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Virulence Potential of *Escherichia coli* Strains Causing Asymptomatic Bacteriuria during Pregnancy[▽]

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We compared the virulence properties of a collection of asymptomatic bacteriuria (ABU) *Escherichia coli* strains to urinary tract infection (UTI) strains isolated from pregnant women in a university hospital over 1 year. The *in vitro* and *in vivo* studies suggest that ABU strains presented a virulence behavior similar to that of strains isolated from cases of cystitis.

Urinary tract infections (UTIs) are bacterial infections (cystitis or pyelonephritis) frequently observed during pregnancy. Urinary tract colonization or asymptomatic bacteriuria (ABU) refers to situations where microorganisms are present at a significant rate (bacteriuria $\geq 10^5$ CFU/ml) in the urinary tract without clinical signs. This situation is very common during pregnancy (3 to 8%), with a peak incidence between the 9th and 17th weeks (26). *Escherichia coli* is the main pathogen found in this situation (27). Several concepts are based on acquired facts about ABU. First, microorganisms are the same in terms of species and virulence compared to ABU in nonpregnant women (24). Second, the main risk of the ABU is the occurrence of pyelonephritis in 30 to 40% of cases (10, 23). Therefore, the ABU must be systematically sought because of the risks of upper tract infection and fetal damage, mainly in the form of premature delivery (17, 25). A properly treated pyelonephritis will heal in a few days without sequelae. Finally, if ABU is not supported in time, it may progress to septic shock and/or a progressive deterioration of renal function to renal failure itself (9, 28). Proof of links between ABU and pyelonephritis are weak and based primarily on observational data. To date, few studies have focused on the virulence of *E. coli* isolated from UTIs or ABU in pregnant women (8, 24). This work has been conducted to assess the *in vitro* and *in vivo* virulence of uropathogenic *E. coli* (UPEC) isolated from pregnant women in different situations (ABU, acute cystitis, acute pyelonephritis, and urosepsis).

The prospective study was initiated on 1 January 2007 and carried out until 31 December 2007 in a French university hospital. During the follow-up of pregnancy, all women with a first episode of an ABU, an acute cystitis, an acute pyelonephritis, or an urosepsis in whom *E. coli* was detected were included. ABU was defined as urine specimens with an *E. coli* culture of $\geq 10^5$ CFU/ml without clinical signs. The cystitis and pyelonephritis strains have been isolated in monocultures from urine specimens of patients diagnosed with acute cystitis and acute pyelonephritis, respectively. The urosepsis isolates were cultured from the blood of patients suffering from UTI-derived sepsis. The genus and species were determined biochemically with the Vitek 2 identification card (bioMérieux, Marcy l'Etoile, France). Susceptibility to antimicrobial agents was tested by using the Vitek 2 card (bioMérieux). Strains were classified as susceptible, intermediately resistant, or resistant to the antibiotics tested according to the recommendations of the Antibiotic Susceptibility Testing Committee of the French Society for Microbiology (http://www.sfm.asso.fr/nouv/general.php?pa=2). Extended-spectrum β-lactamase (ESBL) production was confirmed by the double-disc synergy test (11). The genotypic characterization of ESBL resistance mechanisms was determined by PCR assays targeting bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ and identified by sequencing the PCR products (18). Phylogenetic grouping and serotyping of *E. coli* isolates were determined by a PCR-based method (3, 4). The *in vitro* virulence potential of *E. coli* strains was evaluated by PCR (5, 12–15) with the following genes: *papG* alleles I, II, and III; *papA*; *papC*; *papE*; *sfaS*; *focG*; *afa-draBC*; *fimH*; *hlyA*; *cnf1*; *iutA*; *irp2*; *iroN*; *kpsMT* II; *kpsMK* I; *traT*; and *malX.* Macrorestriction analysis of XbaI-digested chromosomal DNA was performed by pulsed-field gel electrophoresis (PFGE) with the CHEF DRII system (Bio-Rad) and analyzed with Gel Compar computer software (Applied Math, Kortrijk, Belgium) (19). The *in vivo* virulence of *E. coli* strains was investigated by the *Caenorhabditis elegans* model. The nematode infection assay using Fer-15 worms was carried out as described by Lavigne et al. (20). All experiments were conducted in triplicate and repeated at least 5 times for each selected strain. *E. coli* virulence was assessed using the nematode survival curve and calculating the LT_{50} and LT_{100}, the times required to kill 50% and 100% of the worms, respectively. Bacterial counts in the *C. elegans* digestive tract were carried out as described by Garsin et al. (7). For each, virulence factors (VFs), the number of genes (aggregated score), and comparisons

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TABLE 1. VFs associated with *E. coli* strains causing ABU, cystitis, pyelonephritis, and urosepsis in pregnant women[a]

VF	Association [no. present (%)] with strains causing:					*P*[b]		
	ABU (n = 37)	C (n = 26)	P (n = 14)	U (n = 37)	Total (n = 114)	ABU vs C	ABU vs P	ABU vs U
Genes								
Adhesins								
papG allele I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)			
papG allele II	13 (35.1)	10 (38.5)	12 (85.7)	29 (78.4)	64 (56.1)		0.004	0.005
papG allele III	9 (24.3)	7 (26.9)	0 (0)	3 (8.1)	19 (16.7)		0.005	0.05
No *papG* allele	15 (40.6)	9 (34.6)	2 (14.3)	5 (13.5)	31 (27.2)			
papA	12 (32.4)	9 (34.6)	10 (71.4)	25 (67.6)	56 (49.1)		0.03	
papC	7 (18.9)	6 (23.1)	9 (64.3)	23 (62.2)	45 (39.5)		0.005	0.01
papE	9 (24.3)	6 (23.1)	8 (57.1)	22 (59.5)	45 (39.5)		0.04	0.04
sfaS	3 (8.1)	3 (11.5)	0 (0)	3 (8.1)	9 (7.9)			
focG	13 (35.1)	7 (26.9)	9 (64.3)	0 (0)	29 (25.4)			<0.001
afa-draBC	12 (32.4)	8 (30.8)	6 (42.9)	2 (5.4)	28 (24.6)			0.01
fimH	33 (89.2)	25 (96.2)	13 (92.9)	34 (91.9)	105 (92.1)			
Toxins								
hlyA	23 (62.2)	16 (61.5)	11 (78.6)	6 (16.2)	56 (49.1)			<0.001
cnf1	11 (29.7)	11 (42.3)	0 (0)	3 (8.1)	25 (21.9)		0.01	0.04
Siderophores								
iutA	23 (62.2)	17 (65.4)	9 (64.3)	27 (73.0)	76 (66.7)			
irp2	24 (64.9)	21 (80.8)	14 (100)	35 (94.6)	94 (82.5)		0.02	
iroN	17 (45.9)	21 (80.8)	11 (78.6)	29 (78.4)	78 (68.4)	0.02		0.01
Capsules								
kpsMT II	23 (73.0)	21 (80.8)	12 (85.7)	32 (86.5)	88 (77.2)			
kpsMTK I	15 (40.5)	15 (57.7)	10 (71.4)	6 (16.2)	46 (40.4)			0.04
Miscellaneous								
traT	18 (48.6)	11 (42.3)	9 (64.3)	25 (67.6)	63 (55.3)			
malX	32 (86.5)	25 (96.2)	14 (100)	37 (100)	93 (81.6)			
Aggregated gene mean (SD, min–max)[c]	8.1 (3.04, 2–15)	9.2 (3.11, 3–16)	11.2 (2.15, 6–15)	10.5 (2.35, 5–15)	ND		0.001	0.01
Phylotypes								
A	6 (16.2)	1 (3.8)	0 (0)	0 (0)	7 (6.1)			
B1	4 (10.8)	2 (7.7)	0 (0)	3 (13.5)	9 (7.9)			
B2	18 (48.6)	12 (46.2)	9 (64.3)	13 (32.4)	52 (45.6)			
D	9 (24.3)	11 (42.3)	5 (35.7)	21 (45.9)	46 (40.4)			
Serotypes								
O:4	14 (37.8)	12 (46.2)	5 (35.7)	22 (59.4)	53 (46.5)			
O:6	6 (16.2)	3 (11.5)	7 (21.9)	9 (24.3)	25 (21.9)			
O:18	5 (13.5)	2 (7.7)	0 (0)	0 (0)	7 (6.1)			
O:75	3 (8.1)	6 (23.1)	0 (0)	0 (0)	9 (7.9)			
O:2	2 (5.4)	3 (11.5)	0 (0)	0 (0)	5 (4.4)			
Others	7 (18.9)	0 (0)	2 (14.3)	6 (16.2)	15 (13.2)			

[a] C, cystitis; P, pyelonephritis; U, urosepsis.
[b] *P* values (χ^2 test, Fisher's exact test, Wilcoxon test) are shown where *P* is ≤0.05.
[c] min, minimum; max, maximum; ND, not determined.

between the different groups (ABU, cystitis, pyelonephritis, and urosepsis) were evaluated by using the χ^2 test or Fisher's exact test and the Kruskal-Wallis test, respectively. To assess the utility of combining several virulence markers to predict UTIs, we used a logistic regression with a backward procedure to select the most relevant markers; only markers for which the area under the curve for the receiver-operator characteristic (AUC_{ROC}) was greater than 0.80 were initially entered as explanatory variables in the regression analysis. A ROC curve was then generated for the combination derived from the regression model and its area compared with that of every single virulence marker by a nonparametric method adapted to paired data (6). A *P* value of ≤0.05 was considered as reflecting statistical significance. A log rank test was used to compare the entire survival curves in nematode-killing assays. The analysis was carried out using SAS/ETS software release 8.1 (SAS Institute Inc., Cary, NC).

During the studied period, 114 *E. coli* strains were isolated from 114 women (median age, 28.5 [range, 19 to 42]): 37 (32.5%) with ABU, 26 (22.8%) with cystitis, 14 (12.3%) with pyelonephritis, and 37 (32.5%) with urosepsis. All isolates were susceptible to carbapenems. Five strains (4.3%) were resistant to cefotaxime and produced a CTX-M-15 β-lactamase. Susceptibility was observed with amikacin (98.2%), fosfomycin (98.2%), nitrofurantoin (84.2%), ciprofloxacin (80.7%), co-trimoxazole (76.3%), amoxicillin-clavulanic acid (61.4%), and amoxicillin (51.8%). No difference was observed among the different groups. PFGE revealed a high level of genomic diversity between each strain and among each group (data not shown). The virulence profiles of strains isolated in this study are presented in Table 1. The ABU strains mainly belonged to phylotype B2 and serotype O:4, and the *fimH* (89.2%) and *malX* (86.5%) virulence factors were more frequently found in

TABLE 2. LT_{50} and LT_{100} of *C. elegans* infected by *E. coli* isolated from pregnant women[a]

Status/strain	Phylotype	Serotype	No. of VFs	LT_{50} (SD) (days)	LT_{100} (SD) (days)	P^b			
						NECS26375	NECS173730	NECS690406	NECS621464
ABU									
NECS26375	A	O:4	7	5.1 (0.1)	9.7 (0.7)		NS	<0.001	<0.001
NECS173730	B1	O:6	2	5.4 (0.2)	10.0 (0.5)	NS		<0.001	<0.001
NECS690406	B2	O:4	9	3.2 (0.2)	7.4 (0.6)	<0.001	<0.001		NS
NECS621464	D	O:4	7	3.3 (0.1)	7.6 (0.6)	<0.001	<0.001	NS	
Cystitis									
NECS992953	A	O:2	7	5.0 (0.2)	9.4 (0.6)	NS	NS	<0.001	<0.001
NECS698674	B1	O:4	6	5.5 (0.2)	9.9 (0.9)	NS	NS	<0.001	<0.001
NECS978323	B2	O:4	15	3.1 (0.1)	6.8 (0.8)	<0.001	<0.001	NS	NS
NECS55403	D	O:4	7	3.4 (0.1)	7.4 (0.6)	<0.001	<0.001	NS	NS
Pyelonephritis									
NECS914883	B2	O:4	11	3.4 (0.1)	7.7 (0.7)	<0.001	<0.001	NS	NS
NECS37344	D	O:4	11	3.6 (0.1)	8.0 (0.5)	<0.001	<0.001	NS	NS
Urosepsis									
NECS123517	B1	O:6	4	5.3 (0.1)	9.8 (0.8)	NS	NS	<0.001	<0.001
NECS198654	B2	O:4	7	3.3 (0.1)	7.2 (0.8)	<0.001	<0.001	NS	NS
NECS924212	D	O:4	11	3.4 (0.1)	7.7 (0.7)	<0.001	<0.001	NS	NS
Control strain									
OP50				7.1 (0.2)	10.8 (0.8)	<0.001	<0.001	<0.001	<0.001

[a] For each group of isolates (ABU, cystitis, pyelonephritis, urosepsis), strains representative of the results are presented. The data are representative of at least three independent trials for each group of strains. OP50 is an avirulent *E. coli* strain used as a control.
[b] *P* value for LT_{50} (indicated ABU strain versus other strains). NS, not significant.

these strains. The aggregated score was 8.1. Univariate analysis showed that the profile of VFs detected in the ABU group was similar to the profile of VFs found in the cystitis group but was different from the profile of VFs found in the pyelonephritis and urosepsis groups ($P < 0.001$). *papG* allele II, *papG* allele III, *papA*, *papC*, *papE*, *iroN*, *irp2*, and *malX* were more frequently found for pyelonephritis and *cnf1* was more frequently found for ABU ($P < 0.05$) as previously noted (2). The multivariate analysis selected *cnf1* as independent factors linked to a decreased association of pyelonephritis (odds ratio [OR], 0.89; 95% confidence interval [$CI_{95\%}$], 0.54 to 0.9; $P = 0.005$). The AUC_{ROC} for the *cnf1* gene (OR, 0.946; $CI_{95\%}$, 0.863 to 1.000) demonstrated an excellent tool to discriminate between ABU and pyelonephritis (sensitivity, 0.703 [standard deviation (SD), 0.025]; specificity, 1.000 [SD 0.063]). For urosepsis, *papG* allele II and *irp2* were the most prevalent genes as previously observed (1). The low prevalence of the *hlyA* gene in the urosepsis strains was surprising. However, this gene was more prevalent in cystitis than in pyelonephritis (22). The multivariate analysis selected *afa-draBC*, *hlyA*, and *foc* as independent factors linked to a decreased association of urosepsis (OR [$CI_{95\%}$]: 0.69 [0.53 to 0.89], 0.77 [0.62 to 0.95], and 0.65 [0.49 to 0.88], respectively; $P < 0.01$). From the logistic model, the combination of *afa-draBC*, *hlyA*, and *foc* was the most predictive for distinguishing ABU and urosepsis: AUC_{ROC} was 0.875 ($CI_{95\%}$, 0.788 to 0.962). The *afa-draBC* and *foc* genes were previously described as predictors of cystitis, a result similar to our study (16). Concerning the phylotyping groups, no difference could be detected among the groups, suggesting that ABU strains were phylogenetically related to strains that cause symptomatic UTIs as previously noted in other ABU situations (21). The aggregated scores for ABU and cystitis were not different (9.2) ($P = 0.24$, Wilcoxon test), but those for pyelonephritis (11.2) and urosepsis were significantly different (11.5) ($P < 0.001$). To explain this difference, Zdziarski et al. (28) showed that the genomes of ABU strains were related to UPEC but have smaller genome sizes, with alterations in essential virulence genes. In the aim to confirm this low virulence potential of ABU strains, we used a validated *in vivo* nematode model. The *C. elegans* study showed that ABU strains belonging to the two virulent phylotypes (B2 and D) have the same virulence as the strains isolated from cystitis, pyelonephritis, and urosepsis belonging to the same phylotypes (LT_{50}s: 3.2 to 3.3 days ± 0.2 [ABU] versus 3.1 to 3.4 days ± 0.1 [cystitis], 3.4 to 3.6 days ± 0.1 [pyelonephritis], and 3.3 to 3.4 days ± 0.1 [urosepsis]; *P* value nonsignificant) (Table 2). On the other hand, strains isolated from ABU, cystitis, or urosepsis and belonging to the two commensal phylotypes (A and B1) had a low virulence (LT_{50}s: 5.1 to 5.4 days ± 0.2 [ABU], 5.0 to 5.5 days ± 0.2 [cystitis], 5.3 days ± 0.1 [urosepsis]) (Table 2). The number of *E. coli* CFU within the nematode gut varied around 10^5 bacteria per worm for each strain 72 h after ingestion without statistical difference (data not shown), confirming the ingestion and proliferation of *E. coli* isolates in the *C. elegans* intestine. These results show (i) a clear correlation between ABU strains' ability to kill *C. elegans* and the phylotyping origin harbored by *E. coli* strains, whatever the clinical manifestations, and (ii) a clear potential for virulence of the ABU strains.

In conclusion, even if ABU strains have been described to have a reductive evolution of their genomes, the strains isolated from pregnant women remain as virulent as strains isolated from cystitis. Numerous factors (modulation of immune response, variation of adhesion and biofilm formation, envi-

ronment, etc.) could influence the expression of VFs and allow the adaptation of the ABU strains to growth in the urinary tract.

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