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Dr Operon-Associated Invasiveness of *Escherichia coli* from Pregnant Patients with Pyelonephritis

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We used a gentamicin protection assay to assess the ability of gestational pyelonephritis isolates of *Escherichia coli* to invade HeLa cells. The ability to enter HeLa cells was strongly associated with the presence of Dr operons coding for Dr adhesins. In contrast, the noninvasive isolates predominantly expressed *papG*, coding for P fimbriae.

Pyelonephritis in pregnant patients is a serious complication that may lead to severe sequelae such as bacteremia, urosepsis, adult respiratory distress syndrome, and death (16). The course of the pregnancy may be affected, resulting in preterm labor or intrauterine growth retardation (2). *Escherichia coli* remains the primary cause of renal infections in pregnant patients, accounting for 65 to 80% of cases (11). *E. coli* strains isolated from pregnant women with pyelonephritis are genetically closely related and express gestational age-dependent profiles of virulence factors such as Dr and P fimbriae (7, 13). Interaction of recombinant *E. coli* bearing Dr adhesin with receptor decay-accelerating factor (DAF; CD55) on HeLa cells mediates bacterial invasion into epithelial cells (6). Moreover, Dr-positive *E. coli* is able to kill pregnant rats while not affecting nonpregnant animals (12). Mutation of the *E. coli* Dr operon results in abolishment of bacterial invasion and abrogates the development of experimental chronic interstitial nephritis in mice (5). Our general hypothesis is that an experimental lethality of Dr-positive *E. coli* for pregnant animals may account for their epidemiological association with pyelonephritis in pregnant patients and suggest unique gestational virulence. In this report, we evaluated the hypothesis that expression of the Dr family of adhesins in clinical gestational isolates of *E. coli* is associated with invasive properties. We also assessed whether other common virulence traits encountered in uropathogenic *E. coli*, such as P fimbriae and α-hemolysin, may be associated with bacterial entry into HeLa epithelial cells.

In the first set of experiments, we tested the invasive properties of 73 gestational isolates of *E. coli* derived from pregnant patients hospitalized due to pyelonephritis at the University of Texas Medical Branch at Galveston between 1996 and 1999. Strains were selected on the basis of a positive urine culture and clinical symptoms. The standard gentamicin protection assay was performed on human cervical cell line HeLa (ATCC CCL2) as described previously to evaluate the ability of *E. coli* isolates to enter epithelial cells (6). The invasion rate was expressed as the percentage of the initial bacterial inoculum (1.36×10^8) that was recovered after treatment of the HeLa cell monolayer with antibiotic and subsequent lysis with detergent. Isolates which yielded fewer than 0.001% survivors were characterized as noninvasive. This criterion is based on our previous studies, which revealed that a survival rate of <0.001% characterized Dr-negative mutants of clinical or laboratory recombinant strains that are not able to invade HeLa cells as further assessed by electron microscopy (6).

The estimation of HeLa cell-detaching activity was an integral part of the invasion assay. We anticipated that infection of epithelial cells with certain gestational pyelonephritis *E. coli* strains may result in destruction of the monolayer and, by decreasing the number of HeLa cells, artificially lower the rate of invasion. To evaluate the magnitude of detached monolayer, HeLa cells were examined under an inverted microscope after incubation with antibiotic solution and phosphate-buffered saline (PBS) washings but prior to the addition of lysis solution. The intact monolayer in control, noninfected wells was reported as 100% HeLa cells available for lysis. Any deficiencies in the integrity of the monolayer were reported as a percentage of missing monolayer and ranged from 20 to 50% of HeLa cells unavailable for lysis.

The results of the invasion assay for *E. coli* that demonstrated cell-detaching activity were corrected upwards by increasing the number of *E. coli* CFU growing on agar plates proportionally to the percentage of missing monolayer. This adjustment may reflect the invasive potential of cell-detaching positive *E. coli*. It is based on the assumption that bacterial cells can interact and invade epithelial cells within the entire monolayer area, but a concomitant cell-detaching activity might destroy a part of already invaded cells.

All gestational *E. coli* isolates were also tested for adherence to HeLa cells. Briefly, bacterial suspensions made in PBS (optical density at 600 nm [OD_{600}] of 0.1) were incubated with monolayer for 3 h at 37°C in CO_2. Bacterial suspensions were discarded, and cells were fixed with formaldehyde, stained

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TABLE 1. Distribution of fimbrial genotypes between invasive and noninvasive phenotypes of gestational pyelonephritis *E. coli* isolates

Phenotype (no. of isolates)	No. (%) of isolates with isolates with fimbrial genotype:			
	dra	*dra*/*pap*	*pap*	Non-*dra*/non-*pap*
Invasive (23)	14 (61.0)	6 (26.0)	2 (9.0)	1 (4.0)
Noninvasive (50)	3 (6.0)	1 (2.0)	38 (76.0)	8 (16.0)

TABLE 2. Distribution of fimbrial genotypes between hemolytic and cell-detaching gestational pyelonephritis *E. coli* isolates

Phenotype (no. of isolates)	No. (%) of isolates with fimbrial genotype:			
	dra	*dra*/*pap*	*pap*	Non-*dra*/non-*pap*
Hemolysis (23)	0	3 (13.0)	17 (74.0)	3 (13.0)
Cell detachment (23)	4 (17.0)	2 (9.0)	14 (61.0)	3 (13.0)

overnight with Giemsa, and evaluated under the light microscope. The IH4 monoclonal anti-DAF antibody was used to estimate whether blocking of short consensus repeat 3 (SCR3) of the DAF domain might interfere with binding of invasive isolates to HeLa cells.

In the next step we used PCR to investigate whether the presence of Dr and P operons was associated with the internalization of HeLa cells. The *dra*-positive strains were identified with previously described primers that amplify a 750-bp *Pst*I fragment of the *afaB* gene (14). This gene is necessary for biogenesis of the adhesin and is conserved among Dr-related operons. We also used PCR to identify three variants of *papG* genes encoding receptor specificity for *E. coli* PapG adhesins for P antigens. Primer pairs specific for three *papG* classes were selected according to published sequences (8). Amplification reactions were done as described previously (14). Phenotypic expression of P fimbriae was confirmed by hemagglutination (HA) with 3% (vol/vol) human erythrocytes and inhibition of HA by 0.5% Gal-1-4Gal. Hemagglutination with a 3% PBS suspension of human erythrocytes preincubated or not with IH4 anti-DAF antibody was used to confirm phenotypic expression of the Dr family of adhesins. Hemolytic activity was assessed by growing *E. coli* strains for 24 h at 37°C on Trypticase soy agar with 5% defibrinated sheep blood. A zone of clearing around the area of bacterial growth indicated an α-hemolytic strain.

Overall, out of 73 gestational *E. coli* isolates, 61 (84%) displayed binding to the monolayer, with average adherence rate ranging from 1 to more than 20 bacterial cells associated with a single HeLa cell. Invasive isolates occurred only in the group of adherent *E. coli*. We found that 23 (32%) gestational pyelonephritis *E. coli* strains were able to enter HeLa cells. The average adjusted internalization rate for invasive strains was 0.016% of initial CFU (standard deviation [SD] ±0.0104%). Of 50 noninvasive isolates, 46 displayed zero invasion, and four isolates were reported with a detectable rate of invasion of 0.001% of CFU (SD ±0.0003%). The equality of the distribution of fimbrial genotypes between invasive and noninvasive phenotypes was tested with Stata Statistical Software, release 6.0 (Stata Corporation, College Station, Tex.) using a generalization of the Fisher exact test for a 4×2 contingency table (Table 1) (4). The distributions were statistically significantly different ($P < 0.001$). We then combined the first two columns and the last two columns, allowing us to compare the *dra* and non-*dra* genotypes. Again the distributions were significantly different ($P < 0.001$). The analysis indicates that internalization of HeLa cells was strongly associated with the presence of Dr operons, as evidenced by amplification of *dra* sequences. The *dra*-positive *E. coli* isolates comprised 87% of all invasive isolates and displayed an average invasion rate of 0.017% (SD ±0.011). It is worth noting that within the group of invasive *dra*-positive *E. coli*, six isolates that simultaneously carried *pap* sequences demonstrated a different, notably lower invasion rate (0.011% of CFU; SD ±0.007) compared to *dra*-positive, *pap*-negative isolates (0.019% of CFU; SD ±0.011) ($p > 0.05$ as calculated by Student's *t* test) (Table 1). Out of four *dra*-positive, invasion-negative isolates, three were *pap* negative and showed a low level of adherence to the HeLa cell monolayer that correlated with a lack of mannose-resistant agglutination of human erythrocytes. One noninvasive isolate, which was typed as *dra*/*pap*-positive, showed MRHA and moderate binding to HeLa cells. The *pap*-positive isolates prevailed in the group of noninvasive gestational *E. coli*. Only two *pap*-positive isolates demonstrated a low level of invasiveness (Table 1). Preincubation of HeLa cells with anti-DAF IH4 monoclonal antibody completely blocked or significantly reduced adherence of *dra*-positive invasive *E. coli* but not *pap*-positive or non-*dra*/non-*pap* invasive isolates.

We attempted to assess the association of fimbrial genotype with hemolytic activity and the ability to detach the HeLa cell monolayer. Overall, 23 (31.0%) gestational isolates of *E. coli* exhibited hemolysis on blood agar plates. Hemolytic *E. coli* prevailed in the group of *pap*-positive, noninvasive gestational isolates (Table 2). We did not find any hemolysin producers among the *dra*-positive, *pap*-negative isolates. However, the three *dra*-positive, *pap*-positive *E. coli* isolates exhibited this phenotype when grown on blood agar plates. The ability to produce α-hemolysin was usually associated with cytotoxic activity to HeLa cells, manifested as partial detachment of the monolayer during the 3-h invasion assay. Among 23 hemolytic *E. coli*, 19 (78%) caused HeLa cell damage ranging from 20 to 50% of the area of monolayer.

This study shows that the presence of the Dr operon in gestational pyelonephritis *E. coli* isolates is associated with the invasive phenotype. In contrast, the expression of P fimbriae does not appear to contribute to invasiveness in the tested system. Bacterial binding to the DAF receptor and the expression of Dr-related operons appear to be prerequisites for invasion. All nonadherent isolates and the majority of adherent *pap*-positive isolates were unable to enter HeLa cells. The majority of noninvasive *dra*-positive isolates exhibited a low level of adherence; presumably, low expression of Dr adhesin may account for decreased or lack of invasiveness. Moreover, preincubation of the HeLa cell monolayer with anti-DAF antibody significantly reduced adherence of *dra*-positive but not *pap*-positive *E. coli*. This finding reinforces the notion that internalization of *dra*-positive *E. coli* is triggered by interaction of bacterial Dr adhesin with the cell receptor DAF (15).

Interestingly, the reported increase in representation of *dra*-

positive *E. coli* in the group of third-trimester isolates may coincide with increased expression of tissue DAF receptor (13). Temporary changes in the density of DAF have been reported as a response to increased levels of progesterone (9). An elevated level of DAF expressed on the cell surface may thus provide more binding sites for *E. coli* bearing adhesins of the Dr family and increase the risk of colonization and infection late in pregnancy.

The majority of *dra*-positive gestational *E. coli* isolates did not produce hemolysin and did not demonstrate cell-detaching activity. Rare association between hemolytic properties and expression of Dr adhesins has been previously reported for *E. coli* strains isolated from first-time urinary tract infection patients (3). Cell-detaching activity was also identified among fecal isolates and was associated mainly with the ability to lyse sheep erythrocytes and hybridization with an *hly* probe (10). In contrast, *pap* sequences are frequently carried in *E. coli* strains able to produce hemolysin (1). Overall, P-fimbriated strains were noninvasive in HeLa cells. The hypothetical lack of invasiveness due to the masking effect of hemolysin-mediated destruction of HeLa cells was less likely, because for P-fimbriated, hemolysin-positive cells the invasion rate was corrected for the percentage of detached monolayer. One can speculate that hemolysin-associated detachment of superficial epithelial layers may enhance bacterial translocation to otherwise not accessible deeper renal tissue, thus promoting invasiveness during the first and second trimesters, when the P-fimbriated *E. coli* prevail (6).

In conclusion, infections during pregnancy with *E. coli* bearing adhesins of the Dr family may pose a threat for patients due to bacterial invasive potential and pregnancy-associated upregulation of DAF receptor. Further clinical studies are needed to elucidate how infection with Dr-positive *E. coli* affects the outcome of pregnancy.

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