Original Article

Phenotypic Characterization of Escherichia Coli Isolated From Urine and Stool from Short Term and Long Term Catheterized Patients

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ABSTRACT

Background: Uropathogenic strains of *Escherichia coli* possess a number of phenotypic characters which allow them to colonize the catheter and cause infection of the same and the chances of colonization by these micro-organisms increase with the duration of catheterization. **Objectives:** This study was carried out to understand the differences between the phenotypic characters between the strains of *E.coli* isolated from the urine and gut of short term catheterized (STC) and long term catheterized (LTC) patients**. Materials and Methods:** Urine from the catheter and stool sample was processed by standard methods. The following special tests like α-hemolysis, haemagglutination, cell surface hydrophobicity, serum bactericidal assay, biofilm formation and congo red binding assay were performed to detect the phenotypic characters of the isolated *E.coli*. Antibiotic susceptibility testing was performed by Kirby Bauer method as per the Clinical Laboratory Standards Institute. **Results:** Majority (93.75%, 75% and 81.25%) of the *E.coli* isolates from urine of LTC patients were positive for MRHA, cell surface hydrophobicity and resistant to serum killing respectively, while majority (93.75%) of the urine isolates from short term catheterization. On congo red agar, the majority (80% and 60%) of isolates producing rdar type of colony and biofilm respectively,were isolated from urine of long term catheterized patients. ESBL production was noted in 68.75% of the urine isolates from long term catheterized patients. **Conclusion: S**erum resistance and biofilm formation are significant assays in terms of differentiating between the short term and long term catheterization and by elucidating these mechanisms better, it may become easier to combat and prevent such infections.

Keywords: Biofilm, Catheterization, Cell Surface Hydrophobicity, Haemagglutination, Serum Bactericidal Assay

Of all nosocomial infections catheter associated urinary tract infections (CAUTI) are the most common. Catheterization is the most important risk factor predisposing to urinary tract infections. The chances of colonization by micro-organisms increase with the duration of catheterization. If the catheter is present-in-situ to the extent that nearly 50% of the catheters get colonized within a span of 10 days and when this duration gets prolonged, almost all the catheters get colonized. Catheterization increases the risk of developing UTIs and even urosepsis. CAUTIs are major reservoirs of antibiotic-resistant organisms in the hospital [1,2]. Most of the patients with CAUTIs are usually asymptomatic. However, when an episode of catheter associated urinary infection becomes symptomatic; the resulting sequelae can range from mild fever, urethritis, and cystitis to severe acute pyelonephritis, renal scarring, calculus formation, and bacteremia left untreated, these infections can lead to urosepsis and death these complicated infections commonly recur and result in long-term morbidity due to

the presence of encrustation and blockage of the catheter by crystalline biofilms that increase resistance to the host immune response and to antibiotics [2,3].

Escherichia coli is a harmless commensal member of the human intestinal tract. But there are a few pathotypes of *E.coli* which can cause diarrhea while, other few cause disease manifestations in the extra-intestinal sites such as urinary tract infection (UTI), bacteremia, septicemia, and meningitis. Uropathogenic *Escherichia coli* (UPEC), is the major etiological agent of urinary tract infections and can cause both symptomatic and asymptomatic infections in catheterized patients [3,4].

UPEC (Uropathogenic *Escherichia coli*) strains possess a number of phenotypic characters which allow them to colonize the urothelium and cause infection of the same. These include haemolysin, P-fimbria, serum resistance factor, cytotoxic necrotizing factor, capsule, cell surface hydrophobicity and mannose resistant haemagglutination [3-7].

This study was carried out to understand the differences between the phenotypic characters between the strains of *E.coli* isolated from the urine and gut of short term catheterized patients and that of the strains isolated from the urine and gut of long term catheterized patients**.**

MATERIALS AND METHODS

The study was approved by the Institute Ethics Committee. Written and informed consent was obtained from all patients included in the study. Relevant clinical and demographic details were noted in a clinical proforma. Urine was collected for culture using aseptic techniques described to collect the sample from the foley's catheter while the stool sample was collected in a sterile widemouthed screw capped vial. Short term catheterization (STC) was defined as catheterization of 7 days duration while long term catheterization (LTC) was defined as catheterization of 10 days or more duration.

The urine sample was subjected to microscopic examination using the standard methods described earlier [8]. Briefly, the presence of pus cells/ HPF, bacteria, epithelial cells, crystals, casts, RBC's, yeast cells were noted and recorded. The urine samples were inoculated on Cysteine Lactose Electrolyte Deficient (CLED) medium, using a 1µl calibrated loop of 28 SWG nichrome wire without intermittent heating. The following day, colonies grown were counted. The stool samples were plated onto MacConkey's medium and all the inoculated media were incubated at 37ºC overnight. *Escherichia coli* was identified based on the colony morphology and by using the routine biochemical tests namely, Indole test, citrate test, urease test, kligler iron agar, lysine iron agar, mannitol motility test medium. The following special tests were performed to detect the phenotypic characters of *E.coli* isolated.

Antibiotic susceptibility testing: This was done by Kirby Bauer method as per the Clinical Laboratory Standards Institute for the following antibiotics-, ceftriaxone (Ci) 30µg, ceftazidime (Ca) 30µg, ceftazidime/clavulanic acid (30/10µg), nitrofurantoin (F) 300µg, ciprofloxacin (Cf) 5µg, cefoperazone-sulbactam (75/30 µg), cefepime-tazobactam (30/10µg), piperacillin-tazobactam (100/10 µg), Merpenem (M) 10µg, Imipenum (IM)10µg. *E. coli* ATCC 25922 was used as the control and the results were interpreted as per the CLSI criteria [9].

α-hemolysis (β-haemolysin) production: The detection of haemolysin was detected by inoculating bacterial strains on 5% sheep blood agar and the haemolytic zone was observed after overnight growth at 37°C [5].

Haemagglutination: A suspension of human A+ve blood and PBS was mixed on a VDRL (venereal diseases research laboratory - test) cavity slide using a suspension of a single colony of *E. coli*. After incubation at room temperature for 5 minutes, agglutination was looked for. This test was carried out in the presence and absence of D-mannose. ATCC *E. coli* 25922 strain was used as a negative control [5,10].

Cell Surface Hydrophobicity: Bacterial strains were tested for their hydrophobic property by using different molar concentrations of ammonium sulphate in the VDRL slide; 40 ml of bacterial suspension in PBS was added in each of the wells containing 1 M, 1.4 M and 2 M ammonium sulphate. Clumps were examined by naked eyes. Strains were considered hydrophobic, if they aggregated in the PBS concentration of ≤1.4 M [5,10].

Serum Bactericidal Assay: A single colony of *E.coli* was diluted in Hank's balanced salt solution, mixed with human serum in a sterile tube which was then incubated in a water bath at 37°C and inoculated on nutrient agar plates

after 1 hr, 2 hr of incubation in water bath. Growth at 0 hr was taken as control. Any strain of *E. coli* was considered sensitive if the count dropped to 1% and resistant if >90% of the organisms survived after 2 hrs of incubation. ATCC *E. coli* 25922 strain was used as a negative control [5,10].

Biofilm formation: The biofilm assay was performed as described earlier [11]. Stated briefly, 10 ml of Luria-Bertani (LB) medium with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar. The LB broth was incubated at 37° C for 24 hours. The culture was further diluted to 1:100 with fresh medium and flat bottom tissue culture plates (96 wells) were filled with 200µl of diluted cultures individually. Uninoculated sterile broth served as blank. Similarly, control organisms were also diluted and incubated. The culture plates were incubated at 37oC for 24 hours.

After incubation, gentle tapping of the plates was done. The wells were washed with 200 µl of phosphate buffer saline (pH 7.2) four times to remove free-floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionised water and plates were dried properly. Optical densities (OD) of stained adherent biofilm were obtained with a micro ELISA auto-reader at wavelength of 570 nm. Experiments were performed in duplicate and the average of OD values of sterile medium were calculated and subtracted from all test values.

Congo red binding: Congo red binding assay was done as described earlier [12,13]. Briefly, strains were grown on LB agar (37°C, 24h) and seeded onto Congo red agar (trypticase soy agar supplemented with 1 % Congo red dye and 0.15% bile salts) and the cultures were incubated for 24 hours to 48 hours at 37°C. Congo-red-positive *E. coli* isolates were identified by the appearance of red colonies. The results were read as rdar (red colony, expressed curli fimbriae and cellulose), pdar (pink colony, expressed cellulose), bdar (brown colony, expressed curli fimbriae) and saw (no expression of curli fimbriae nor cellulose), ras (red and smooth), bas (brown and smooth) or pas (pink and smooth) and orange colonies which signified that strain was highly virulent [12,13].

RESULTS

Present study included 64 *Escherichia coli* strains isolated from urine and stool of catheterized patients. Among 64 isolates 32 strains were from short term (16 each from urine and stool samples) and 32 strains from long term catheterized patients (16 each from urine and stool samples) respectively.

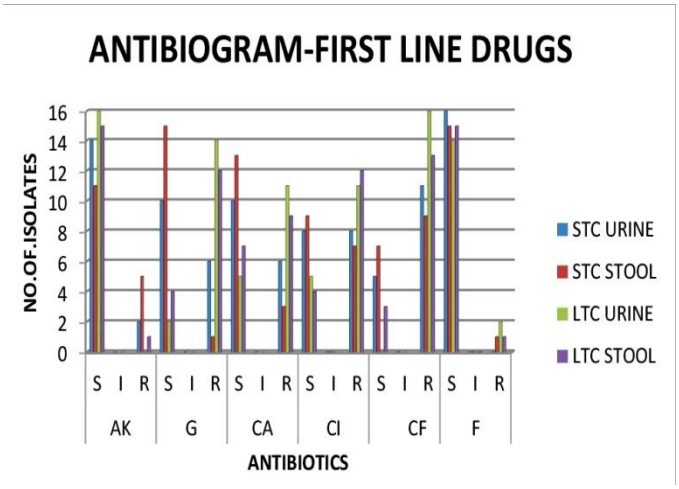

Figure 1: Antibiotic susceptibility testing profile of the *E. coli* isolated from urine and stool specimens to the first line antibiotics

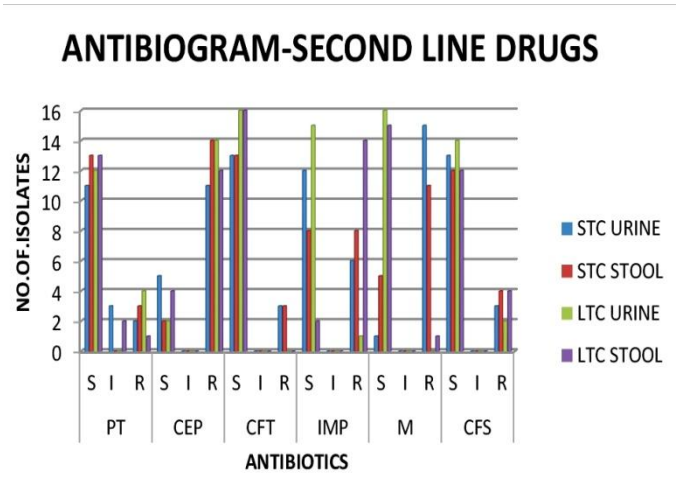

Figure 2: Antibiotic susceptibility testing profile of the *E. coli* isolated from urine and stool specimens to the second line antibiotics

Hemagglutination: Among the urinary isolates, 7/32 (21.87%) demonstrated mannose sensitive hemagglutination (MSHA) while, 25/32 (78.12%) demonstrated mannose resistant haemagglutination (MRHA). Of these 7 MSHA positive strains, 6/16 (37.5%) were from short term and 1/16 (6.25%) was from a long

term catheterized patient. Among the MRHA positive strains from urine, 10/16 (62.5%) were from STC patients while, 15/16 (93.75%) were from LTC patients.

Among stool isolates, 8/32 (25%) strains demonstrated mannose sensitive hemagglutination (MSHA) while, 24/32 (75%) strains demonstrated mannose resistant haemagglutination (MRHA). Of these 8 MSHA positive strains, 6/16 (37.5%) were from short term and 2/16 (12.5%) were from a long term catheterized patient. Among the MRHA positive strains, 10/16 (62.5%) were from STC patients while, 14/16 (87.5%) were from LTC patients (**Fig. 3**).

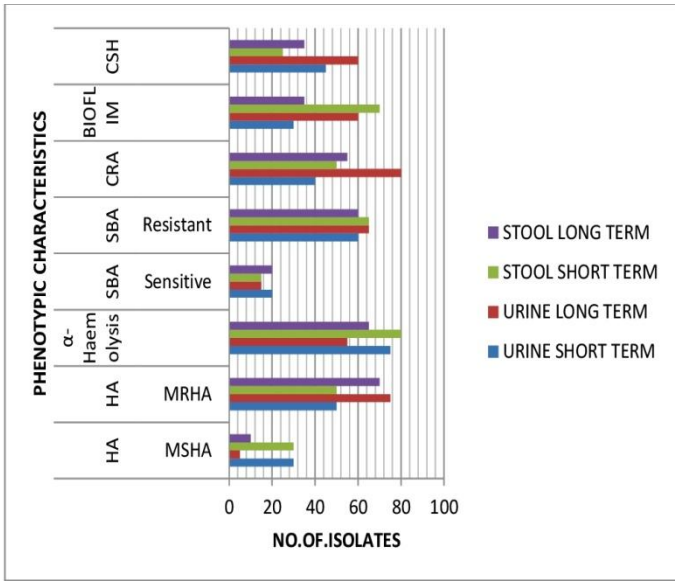

Figure 3: Phenotypic characteristics of the *E.coli* isolated from urine and stool

β haemolysin production: Among the urinary isolates, 26/32(81.25%) demonstrated haemolysis. Among these 26 strains, 15/16 (93.75%) were from STC and 11/16 (68.75%) were from LTC patients. Among the stool isolates 29/32 (90.62%) demonstrated haemolysis. Among these 29 strains 16 (100%) strains were from STC while, 13/16 (81.25%) strains were isolated from LTC patients (**Fig. 3**).

Cell surface hydrophobicity: Out of 32 urine isolates, 21 (65%) were positive. Of these, 9/16 (56.25%) were from short term and 12 (75%) strains were from long term catheterized patients. Out of 32 stool isolates, 12 (37.5%) strains were positive. Among these, 5/16 (31.25%) strains were from short term and 7/16 (43.75%) were from long term catheterized patients (**Fig. 3**).

Serum bactericidal assay: Out of 32 urine isolates 7(21.8%) strains were sensitive to the cidal effect of serum antibodies and 25(78.1%) strains were resistant. Among these 7sensitive strains, 4/16(25%) strains were isolated from short term and 3/16 (18.75%) were isolated from long term catheterized patients. Among the stool isolates, 3/16 (18.75%) were from short term and 4/16 (25%) were from long term catheterized patients (**Fig. 3**).

Out of 32 urine isolates 25 (78.12%) were resistant to the cidal effects of serum, of which 12/16 (75%) were from short term while, 13(81.75%) were from long term catheterized patients. Among the stool isolates, 13(81.75%) were from short term and 12 (75%) were from long term catheterized patients (**Fig. 3**).

Congo red binding: Out of 32 urine isolates, 24 (75%) produced rdar (expressed curli fimbriae and cellulose) type colonies, 2 (6.2%) strains produced ras (cellulose only) type, and 6 (18.7%) strains produced orange coloured colonies which indicated that they were highly virulent. None of strains produced pdar, saw, bas or pas type of colonies. Among 24 rdar strains, 8/16 (50%) were from short term and 16 (100%) were from long term catheterized patient strains.

Among 6 virulent strains (produced orange coloured colonies) 4 (66.6%) strains were short term and 2(33.3%) strains were isolated from long term catheterized patients. Out of 32 stool isolates 21(65.62%) strains produced rdar type of colonies, 5(15.6%) produced ras type, only 1 strain produced pdar and 4 (12.5%) strains produced orange coloured colonies. None of the strains produced saw, bas or pas type of colonies. Of these 21, 10/16(62.5%) were from short term while, 11/16 (68.75%) were from long term catheterized patients (**Fig. 3**).

Biofilm production: Of the 32 urine isolates, 18(53.1%) formed biofilm. Of these 6/16 (37.5%) were from short term and 12/16 (75%) were from long term catheterized patients. Out of 32 stool isolates 21 (65.6%) strains were biofilm producers. Of these 14/16(87.5%) strains were from short term and 7/16 (43.75%) strains were from long term catheterized patients (**Fig. 3**).

DISCUSSION

Urinary tract infections (UTIs) are one of the most common infections in clinical practice mainly being associated with different members of the family *Enterobacteriaceae* and among which *Escherichia coli* (*E. coli*) is the most frequently isolated pathogen. Certain serotypes of *Escherichia coli* are consistently associated with uropathogenicity and are designated as uropathogenic *E. coli* (UPEC). Uropathogenic strains of *E.coli* account for 90% of all UTIs among ambulatory patients and up to 50% of all nosocomial UTIs [1,2]. Uropathogenic *E. coli* strains consists of a variety of virulence properties that help them to colonize host mucosal surface and circumvent host defense to allow invasion of the normally sterile urinary tract. They are known to secrete a cytolytic protein called haemolysin [3,11-13]. Haemolysin production was noted more in strains isolated from short term catheterized patients than long term catheterized patients but the difference was not statistically significant (P=0.1719).

Haemagglutination is mediated by fimbriae and mannose resistant haemagglutination (MRHA) is mediated by "P" fimbriae and also X, FIC, Dr Fimbriae. Hence, it is evident that MRHA positive strains can be considered as uropathogenic *Escherichia coli* as they are likely to possess "P" fimbriae [11-13]. In the present study we found that 78% of urine and 75% faecal strains demonstrated mannose resistant haemagglutination indicating that expression of "P" fimbriae did not differ in *Escherichia coli* strains isolated from urine and stool. These results were similar to earlier studies [11,14] and different from the other studies [5-7]. This reiterates the fact that "P" fimbria plays an important role during colonization of the intestine and hence, the similar rate in urine and stool isolates. We also found that MRHA strains were isolated more frequently from long term catheterized cases than short term catheterized ones but this was statistically not significant (P=0.0829), similar to earlier reports [11,15]. More studies are required to assess the role of MSHA in the pathogenecity of UPEC.

Cell surface hydrophobicity is one of the important virulence factors of *Escherichia coli.* It mediates adherence to mammalian cells [11,12]. In this study more than 65% of urine and 37% faecal strains demonstrated positive hydrophobic character. In the present study, although there was no significant difference in cell-surface hydrophobicity between the short term and long term catheterization (P=0.4578), the isolates from the long term catheterized urine samples were more hydrophobic than those from the short term ones. Adherence to catheter is dependent on hydrophobicity and this factor contributes to the formation of biofilms [11].

Bacteria are killed by normal human serum through the lytic activity of the alternative complement pathway. Some strains of *Escherichia coli* are resistant to killing by serum antibodies due to capsular O polysaccharides and proteins. In this study, no significant difference was noted in the urine and stool isolates in this aspect (P=0.6130). The serum resistance among the isolates from urine of long term catheterization was relatively more than in the short term catheterized ones.

Curli fimbriae and cellulose are the important markers of virulence in *Escherichia coli.* Biofilm formation and expression of curli fimbriae and cellulose are those virulence markers which are expressed as extracellular matrix components. While the contribution of cellulose to virulence remains less studied, several virulence-associated features have been assigned to curli fimbriae [11,12]. Though curli fimbriae are not consistently expressed by *E. coli* pathovars, the majority of sepsis isolates have been found to express curli fimbriae and uropathogenic *E. coli*, the latter shows expression only at ambient temperature [12].In this study we used congo red agar (containing 1% congo red) to detect the curli fimbriae and cellulose. Detection of curli fimbriae and cellulose is dependent on type of colony produced on congo red agar. More than 75% of the urine and 68% of the faecal isolates produced rdar (red, dry and rough) colonies which was indicative of expression of curli fimbriae and cellulose. Strains isolated from long term catheterized patients demonstrated curli fimbriae and cellulose (rdar) more frequently when compared to short term catheterized patients and this was statistically significant (P=0.0024). Some urine and fecal strains produced orange colored colonies indicating that they were highly virulent strains.

Biofilm production is an important virulence factor of *Escherichia coli* and is important in initiating as well as maintaining CAUTIs. It protects the bacteria from the

hydrodynamic forces of urine flow, host defenses and antibiotics. Several different factors are associated with biofilm formation with *Escherichia coli* including type1 fimbriae, flagella, and antigen. Biofilm formation was more in strains isolated from long term catheterized patients than short term catheterization and the difference was found statistically significant(P=0.0732) (Figure 3). The findings of the biofilm assay and the congo red assay emphasize the role of curli fimbria in the formation of biofilm in long-term catheterization, which has been mentioned earlier [11]. We correlated the findings of the biofilm assay and that of the serum resistance assay, we observed that biofilm producing strains were resistant to the cidal effect of serum, a fact which has been documented in many studies earlier [11,16]. The serum sensitive strains i.e. the isolates from urine of short term catheterization were less associated with biofilm (Figure 3), while most of the serum-resistant strains i.e. the isolates from the urine of the long term catheterization produced biofilm.

In this study most of the *Escherichia coli* strains (urine & stool) were resistant to 3rd generation cephalosporin like ceftazidime and 4th generation cephalosporin cefepime. The combination of cefoperazone-sulbactum and cefepime-tazobactum were as effective as nitrofurantoin, aminoglycosides and meropenem in short term as well as long term catheterized patients (more than 80% strains were sensitive). Hence, beta- lactam / beta-lactamase inhibitor combination can be an alternative choice of therapy in CAUTI among others. Among the isolates obtained from the urine samples 28% were ESBL producers while 9% of the faecal isolates were ESBL producers. ESBL production was more among strains isolated from long term catheterized patients (55.5%) than the short term catheterized patients. More long term studies including a larger population are required in this regard. There appears to be an association between biofilm formation and antibiotic sensitivity, as has been described previously [10,11,16].

Some of the virulence factors like haemolysin production and MSHA were more in urinary isolates in short term catheterized patients**.** On the contrary, biofilm production, cell surface hydrophobicity, ESBL production, curli fimbriae and MRHA were more in strains isolated from long term catheterized patients. We conclude that the characterization of UPEC is important and that the virulence factors like fimbrial expression and cell surface hydrophobicity, leading to biofilm formation which in turn leading to increased antibiotic resistance and MRHA are probably the most important and consistent characters required to understand UPEC and it behavior with the duration of catheterization when compared to the others. In contrast, the faecal isolates did not conform to the biofilm assay, the haemolysin and serum bactericidal resistance assays indicating that the behavior of the gut E.coli may not be reflective of that in urine. Our understanding is that this difference is limited by the few numbers of isolates and the short time of study period. Hence, this needs to be further assessed using larger number of isolates studied over a longer period of time.

CONCLUSION

The pathogenesis of CAUTI by UPEC is complex and involves the interplay of several virulence factors. While many studies have been done on the virulence factors, it is interesting to know that very few have shed light on the differences between long term and short term catheterization, and CAUTI continues to be the foremost infection in the hospitalized patients. Serum resistance and biofilm formation are significant contributors to pathogenesis and by elucidating these mechanisms better, it may become easier to combat and prevent such infections.

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