Uropathogenic *Escherichia coli* Isolates from Pregnant Women in Different Countries

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Urinary tract infection (UTI) is common during pregnancy and can be associated with negative outcomes for both the mother and fetus. Increased risk of infection among these patients has been attributed to physiological changes, and less focus has been placed on *Escherichia coli*, the most frequent causative agent. We investigated the virulence properties of isolates causing UTI in pregnant women in Sweden, Uganda, and Vietnam, as well as nonpregnant women in Sweden. Although phylogenetic group B2 was the most prevalent group, more Ugandan isolates belonged to group B1, associated with commensal strains, than isolates from other countries. Adherence to and invasion of urothelial cells, key events in the infection process, were low among group B1 isolates from pregnant Swedish women compared to those from nonpregnant patients. Similar levels of adherence and invasion were seen in isolates from pregnant women in Uganda and Vietnam. More biofilm was formed by group B2 isolates than by those belonging to group B1 and by Ugandan group B2 isolates than by those from pregnant Swedish and Vietnamese women. The antigen 43a-encoding gene, *fluA* tipF073, was most prevalent among Ugandan isolates. Expression of the biofilm components, curli and cellulose, was low among all isolates. Multidrug resistance was more common among isolates from Uganda and Vietnam than among those from Swedish patients. We suggest that while bacterial virulence properties play an important role in UTI during pregnancy, physiological changes in the host may contribute more to the incidence of infection caused by less virulent *E. coli*.

**MATERIALS AND METHODS**

**Patients.** Midstream urine samples from pregnant women with acute UTI caused by *E. coli* were collected at antenatal clinics at Karolinska University Hospital, Stockholm, Sweden; Mulago Hospital, Kampala, Uganda; and the National Hospital of Obstetrics and Gynecology, Hanoi, Vietnam, between September 2009 and January 2012. Patients were 17 to 48 years old and had gestational ages ranging from 4 to 40 weeks (Table 1). As controls, samples from nonpregnant age-matched women with UTI caused by *E. coli* were collected from outpatient clinics in Stockholm, Sweden. Only patients not taking antibiotic treatment at the time of sampling were recruited for the study.

**Bacterial species identification and culture.** In all, 148 *E. coli* isolates were obtained from urine samples from pregnant patients and 50 isolates were collected from nonpregnant patients (Table 1). Species identification was first performed at the hospitals at which samples were collected. Thereafter, *E. coli* identification was confirmed using the Vitek 2 Gram-Negative Identification Card (bioMérieux, Marcy l’Etoile, France), as previously described (21). Vitek screening and all subsequent tests in this study were performed at the Department of Clinical Microbiology, Karolinska University Hospital.

*E. coli* isolates were grown overnight at 37°C on blood agar or for 24 h on LB agar without salt for infection or biofilm assays, respectively. Colonies were suspended in phosphate-buffered saline (PBS) and centrifuged.

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**Urinary tract infection (UTI)** is the predominant type of bacterial infection among pregnant women (7, 18). As many as 90% of UTIs are caused by *Escherichia coli* (5). Hormonal and physiological changes in the urinary tract, including ureteral dilation and changes in bladder volume and tone, may promote infection in pregnant women (18). Interestingly, the incidence of UTI during pregnancy is higher among women who have had childhood infections (13). Furthermore, it has been observed that pregnant women have a propensity to develop recurrent UTIs (18).

Overall, UTI can be dangerous for both the mother and fetus. Complications that can arise include preterm delivery and increased incidence of intrauterine growth restriction. To a lesser degree, preeclampsia, caesarean delivery, anemia, sepsis, and septic shock may also be associated with UTI in these patients (14). Among patients suffering acute pyelonephritis, those who are pregnant are more likely than nonpregnant women to develop renal scars (22).

The possession of virulence factors that enable colonization is important in the pathogenesis of uropathogenic *E. coli* (UPEC). It has been demonstrated that among isolates causing acute pyelonephritis in pregnant women, expression of type 1, P, and Dr fimbriae can vary by gestational age (19). However, little is known about the prevalence of other *E. coli* virulence-associated factors, including those known to play a role in long-term survival in the human host.

The aims of this study were to investigate the impacts of virulence properties of *E. coli* isolates causing UTI in pregnant women in different countries. Therefore, the ability to cause infection and the prevalence of virulence factors associated with persistence and survival, as well as antibiotic resistance levels, were investigated.
TABLE 1 Patient and gestational ages of pregnant and nonpregnant women who presented with UTI caused by E. coli in Sweden, Uganda, and Vietnam

<table>
<thead>
<tr>
<th>Patient group and country</th>
<th>Patient age (yr)</th>
<th>Gestational age range (wk)</th>
<th>No. (%) of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[median (range)]</td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Pregnant</td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Sweden</td>
<td>32 (19–42)</td>
<td>6–40</td>
<td>56</td>
</tr>
<tr>
<td>Uganda</td>
<td>23 (17–45)</td>
<td>8–32</td>
<td>42</td>
</tr>
<tr>
<td>Vietnam</td>
<td>31 (21–48)</td>
<td>4–39</td>
<td>50</td>
</tr>
<tr>
<td>Nonpregnant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweden (control)</td>
<td>29 (18–45)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

at 300 × g for 10 min to remove bacterial aggregates. The concentrations of bacterial suspensions were adjusted in PBS spectrophotometrically and confirmed by viable count.

In vitro assays. (i) Cell culture. Human bladder T24 (HTB-4; ATCC) cells were grown in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS). For experiments, cells were grown to confluence in 24-well cell culture plates (Costar, Corning, NY) in a humidified incubator with 5% CO₂.

(ii) Infection experiments. Adherence and invasion were analyzed using a modification of the previously described method (12). Cells were infected with 10⁶ CFU/ml of bacteria, centrifuged (600 × g for 5 min), and incubated for 30 min at 37°C with 5% CO₂. For adherence assays, cells were washed with PBS, lysed with 0.1% Triton X-100–0.5% trypsin in PBS, serially diluted, and plated on blood agar plates. To test invasion, cells were infected and washed as for the adherence assay. Thereafter, fresh medium was added, and the cells were incubated for a further 30 min. Next, the cells were washed with PBS and incubated for 30 min in fresh medium containing 100 μg/ml gentamicin. The cells were then washed, lysed, and plated as described above. Bacterial colonies were counted following overnight culture at 37°C. Adherence efficiency was calculated as the proportion of total cell-associated bacteria in the viable count of the original bacterial suspension. The invasion efficiency was assessed by calculating the proportion of intracellular bacteria in the total cell-associated bacteria in parallel experiments. Isolates that were gentamicin resistant were excluded due to the use of a gentamicin protection invasion assay. Therefore, the group B1 isolates that were tested included those from pregnant women in Sweden (n = 3), Vietnam (n = 2), and Uganda (n = 12) and nonpregnant Swedish controls (n = 3), as well as 3 group B2 isolates, with virulence factor gene profiles similar to those of group B1 isolates from each country/patient group.

(iii) Biofilm formation. The ability to form biofilm was assessed using a microtiter plate method. All group B1 and B2 isolates that were tested for adherence and invasion, as well as two more group B1 isolates that were gentamicin resistant (one each from Uganda and Vietnam), were tested for biofilm formation. Bacterial suspensions in PBS (10⁸ CFU/ml) were further diluted 1:100 in LB broth without salt. Thereafter, 200 μl of suspension was aliquoted in triplicate into 96-well microtiter plates (Costar; Corning). The plates were incubated for 24 h at 37°C. Bacterial growth was measured at 630 nm. The bacterial suspensions were aspirated, and the wells were carefully washed with PBS and air dried. The biofilm was stained with crystal violet (3%) (BD, Franklin Lakes, NJ). Next, the dye was solubilized using 20% acetone–80% ethanol, and the optical density was read at 550 nm. Biofilm formation was calculated as the proportion of the optical density of the solubilized crystal violet to the optical density of the bacterial growth.

(iv) Expression of type 1 fimbriae. To elucidate the possible effects of type 1 fimbriae during adherence, invasion, and biofilm testing, type 1 fimbrial expression was investigated under the specific growth conditions for each assay. Briefly, isolates were grown on blood agar or LB agar without salt, as described above. The bacteria were then suspended in PBS (approximately 10¹⁰ CFU/ml). Mannose-sensitive agglutination was tested by mixing bacteria in equal parts with a suspension of baker’s yeast (Saccharomyces cerevisiae, 3% in PBS), as previously described (10). Inhibition of agglutination by mannose (5% in PBS) was used to confirm the specificity of the reaction.

PCR amplification of virulence factor genes. A modified boiling method was used to extract whole-cell DNA from E. coli isolates (21). One or two colonies of bacteria grown on blood agar were suspended in 100 μl sterile deionized water and boiled at 99°C. Following centrifugation, the supernatant, containing the DNA, was stored at −20°C.

A previously described triplex PCR was used to phylogenetically group isolates (1, 21). Genes, including flu, the allelic variants fluA_CFT073 and fluB_CFT073 (21), tcpC (2), and iroN_E.coli (1), were investigated using previously described primers. In uniplex PCRs, amplification reaction mixtures contained DreamTaq Green PCR Master Mix (Fermentas, Thermo Fisher Scientific, Waltham, MA), 0.4 μM each appropriate forward and reverse primer, and 1 μl of DNA template. The amplification conditions for the flu gene consisted of initial denaturation at 95°C for 1 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min; and one final extension cycle of 72°C for 5 min. Amplification conditions for the genes fluA_CFT073, fluB_CFT073, and tcpC were as for the flu gene except for the annealing temperatures, which were 65°C, 63°C, and 53°C, respectively. For the iroN_E.coli gene, amplification consisted of heating to 95°C for 1 min; 25 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 1 min; and one final extension cycle of 72°C for 5 min. Strain UPEC CFT073 was used as the positive control for all virulence factor genes tested. PCRs, gel electrophoresis, and visualization following GelRed (Biotium, Hayward, CA) or ethidium bromide staining were performed as previously described (21).

Expression of curli and cellulose. The expression of curli and cellulose was determined by inspection of the colony morphology of isolates after 48 h of growth on Congo red and Calcofluor agar, as previously described (21).

Antibiotic susceptibility testing. Antibiotic susceptibility testing of all isolates was performed using the Vitek 2 Antimicrobial Susceptibility Tests (AST-N106) (bioMérieux) as previously described (21). Extended-spectrum beta lactamase (ESBL)-producing isolates were identified by the Vitek 2 Susceptibility Tests. Isolates that were resistant to antibiotics belonging to three or more different classes were classified as multidrug resistant (MDR) (4).

Statistical analysis. Differences between countries were assessed using a combination of χ² and pairwise Fisher’s exact two-tailed tests, as previously described (21); a P value of <0.01 was considered significant. For individual countries, correlations between antibiotic resistance, phylogenetic group, and virulence factor genes were assessed using Fisher’s exact two-tailed test. Data from in vitro experiments were compared by Kruskal-Wallis analysis of variance (ANOVA), followed by multiple comparisons of mean ranks, as appropriate. A P value of <0.05 was considered significant. Statistical analyses were performed using GraphPad (San Diego, CA) Prism, version 5.02, and Statistica (Tulsa, OK) Statsoft version 7.0.
RESULTS

Phylogenetic groups among isolates from different countries. *E. coli* isolates causing UTI in pregnant and nonpregnant women belonged to all four phylogenetic groups; however, group B2 was the most prevalent group among isolates from Sweden and Vietnam (Fig. 1). Conversely, fewer Ugandan isolates belonged to group B2 (*P*/H11005 0.03) while more belonged to group B1 (*P*/H11005 0.02) in comparison to isolates from pregnant women in Sweden (Fig. 1). However, these differences did not meet the strict criteria for statistical significance employed in this study. In all, 9 (6%) isolates did not carry any of the phylogenetic grouping genes and were therefore classified as nontypeable. No associations were found between phylogenetic groups and the trimester of pregnancy during which isolates were collected (data not shown).

Adherence and invasion abilities among UPEC isolates. In view of our finding of increased prevalence of group B1 isolates but fewer group B2 isolates from Ugandan patients (Fig. 1), we next investigated the isolates’ abilities to cause infection according to their phylogenetic groups. Interestingly, group B1 isolates from pregnant women in Sweden adhered and invaded less than corresponding control group B1 isolates from nonpregnant Swedish women (*P*/H11021 0.05) (Fig. 2A and B). While group B1 isolates from Swedish pregnant women also adhered less than those from Ugandan women (*P*/H11021 0.05) (Fig. 2A), no differences in adherence and invasion efficiencies were seen among group B1 isolates from Uganda and Vietnam (Fig. 2A and B). Among isolates from pregnant Swedish women, those belonging to group B2 invaded more than group B1 isolates. However, adherence and invasion abilities of isolates from Uganda and Vietnam were similar between the two phylogenetic groups (Fig. 2A and B). Overall, no differences in adherence and invasion were seen among group B2 isolates (Fig. 2A and B).

Differences in biofilm formation among UPEC isolates. The ability to form biofilm has been associated with UPEC persistence in the urinary tract (24). Therefore, we next investigated biofilm formation among isolates belonging to phylogenetic groups B1 and B2. Our results revealed that group B1 isolates from pregnant Swedish women formed less biofilm than those from nonpregnant Swedish controls (*P* < 0.001); however, this difference was not seen among group B2 isolates (Fig. 2C). Additionally, more biofilm was formed by group B2 isolates from pregnant Ugandan and Swedish patients than by group B1 isolates (*P* < 0.05) (Fig. 2C). Overall, among group B2 isolates from pregnant women, those from Ugandan women formed more biofilm than those from Swedish and Vietnamese women (*P* < 0.05) (Fig. 2C).
Our results revealed that no isolates expressed type 1 fimbriae under the conditions of growth that were employed in the in vitro assays. This therefore suggested that type 1 fimbriae did not play a major role in the differences in adherence, invasion, and biofilm formation that were seen in the present study.

The fluA<sub>CFT073</sub> gene is highly prevalent among Ugandan isolates. Due to differences in biofilm formation, we next investigated the prevalence of the gene encoding antigen 43 (Ag43). In particular, one of the allelic variants, Ag43a, has been shown to promote biofilm formation, as well as cell aggregation and UPEC persistence (26). Prevalence of the flu gene, which amplifies all 5 Ag43 allelic variants (23), was high among isolates from pregnant women in Sweden (n = 50), Uganda (n = 56), and Vietnam (n = 42) and nonpregnant Swedish controls (n = 50). Comparisons were made by χ² and pairwise Fisher’s exact two-tailed tests.

Widespread antimicrobial resistance among isolates from Uganda and Vietnam. Antibiotic resistance levels were generally low and similar among isolates from pregnant and nonpregnant patients in Sweden (Fig. 4). However, among isolates from pregnant Ugandan and Vietnamese women, resistance and the prevalence of ESBL-producing and MDR isolates were more common than among pregnant Swedish patients (Fig. 4). The incidence of resistance could not be linked to any of the trimesters of pregnancy (data not shown). Overall, few or no isolates were resistant to other antibiotics, including aztreonam, cefazadime, ertapenem, nitrofurantoin, aminocillin, and tobramycin (data not shown).

Interestingly, the presence of the flu gene was associated with antibiotic resistance. More flu-positive isolates than flu-negative isolates from Uganda were resistant to ampicillin (29 [88%] versus 13 [39%]; P < 0.05). Similarly, among isolates from nonpregnant controls, 11 (34%) that were flu positive were ampicillin resistant compared to only 1 (6%) isolate lacking the gene (P < 0.05). The flu gene was also associated with ceftazolin resistance among Ugandan isolates, with 10 (37%) flu-positive isolates resistant compared to just 2 (10%) flu-negative isolates (P < 0.05). Among Vietnamese isolates, more that carried the flu gene than

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### TABLE 2 Prevalences of tcpC and iroNe<sub>E.coli</sub> genes among phylogenetic group B2 and non-B2 E. coli isolates from pregnant and nonpregnant women with UTI in different countries

<table>
<thead>
<tr>
<th>Characteristic for patient group and country</th>
<th>No. of isolates (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B2</td>
<td>Non-B2 (A + B1 + D)</td>
</tr>
<tr>
<td>Pregnant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweden (n = 50)</td>
<td>27 (54)</td>
<td>23 (46)</td>
</tr>
<tr>
<td>tcpC positive</td>
<td>13 (48)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>iroNe&lt;sub&gt;E.coli&lt;/sub&gt; positive</td>
<td>13 (48)</td>
<td>6 (26)</td>
</tr>
<tr>
<td>Uganda (n = 56)</td>
<td>18 (32)</td>
<td>38 (68)</td>
</tr>
<tr>
<td>tcpC positive</td>
<td>5 (28)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>iroNe&lt;sub&gt;E.coli&lt;/sub&gt; positive</td>
<td>3 (18)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Vietnam (n = 42)</td>
<td>23 (55)</td>
<td>19 (45)</td>
</tr>
<tr>
<td>tcpC positive</td>
<td>13 (57)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>iroNe&lt;sub&gt;E.coli&lt;/sub&gt; positive</td>
<td>16 (70)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Nonpregnant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweden control (n = 50)</td>
<td>28 (56)</td>
<td>22 (44)</td>
</tr>
<tr>
<td>tcpC positive</td>
<td>6 (21)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>iroNe&lt;sub&gt;E.coli&lt;/sub&gt; positive</td>
<td>14 (50)</td>
<td>3 (14)</td>
</tr>
</tbody>
</table>

* Data were analyzed using Fisher’s exact two-tailed test with a P value of <0.05 considered significant. NS, not significant.
those lacking the gene were trimethoprim resistant (26 [79%] versus 3 [38%], respectively; P < 0.05).

**DISCUSSION**

It is commonly accepted that a high frequency of UTI during pregnancy is due to physiological changes that the human body undergoes in the pregnant condition (9). In this study, we demonstrate that differences exist in the virulence properties, including the ability to adhere to and invade urothelial cells and to form biofilm, among *E. coli* isolates causing UTI in pregnant and nonpregnant women, as well as among isolates from pregnant women in different countries.

Adherence and invasion are key virulence mechanisms employed by UPEC (16). We found here that group B1 isolates from pregnant Swedish women were less efficient in both adherence and invasion than isolates from nonpregnant Swedish women. It has previously been reported that *E. coli* isolates causing UTI in pregnant women and those causing infection in nonpregnant women have similar adherence abilities (25). Our results among group B2 isolates generally confirm this. However, the differences seen among phylogenetic group B1 isolates suggest that physiological changes in the pregnant condition play a role in facilitating the infection process. The similar abilities to adhere and invade that were seen among group B1 and B2 Ugandan isolates suggest that group B1 isolates, which may normally be regarded as commensals, are as well equipped as virulent strains to cause infection in the pregnant host. Furthermore, higher adherence efficiency among Ugandan group B1 isolates than among those from pregnant Swedish women indicates that Ugandan group B1 isolates may have a greater capacity to cause infection than group B1 isolates from other countries. This may explain the higher prevalence of group B1 among Ugandan isolates than among those from other countries (Fig. 1).

Exploring the ability to persist in the human host, we next investigated biofilm formation. Group B1 isolates from pregnant women formed less biofilm than isolates from nonpregnant women. Furthermore, isolates belonging to group B1 formed less biofilm than those belonging to group B2. Collectively, these findings further emphasize the role of physiological changes, rather than bacterial virulence properties, in UTI caused by these less virulent isolates. While differences in biofilm formation efficiencies were seen among isolates, including increased biofilm formation among Ethiopian group B2 isolates compared to those from other countries, these results could not be linked to the biofilm-associated virulence factors *fluA* and *CFT073*, curli, and cellulose. Thus, it seems likely that alternative virulence factors may play a role in biofilm formation among these isolates.

Our results indicate that isolates from Uganda are equipped to persist in the pregnant host due to higher prevalence of *fluA* and pairwise Fisher’s exact two-tailed tests. AM, ampicillin; AMC, amoxicillin-clavulanic acid; CIP, ciprofloxacin; CN, cefalexin; CTX, cefotaxime; GM, gentamicin; TMP, trimethoprim.

**FIG 4** Prevalence of antibiotic resistance among uropathogenic *E. coli* isolates causing urinary tract infection in pregnant patients in Sweden (n = 50), Uganda (n = 56), and Vietnam (n = 42) and nonpregnant patients (Sweden control; n = 50). Comparisons were made by $\chi^2$ and pairwise Fisher’s exact two-tailed tests.

Antibiotics that were used to determine antimicrobial resistance are as follows: AM, ampicillin; AMC, amoxicillin-clavulanic acid; CIP, ciprofloxacin; CN, cefalexin; CTX, cefotaxime; GM, gentamicin; TMP, trimethoprim.
isolates from Sweden may be a reflection of clinical practices that are in place to reduce the use of antibiotics and the development of resistance (15). Treatment of UTI in pregnancy is crucial for preventing pyelonephritis and complications that can endanger both the mother and fetus (18). In view of this, the high prevalence of antibiotic resistance among isolates in the present study is of great concern. Furthermore, our finding of an association between resistance and antigen 43, linked to UPEC persistence, indicates increased potential for development of difficult-to-treat upper UTI.

In conclusion, our results demonstrate that the ability to adhere, invade, and form biofilm are important in UPEC pathogenesis among isolates causing UTI in pregnant women in different countries. However, where *E. coli* isolates causing infection in pregnant women have low capacity to colonize the urothelium, physiological changes that occur in the host may play a more dominant role in facilitating the occurrence of UTI during pregnancy.

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REFERENCES