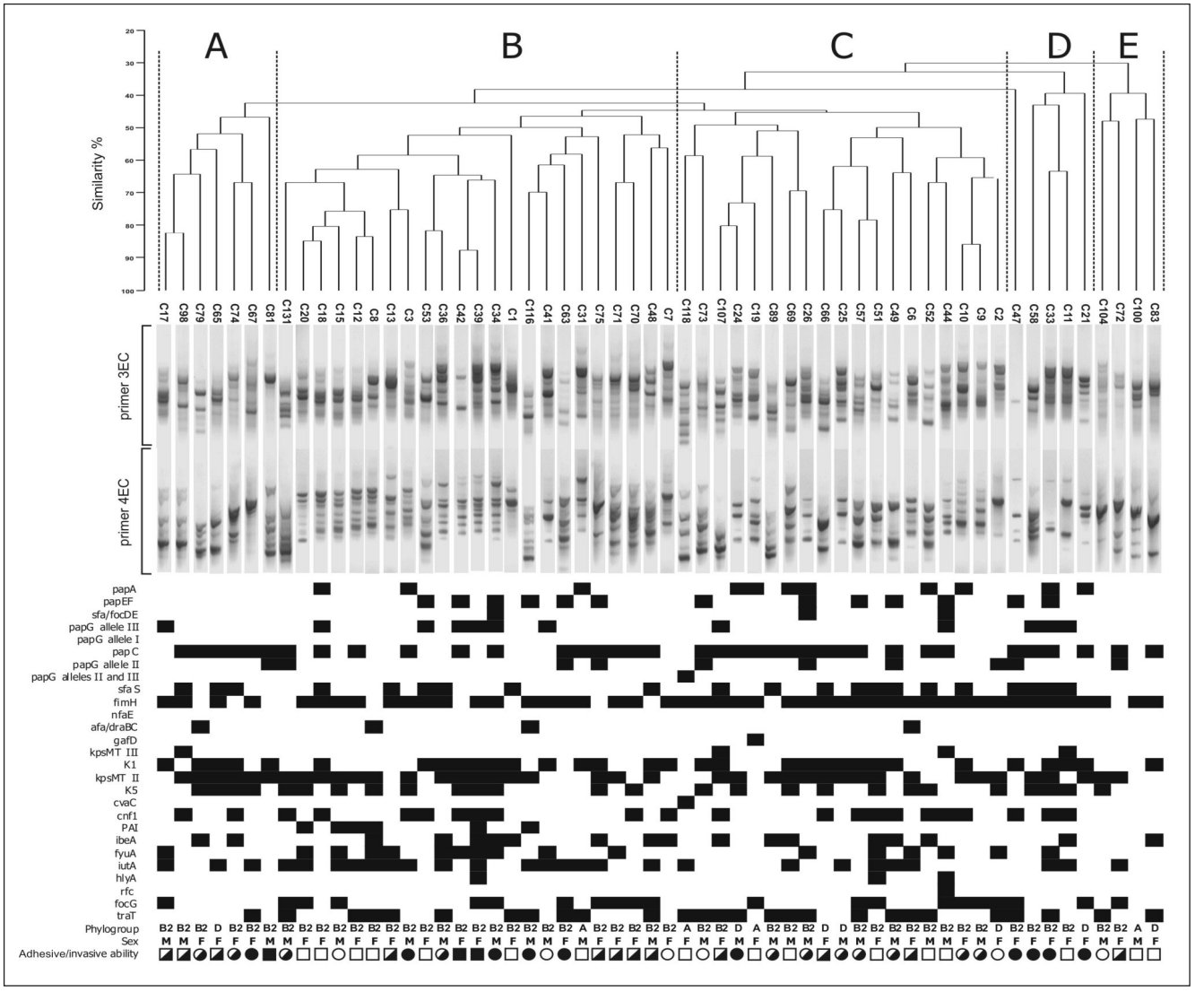

Figure 1 - *RAPD based dendrogram of E.coli isolates. Cluster analysis based on RAPD profiles using Pearson's correlation as the numeric coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm. Squares refer to non invasiveness, circles to invasiveness. Adhesion percentage is presented as symbol filling: solid ≥51%; solid/open between 11 and 50%; open ≤10%.*

Table 1 - *Age distribution of patients and characteristics of E.coli isolates.*

Patients		*E.coli invasiveness*				*Biofilm a*			*Antibiotic resistance b*																	
Age	N(M)	*Negative*		*Positive*					*AmC*		*KF*		*CTX*		*ATM*		*GM*		*CIP*		*SXT*		*NIT*		*FOS*		*None*	
		M	F	M	F	W	Me	S	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
<17	7(4)	1	0	3	3	4	1	2	2	2	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	2	1
						3	**1**	**2**	**2**	**2**	**1**	**1**					**1**			**1**			**1**				**1**	**1**
17-30	6(0)	0	3	0	3	1	4	1	N/A	3	N/A	1	N/A	0	N/A	0	N/A	1	N/A	0	N/A	1	N/A	1	N/A	0	N/A	2
							3			**1**		**0**						**0**				**1**		**0**				1
31-60	18(6)	1	8	5	4	6	7	5	1	4	2	0	1	0	1	0	2	1	1	2	1	3	1	0	0	0	3	7
						3	**3**	**2**	**1**	**2**	**2**		**1**		**1**		**2**	**0**	**1**	**0**	**1**	**2**	**1**				**2**	**2**
>60	27(15)	7	11	8	1	10	8	9	11	5	4	3	1	1	0	1	2	1	7	2	7	2	3	1	1	0	3	7
						1	**2**	**7**	**6**	**0**	**2**	**0**	**1**	**0**		**0**	**2**	**0**	**3**	**0**	**4**	**0**	**3**	**0**	**1**		**1**	**1**
Tot	58(25)	9	22	16	11	21	20	17	28		11		3		2		8		13		14		7		1		25	
						7	**9**	**11**	**14**		**6**		**2**		**1**		**5**		**5**		**8**		**5**		**1**		**9**	

N total number of patients; M male; F female.

[a]Weak (W), Medium (Me), Strong (S) E. coli biofilm producers. [b]AmC Amoxycillin- clavulanic acid, KF cephalexsin, CTX Cefotaxime, ATM aztreonam, GM gentamicin, CIP ciprofloxacin, SXT Sulfamethoxazole/Trimethoprim, NIT Nitrofurantoin, FOS fosfomycin, N/A not applicable. [a,b]Bold italics refer to invasive positive fraction of strains.

ters generated by RAPD profile analysis, a bilateral Mann-Whitney U-test was used. A p value ≤0.05 was considered statistically significant. In this study, we first analyzed the capacity of 58 UPEC strains to adhere and to invade prostate cells. The adhesion assays of UPEC strains to cell line, showed that the strains were able to adhere to RWPE-1 cells with different efficiency. The percentage of adhesive strains, in relation to the initial inoculum, was 97% (56/58), and of these 36% (20/56) showed a low efficiency of adhesion (≤10%), 41% (23/56) in the range of 11-50% and 23% (13/56) of the strains had adhesive capacity more than 51% (*Figure 1*). Regarding invasive ability, isolates that showed invasion level ≥0.1% were 47% (27/58) of the strains (*Table 1*).

Moreover, the adhesive ability was significantly associated with invasive phenotype (p=0.02): the strains able to adhere with an efficiency >11% to monolayers were 78% (21/27) in invasive strains and 48% (15/31) in non-invasive strains (Figure 1).

Respect to the age groups, invasive strains showed an association with patients less than 17 years old (6/7; 86%) (p=0.03). In the elders group a higher percentage of invasive strains was present in males (8/15) more than in female (1/12) (53% vs 8%, p=0.01), furthermore strong invasion ability (≥0.5%, data not shown) was associated with male patients (11/16 *vs* 3/11 in female, p=0.03), although the sample number was low. In invasive strains full susceptibility to antibiotics was not statistically significant with respect to non-invasive strains (33% *vs* 52%) (*Table 1*).

Multiple resistance was similarly present in invasive or non-invasive strains (22% and 29%); resistance to three or more drug classes was present in invasive strains from male patients (6/16) and absent from women (0/11), although not significant because of the sample size (data not shown). In biofilm formation assays, 17 out 58 (29%) strains were classified as strong biofilm producers, and 21 out 58 isolates (36%) were weak or non-biofilm producers. The invasive group displayed stronger biofilm formation capability 41% (11/27) with respect to non-invasive strains 19% (6/31) (*Table 1*). In the elderly (27/58), invasive strong biofilm producer strains were 70% (7/9) *vs* 12% (3/18) invasive low/medium biofilm producers (p=0.002), although the sample size was low. Among adhesion factors, *fim*H adhesin was the prevalent VF detected, occurring in about 81% of strains (47/58). The presence of papC and kpsMTII genes was statistically correlated to different degree of adhesive ability: among UPEC with a low (≤10%) adhesive ability papC and kpsMTII were both present in 14% (3/22) of the strains, and in 69% (9/13) among isolates with a high adhesive ability (p<0.01) (*Figure 1*). No substantial differences in the pattern of virulence factors were found between invasive and non-invasive strains. Genes such as *fim*H, *pap*C, *sfa*S were present equally in both invasive and non-invasive strains, whereas the percentage *pap*EF (33% *vs* 13%) and *pap*G allele II (26% *vs* 10%) was slightly different but not statistically significant. Prevalence of serum resistance-associated *tra*T gene and invasion of brain endothelium *ibe*A gene was 58% and 26% respectively in invasive strains *vs* 39% and 35% in non-invasive strains. Distribution of the VF genes among RAPD types showed that 52 strains belonged to three major clusters (i.e. A, B and C) (*Figure 1*).

No significant difference in the distribution of VF genes among the three clusters was observed except for genes *fyu*A (p=0.002) and *tra*T (p=0.04) that were predominant in clusters B and C respectively. Furthermore, no significant difference was found in the distribution of phylogroups, adhesiveness, invasiveness and VF score among the three major clusters. The results obtained from this study confirm the heterogeneity of UPEC strains regarding the adhesive/invasive ability and virulence factors occurrence although in our cell model most of the adhesive strains showed the ability to invade the prostate cells and to produce a strong biofilm (Mobley, 2016, Saldaña *et al*., 2014). It has been reported that a higher percentage of biofilm-producing *E. coli* strains is involved in acute prostatitis than in cystitis or pyelonephritis. This evidence could support the notion that bacterial colonization of the prostate may occur after bladder infection, and in turn, bacteria may spread from the prostate into the urinary tract resulting in recurrent infections (Soto *et al*., 2007). We

observed that the highest percentage of invasive strains with better invasive efficiency was found among *E. coli* isolated in male patients. Although the limitations of our study are the sample size and the lack of data due to the community origin of the patients, the results support the findings of Rudick *et al*. who described a different RWPE-1 cells invasion efficiency of a single *E. coli* isolated from a patient with chronic prostatitis with respect to a *E. coli* strain isolated from the urine of a woman with acute cystitis (Rudick *et al*., 2011).

Other studies regarding UTI pediatric patients reported that *E. coli* infections were more prevalent among males during the first year of life and among females thereafter. Although based on small sample, the invasive features of our strains were significantly associated with the pediatric cohort age group.

Moreover, in this study, it was not possible to associate a peculiar virulence spectrum to invasive ability, as also confirmed by RAPD analysis, supporting the hypothesis that multiple different factors, working in concert, may contribute to the invasive process (Lo *et al*., 2015).

Furthermore, it should be considered that not all bacterial genes putatively involved in the adhesion/invasion process were screened in this study and a number of unknown genes correlated to virulence undoubtedly remain uncharacterized. As regards susceptibility to antibiotics, we could not find any significant difference between invasive and non-invasive strains.

Although the resistance rate of *E. coli,* especially against aminopenicillins, antimicrobials that are commonly prescribed in children, is currently a matter of concern, in our study, the amoxicillin-clavulanic acid resistance rate was similar to that reported in other age groups. Jointly, our data suggest that some subtypes from the heterogeneous population of UPEC strains possess the ability to colonize and probably to persist in an intracellular milieu and that RWPE-1 cells could represent a useful cell model to study *E. coli*-epithelial cell interactions.

The identification of mechanisms that may facilitate UPEC adherence to and invasion of urinary cells may help to explain the susceptibility of some individuals to UTIs, potentially highlighting additional targets for therapeutic interventions. The determinants and strategies involved in the UPEC invasive step in prostate cells require further study and are now under our investigation.

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