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Original Research Article

Characterisation of uropathogenic E.coli by detecting the virulence factors and its drug resistance pattern in a tertiary care hospital in India

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ABSTRACT

Background: Urinary tract infections (UTIs) are among the most prevalent nosocomial and community-acquired bacterial diseases in humans, with E.coli being the most typical pathogen isolated.
Aim: To detect the prevalence of virulence factors like haemolysin, haemagglutination of human erythrocytes with its effect of D-mannose, and cell surface hydrophobicity, the antibiotic sensitivity pattern and ESBL production in urinary isolates of E.coli obtained from clinical samples.
Materials and Methods: We included the E.coli isolates obtained from a midstream urine sample for the study. Virulence factors like haemolysin, hemagglutination and salt aggregation were detected as per standard protocols. Antibiotic sensitivity testing was performed by the Kirby Bauer disc diffusion method. Extended-spectrum beta-lactamase (ESBL) production was seen by the combined disc diffusion method on Muller Hinton agar as per CLSI guidelines.
Results: A total of 103 E.coli isolates were tested, and among them, 24(23.30%) produced haemolysin, 65(63.10%) produced hemagglutination and 38(36.89%) had salt aggregation properties. Most isolates obtained were resistant to beta-lactam antibiotics but showed high sensitivity towards antibiotics like chloramphenicol, meropenem, amikacin, imipenem and nitrofurantoin. Around 48% of them were ESBL producers.
Conclusions: The common virulence factors associated with UTI were P-fimbriae (MRHA), haemolysin production, cell surface hydrophobicity and type-1 fimbriae. Because of the emerging drug resistance among UPEC, therapy should be advocated as far as possible after obtaining the culture and sensitivity results to determine exact aetiology and susceptibility patterns.
Key messages: The sensitivity to nitrofurantoin is very high, suggesting that antibiotic recycling will help clinicians treat UPEC.

1. Introduction

Urinary Tract infections(UTI) are the most common bacterial infections that lead patients to seek medical care. UTIs are also significant among hospital-acquired infections, accounting for as many as 35% of nosocomial infections. UTIs are important complications of diabetes, renal disease, renal transplantation, and structural and neurologic abnormalities that interfere with urine flow.

E.coli is the most common etiological agent in both community and hospital-acquired urinary tract infections. About 150 million UTI cases occur worldwide annually[1] and E.coli accounts for 50-90% of all uncomplicated urinary tract infections.

There are subsets of faecal E.coli which can colonise the periurethral area, enter the urinary tract and cause

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symptomatic disease. These are currently defined as uropathogenic E.coli (UPEC).[1] UPEC strains possess an arsenal of virulence factors that specifically contribute to their ability to cause disease in the human urinary tract. Uropathogenic E.coli has chromosomally encoded virulence markers, including haemolysin, cell surface hydrophobicity and fimbriae (haemagglutination).

Considering the high degree of morbidity and mortality of urinary tract infections, the subject of uropathogenic E.coli is receiving increasing attention. Multidrug-resistant strains of E. coli are widely prevalent, and isolation of the same in the community-acquired urinary tract infection is a matter of grave concern.[2]

Various studies have reported the production of extended-spectrum beta-lactamase (ESBL) and concomitant multidrug resistance among uropathogenic E.coli.[2] ESBL are enzymes secreted by bacteria that can hydrolyse all beta-lactam drugs except cephamycin and carbopenem.[3] Inappropriate overuse of antibiotics has led to the emergence of drug resistance mechanisms like extended-spectrum beta-lactamase, AmpC beta-lactamase, Metallo-beta-lactamases and carbapenemases.

This study was carried out to detect the virulence factors of uropathogenic E.coli, also to know the resistance pattern of E.coli responsible for urinary tract infections. The prevalence of multidrug resistance and extended-spectrum beta-lactamase production among E.coli isolated from UTI. These measures help better understand the organism and establish a regimen for the antibiotic policy of urinary tract infections based on the drug sensitivity profile of the isolates.

2. Methodology

2.1. Study design

Prospective cross-sectional study.

All midstream urine samples showing a single morphotype colony of E.coli with a count of 10^4-10^5 CFU/mL from the microbiology laboratory were included. A Midstream urine sample with a count of $<10^3$CFU/mL was considered insignificant.

E.coli isolates obtained from urine specimens received from the microbiology laboratory of Yenepoya medical college hospital, identified by standard biochemical reactions, were taken for the study. The patient's detail was delinked. A total of 103 samples were collected. The additional features noticed in terms of colony morphology were mucoid or nonmucoid and biochemical reactions. They showed haemolysis on sheep blood agar after overnight incubation, whether they were typical and atypical isolates. An isolate was considered typical if it was a lactose fermenter and aerogenic and atypical if it was a non-lactose fermenter and anaerogenic. E.coli thus obtained from cases with significant or probably significant counts were screened for the following virulence markers.

2.1.1. Haemolysin

The cytolytic protein toxin secreted by haemolytic E.coli isolates is alpha-haemolysin. Haemolysin was detected by inoculating the strains on 5% sheep blood agar plates and kept for overnight incubation at 37^0C[4].

The test is haemolytic when a zone of lysis is seen around each colony on blood agar plates (Figure 1).

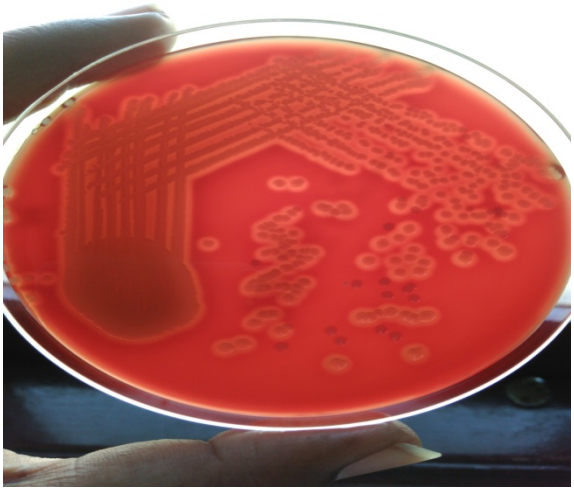

Fig. 1: Haemolysis**:** showing beta lytic colony on 5% sheep blood agar.

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2.1.2. Haemagglutination

Fimbriated E. coli produces haemagglutination, can be detected by demonstrating clumping of erythrocytes. Blood was washed three times with normal saline, and 3% erythrocyte suspension was later made with phosphate buffer saline (pH 7.4). E.coli grown on nutrient agar is inoculated into 5 ml phosphate-buffered saline pH 7.4. The procedure was performed on VDRL slides. 40 μl of bacterial suspension was mixed with 40 μl of human blood and 40 μl of PBS with and without 3% D. mannose. The slide was then placed on a VDRL rotator and rotated for four minutes, and hemagglutination reaction with saline and mannose were recorded (Figure 2). Hemagglutination inhibited in D-mannose was labelled as Mannose Sensitive Hemagglutination (MSHA), indicating type 1 fimbriae. If agglutination occurs in the presence of D-mannose, it is called Mannose Resistant Hemagglutination (MRHA), indicating the presence of P-fimbriae.[4]

2.1.3. Salt aggregation test (cell surface hydrophobicity

The hydrophobic property of E. coli was tested using different molar concentrations of ammonium sulphate. Those which aggregated with salt particles and

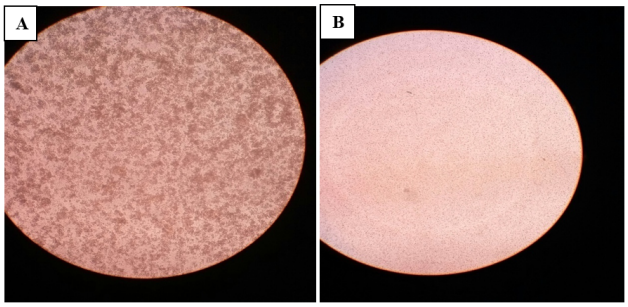

Fig. 2: Haemagglutination: (**A**) Showing haemagglutination under 10x; **(B):** Showing no reaction

formed clumps were considered hydrophobic. E.coli grown on nutrient agar plates was inoculated into 1mL of PBS (pH 6.8), and turbidity were matched with McFarland tubes 6 to get a colony count of $5x10^9$ colonies/ml. Different molar concentrations of ammonium sulphate, namely 1M, 1.4M and 2M concentrations, were prepared by adding 13.21 gm, 18.48 gm, and 26.4 gm of ammonium sulphate to 100 ml distilled water, respectively. Forty microliters of 0.2 M PBS of 6.8 pH were taken in the VDRL slide's first column. 40 μl of 1M, 1.4M and 2M concentrations of ammonium sulphate added in each well of other columns of the VDRL slide. Later, forty microliters of E.coli suspension was added to each of these wells. The slides were gently rotated by hand and observed after 2 min for the presence of bacterial aggregation visually (Figure 3).[5]

In this assay, surface hydrophobicity is inversely correlated with the salt concentration required to mediate agglutination of bacteria. Bacteria agglutinated by concentrations of ammonium sulphate of 1.4M or less are defined as hydrophobic. Isolates that gave inconclusive results were retested.

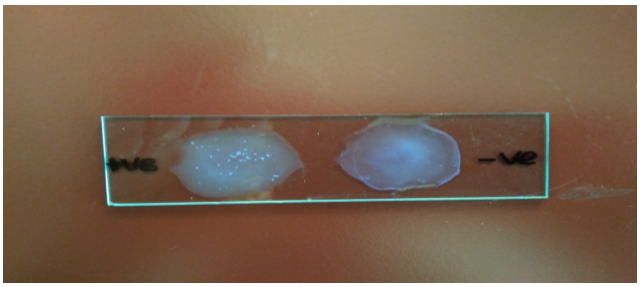

Fig. 3: Salt aggregation test: showing a positive and negative test of salt aggregation test.

2.1.4. Antibiotic susceptibility test

The Kirby Bauer disk diffusion method was used for Antibiotic susceptibility testing on Muller Hinton Agar with 0.5 McFarland standard inoculum. The tested antibiotics were amikacin (10μg), amoxiclav (20/10μg), ampicillin (10μg), chloramphenicol (30μg), cotrimoxazole (25 μg), gentamicin (10μg), cefepime (30μg), cefotaxime (30 μg), Ceftazidime (30μg), imipenem (10 μg), piperacillin-tazobactam (100/10μg), and nitrofurantoin (300 μg), norfloxacin (10 μg), and meropenem (10μg). The E. coli (ATCC 25922) strain was used as quality control for Antibiotic susceptibility tests as per CLSI guidelines. The inhibition zone size was interpreted using the standard recommendation of the Clinical Laboratory Standard Institute M100, and their sensitivity patterns were noted down. An isolate was considered multidrug-resistant if it was resistant to ≥3 groups of antibiotics.[6,7]

Combined Disc Diffusion Method to detect ESBL production: From the E. coli isolates, 0.5 McFarland's turbidity standard suspension was prepared. On the Muller Hinton Agar plate, lawn culture was made with the prepared inoculum. Ceftazidime and Ceftazidime + Clavulanic acid (30 mcg/10 mcg) discs were placed aseptically on the surface of MHA. The distance of 15mm was kept between the discs, and overnight incubation was done at 37 ^{0}C. ESBL production is confirmed when an increase of ≥ 5mm in zone diameter of Ceftazidime + Clavulanic acid in comparison to the zone diameter of Ceftazidime alone is seen (Figure 4).[6]

Fig. 4: Combined disc diffusion method for ESBL production

Ethical clearance was taken from the institutional ethical committee with protocol number 2017/018 dated Feb 2017.

3. Results

Among 103 E.coli isolated from patients with urinary tract infections were tested for the ability to cause lysis of human erythrocytes. Among 103 isolates, 24 (23.31%) showed haemolysis on blood agar. A total of 36 (34.9%) among 103 isolates showed mannose-resistant haemagglutination (MRHA), and 29 (28.1%) showed mannose-sensitive haemagglutination (MSHA). Hence, a total of 65(63.1%)

isolates were positive for haemagglutination, and 38(36.8%) isolates were non-haemagglutinating types (NHT).

A total of 38 (36.8%) were positive for salt aggregation among 103 cases of urinary tract infections, and the rest, 65 (63.1%), showed no salt aggregation. The distribution of the virulence factors tested among E.coli isolates in this study is tabulated in Table 1.

Table 1: Distribution of virulence factors among E.coli isolate

Virulence factors		Number (%)
Isolates having all the three virulence factors		7 (6.7%)
Isolates having Two virulence factors	Haemolysin and haemagglutination	12 (9.7%)
	Haemolysin and salt aggregation	8 (7.7%)
	Haemagglutination and salt aggregation	15 (14.5%)
Isolates having One virulence factor	Haemolysin	6 (5.8%)
	Haemagglutination	29 (28.1%)
	Salt aggregation	7 (6.7%)
Isolates having no virulence factors		19 (18.4%)
Total		103 (100%)

3.1. Antibiotic sensitivity test

The antibiotic susceptibility test was done by using the Kirby Bauer disk diffusion method. The inhibition zone size was interpreted using the standard recommendation of the Clinical Laboratory Standard Institute, and their sensitivity patterns are enlisted in Table 2.

Table 2: Antibiotic sensitivity pattern of 103 *E.coli* isolates

Antibiotics	Sensitive	Resistant
Ampicillin	10 (9.7%)	93 (90.2%)
Amoxiclav	8 (7.7%)	95 (92.2%)
Piperacillin-Tazobactam	75 (72.81%)	28 (27.1%)
Cefepime	31 (30%)	72 (69.9%)
Cefotaxime	27 (26.21%)	76 (73.7%)
Ceftazidime	29 (28.15%)	74 (71.84%)
Gentamicin	62 (60.19%)	41 (39.8%)
Amikacin	92 (89.32%)	10 (9.7%)
Norfloxacin	74 (71.84%)	29 (28.1%)
Cotrimoxazole	43 (41.74%)	57 (55.3%)
Chloramphenicol	99 (96.1%)	4 (3.8%)
Nitrofurantoin	77 (74.75%)	26 (25.2%)
Imipenem	87 (84.46%)	16 (15.53%)
Meropenem	92 (89.32%)	11 (10.6%)

The isolates showed a wide range of susceptibility to the tested antibiotics. Chloramphenicol (96%), Amikacin (92%), Meropenem (92%), Imipenem (87%) and Nitrofurantoin (77%) showed high sensitivity. The tested UPEC isolates exhibited high resistance to antibiotics such as ampicillin (90.2%) and Amoxicillin / Clavulanic acid (92.2%).

The phenotypic test for the ESBL confirmation indicated that 50(48.54%) out of the 103 isolates were ESBL producers. Amikacin(88%), Meropenem(84%), Imipenem(82%) and Nitrofurantoin(66%) showed high sensitivity towards the ESBL producers.

High susceptibility to Chloramphenicol, Amikacin, Meropenem, Imipenem and Nitrofurantoin is seen consistently among isolates with various tested virulence factors, as shown in Table 3. Among the haemolysin producers, 17(56.6%) were ESBL producers and around 18 (50%) of the MRHA and 14 (48.2%) MSHA isolates were ESBL producers. Among the 38 salt aggregation positive isolates, half of the 19 (50%) produced ESBL.

4. Discussion

Cell morphology and molecular biology studies have revealed that uropathogenic E. coli express fimbriae, haemolysins, and other virulence factors. The occurrence of virulence factors in UPEC strains strengthens the concept of association of UPEC with urinary pathogenicity.

4.1. Virulence factors

4.1.1. Haemolysis

E.coli produces three types of haemolysin, namely α, β and γ. The cytolytic protein toxin secreted by most haemolytic E.coli is alpha haemolysin.[2] Among the 103 urinary E.coli isolates, about 29% were haemolytic in our study. In earlier studies conducted by Seema et al.[8] and Desai et al.,[9] shows 47% and 54%, respectively. The present study showed a lower percentage of haemolysin production in comparison to other studies.

4.1.2. Haemagglutination

Agglutination of human erythrocytes by E. coli strain is indirect evidence of the presence of fimbriae on that strain. Based on the agglutination of human erythrocytes, isolates are divided into MRHA, MSHA and NHT. Mannose Resistant Haemagglutination positive strains can be considered as UPEC having P fimbriae. These MRHA possessing E. coli strains are associated with severe forms of UTIs. Type I fimbriae bind to mannose-containing receptors are found in most E. coli urinary isolates, which are indicated by MSHA.[10] The present study showed 34.9% MRHA and 28.1% MSHA, while the survey conducted on virulence factors by Vagarali et al.[1] and Desai et al.[9] shows 34% and 30% respectively for MRHA and 25% and 36% for MSHA, which agrees with our study.

4.1.3. Salt aggregation test

Adherence of bacteria to mucosal surfaces is an important virulence factor in infections of the urinary tracts. The ability of organisms to attach to these surfaces is often

Table 3: Comparison between all the three virulence factors and antibiotic sensitivity pattern

Antibiotics	Hemolysin producers		Haemagglutination				Salt aggregation	
			MRHA		MSHA			
	S	R	S	R	S	R	S	R
Ampicillin	2 (6.6%)	28 (93.3%)	3 (8.3%)	33 (91.6%)	4 (13.7%)	25 (86.2%)	7 (18.1%)	31 (81.5%)
Amoxyclav	1 (3.3%)	29 (96.6%)	2 (5.5%)	34 (94.4%)	1 (3.4%)	28 (96.5%)	2 (5.2%)	36 (94.7%)
Piperacillin-Tazobactam	20 (66.6%)	10 (33.3%)	25 (69.4%)	11 (30.5%)	24 (82.7%)	5 (17.2%)	27 (70.1%)	11 (28.9%)
Cefepime	9 (30%)	21 (70%)	12 (33.3%)	24 (66.6%)	10 (34.4%)	19 (65.5%)	16 (24.1%)	22 (57.8%)
Cefotaxime	7 (23.3%)	23 (76.6%)	10 (27.7%)	26 (72.2%)	9 (31%)	20 (68.9%)	14 (36.8%)	24 (63.1%)
Ceftazidime	6 (20%)	24 (80%)	10 (27.7%)	26 (72.2%)	9 (31%)	20 (68.9%)	14 (36.8%)	24 (63.1%)
Gentamicin	18 (60%)	12 (40%)	23 (63.8%)	13 (36.1%)	17 (58.6%)	12 (41.3%)	24 (63.1%)	14(36.8%)
Amikacin	28 (93.3%)	2 (6.6%)	34 (94.4%)	2 (5.5%)	26 (89.6%)	3 (10.3%)	36 (94.7%)	2 (5.2%)
Norfloxacin	18 (60%)	12 (40%)	27 (75%)	9 (25%)	21 (72.4%)	8 (27.5%)	27 (70.1%)	11 (28.9%)
Cotrimoxazole	10 (33.3%)	20 (66.6%)	17 (47.2%)	19 (52.7%)	15 (51.7%)	14 (48.2%)	17 (44.7%)	21 (55.2%)
Chloramphenicol	28 (93.3%)	2 (6.6%)	33 (91.6%)	3 (8.3%)	28 (96.5%)	1 (3.4%)	35 (92.1%)	3 (7.8%)
Nitrofurantoin	19 (63.3%)	11 (36.6%)	27 (75%)	9 (25%)	23 (79.3%)	6 (20.6%)	28 (73.6%)	10 (26.3%)
Imepenem	24 (80%)	6 (20%)	29 (80.5%)	7 (19.4%)	26 (89.6%)	3 (10.3%)	32 (83.2%)	6 (15.7%)
Meropenem	26 (86.6%)	4 (13.3%)	33 (91.6%)	3 (8.3%)	24 (82.7%)	5 (17.2%)	34 (89.4%)	4 (10.55)

critical for initiating bacterial surface colonisation. Bacteria can bind to mucosal surfaces by a less specific mechanism mediated by the interaction of hydrophobic domains, which is detected by the salt aggregation test.

The study by Raksha R et al[11] and Fatima Net al.[12] showed similar results of 26% and 22%, respectively, as the present study(30%). Desai S et al.[9] showed a 76% cell surface hydrophobicity which is very high compared to the present study.

4.2. Antibiotic susceptibility test

In this study, the E. coli isolates manifested a wide range of susceptibility to most tested antibiotics. Chloramphenicol (96%), amikacin (92%), meropenem (92%), imipenem (87%) and nitrofurantoin (77%) can be considered as the most effective antibiotics against these isolates. Other studies were done by Mandira Mukerjee et al.[13] (72%), and Stephenson et al.[14] (98%) showed high sensitivity to nitrofurantoin similar to this study

The tested UPEC isolates exhibited high resistance to antibiotics like Ampicillin, Amoxicillin / Clavulanic acid, and ciprofloxacin with a resistant rate of 92.2%, 90.2%, and 83.4%, respectively. Trimethoprim/ sulfamethoxazole, traditionally considered a frontline therapy for UTIs, showed a negligible effect on these isolates with a resistance rate of 57%. These isolates also showed high resistance to the Cephalosporins including; Cefepime, Ceftazidime, and Cefotaxime, with resistance rates of 73.7%, 71.8% and 69.9%, respectively. Various studies also support the findings with Ampicillin (97%), Cotrimoxazole (82%), and Ciprofloxacin (80%) resistance in Mandira et al.[13] study and Ampicillin (100%) and Ceftazidime (100%) resistance seen in Ghadiri et al[15] study.

A high rate of ESBL producers(48%) is seen when compared to the other studies like Stephenson et al.[14] study shows 31% ESBL producers, and Vasumathi et al.[3] shows 29%, ESBL producers.

Our study found out that compared with virulence factors and antibiotic sensitivity pattern, the high susceptibility was shown only to Chloramphenicol, Amikacin, Meropenem, Imipenem, and Nitrofurantoin. Hence, a significant difference was not seen with virulence factors and drug susceptibility.

5. Conclusion

This study showed that most urinary isolates from cases had at least one virulence marker, and some of the isolates had multiple virulence factors. The occurrence of numerous

virulence factors in the UPEC strains further strengthens the concept of the association of UPEC with urinary pathogenicity.

It is better to avoid antibiotics like penicillins and fluoroquinolones though they are the first line of treatment as we have not found any efficacy for these antibiotics. In our study, the sensitivity to nitrofurantoin is very high, suggesting that antibiotic recycling will help clinicians treat these cases and prevent antibiotic resistance by overuse of beta-lactam antibiotics. This study exposes the emergence of ESBL producers among E. coli isolates, making the treatment failure and more extended hospital stays. Hence, the clinician must be aware of the antibiotic resistance and sensitivity knowledge in their locale, which prevents treatment failure and imparts proper infection control practices, reflecting on good patient outcomes. Targeted antibiotic therapy administered after the culture report may prevent the emergence of drug resistance.

6. Limitations of the Study

In the present study, the other virulence factors like siderophore production, serum resistance, and Congo red dye uptake were not included. Other than ESBL, E.coli produces other enzymes such as MBLs, and carbapenemases were not tested in this study.

7. Source of Funding

None.

8. Conflict of Interest

None.

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