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RESEARCH ARTICLE

STUDY OF UROPATHOGENIC *ESCHERICHIA COLI* WITH SPECIAL REFERENCE TO VIRULENCE FACTORS AND ANTIBIOTIC SUSCEPTIBILITY PATTERN IN A TERTIARY CARE HOSPITAL

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ABSTRACT

Escherichia coli is the most common pathogen causing UTI. Various virulence factors play role in pathogenesis of uropathogenic *E. coli.* The common virulence factors include surface hydrophobicity, colonization factor, capsule, serum resistance, resistance to phagocytosis, hemolysin, enterotoxin, siderophore production, fimbriae and hemagglutination. Multidrug resistance is increasing in *E. coli* and production of various beta lactamases pose a major problem for clinical therapeutics and contributes to the virulence.

Method: In the present study various virulence factors of UPEC like serum resistance, hemolysin, protease (gelatinse) and hemagglutination was detected. Antibiotic susceptibility profile and detection of ESBL, carbapenemase, Metallo-beta-lactamase, AmpC beta lactamase production by phenotypic methods was done.

Result: 75 UPEC isolates with significant colony count were included in the study. Hemolysis was seen in 32% of the isolates; Hemagglutination in 40% of the isolates, 24% of the isolates were MSHA and 16% of the isolates were MRHA; 47% of the isolates were showing serum resistance and 13% of the isolates produced gelatinase. 32(43%) of them had more than one virulence factors. Maximum sensitivity was seen with Imipenem (92%), Nitrofurantoin (85%) Amikacin (80%) and least with Ampicillin (2.6%) and Amoxicillin- Clavulanate (6%). 53% of the isolates were ESBL producers, 8% of the isolates produced AmpC beta lactamase. 65% of the isolates having virulence factors were ESBL and/or AmpC beta lactamase producers. None of the isolates were Carbapenamase or MBL positive by phenotypic methods.

Conclusion: Presence of virulence factors help in the pathogenesis of *E. coli* causing UTI. And along with it production of beta lactamases possess major problem in treatment.

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INTRODUCTION

Urinary tract infection is the most common infection encountered both in community and hospital acquired infections (Jack d. Sobel and Donald Kaye, 2010). *Escherichia coli* is the most common pathogen causing UTI in all ages, accounting for about 85% of community acquired and 50% of hospital-acquired infections. Serotypes causing UTIs are designated as uropathogenic *Escherichia coli* (UPEC) (Mittal *et al*., 2014). Various virulence play role in pathogenesis of UPEC. The common virulence factors include surface hydrophobicity, colonization factor, capsule, serum resistance, resistance to phagocytosis, hemolysin, enterotoxin, siderophore production, fimbriae and hemagglutination (Jack d. Sobel and Donald Kaye, 2010; Mittal *et al*., 2014). These markers of UPEC are expressed with different frequencies in different diseases states ranging from asymptomatic bacteriuria to chronic pyelonephritis (Mittal *et al*., 2014). There are various virulence factors determining genes which are responsible for expression of virulence factors. By acquiring such properties E.coli can cause extraintestinal infections especially UTI. The adhesion to uroepithelial cells is increased with the help of fimbriae. Unique properties of fimbriae, are some can agglutinate RBCs by which it can be demonstrated. Type 1 fimbriae are inhibited in the presence of mannose and P fimbriae are not affected in the presence of mannose. Mannose resistant fimbriae is associated with increased pathogenicity and ability to cause pyelonephritis. Hemolysins exhibit cytotoxicity to various host cell and involved in inflammatory reactions helping in causing kidney infection (Slavchev *et al*., 2008-2009). Serum resistance is due to the presence of various surface antigens such as O and K lipopolysaccharide antigens which can inhibit the bacteriolysing factors present in the

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serum (Hughes *et al*., 1982). Presence of proteinases helps in spreading of infection. And other factors such as iron sequestrating systems helps in the growth of bacteria, presence of capsule inhibits phagocytosis (Slavchev *et al*., 2008-2009). Incidence of multidrug resistance is increasing in *E.coli*. Extended spectrum β -lactamase (ESBL), Carbapenemase, Metallo-beta-lactamase, AmpC beta lactamase producing organisms pose a major problem for clinical therapeutics. And, incidence of multidrug resistance is increasing (Sharma *et al*., 2007). Therefore, the present study is carried out with aim to know the prevalence of various virulence factors like serum resistance, hemolysin, protease (gelatinse) and hemagglutination in UPEC, to study their antibiotic susceptibility profile with reference to various phenotypic resistance like ESBL, carbapenemase, Metallo-beta-lactamase, AmpC beta lactamase production for epidemiological purpose.

MATERAIALS AND METHODS

A prospective study was conducted on clinical isolates of *Escherichia coli* from urine sample from cases of UTI during June 2016-January 2017, in the Department of Microbiology attached to Victoria Hospital, Bowring and Lady Curzon Hospital, Minto and Vani Vilas Hospital, Bangalore Medical College & Research Institute, Bangalore. Any Gram-negative bacilli other than *Escherichia coli*, Gram positive cocci, bacilli and fungi were excluded from the study. A mid-stream clean catch urine or urine collected with sterile syringe after clamping of Foleys catheter in catheterised patients were cultured withhalf an hour of collection. Culture of urine samples was done by semi quantitative (Mayo's technique) method on Mac Conkey agar and Blood Agar plates, incubated aerobically at 37˚C for 18-24 hours. A significant colony count and colonies with morphology of *Escherichia coli* confirmed by standard biochemical reactions (Pamela B. Crichton, 2010) was processed.

Detection of Virulence factors

1. **Hemolysin:** The *E. coli* isolates was inoculated on 5% sheep blood agar and incubated overnight at 37°C. Presence of a zone of complete lysis of erythrocytes around the colony and clearing of the medium indicates presence of hemolysin (Fatima *et al*., 2012).
2. **Hemagglutination:** Direct bacterial hemagglutination test-slide method: One drop of red blood cell (RBC) suspension was added to a drop of broth culture and the slide rocked at room temperature for 5 min. Presence of clumping is taken as positive for hemagglutination. Mannose-sensitive hemagglutination (MSHA) was detected by the absence of hemagglutination in a parallel set of tests in which a drop of 2%D-mannose added to the red cells and a drop of broth culture. Mannose resistant hemagglutinating (MRHA) was detected by the presence of hemagglutination of 3% 'O' blood group human RBCs in the presence of 2% D-mannose (Fatima *et al*., 2012).
3. **Serum resistance:** Overnight culture of *E. coli* on blood agar plates was suspended in Hank's balanced salt solution. Equal volume of this bacterial suspension and serum (0.05 ML) incubated at 37°C for 3 h. Then 10 µl of this mixture was inoculated on agar plate and incubated at 37°C for 24 h and viable count was determined. It is termed as sensitive when colony count drops to <1% of initial value (Fatima *et al*., 2012).
4. **Gelatinase test:** Gelatinase production was tested by gelatine liquefaction test.

Antibiotic susceptibility pattern of the isolates is studied with respect to Ampicillin (10 µg), Amikacin (30 µg), Co-trimoxazole (1.25/23.75 µg), Cefotaxime (30 µg), Ciprofloxacin (5 µg), Gentamicin (30 µg), Cefoxitin (30 mcg), Imipenem (10 mcg), Amoxiclav (20/10 mcg), Nitrofurantoin (300 mcg) and Norfloxacin (10 mcg) according to CLSI 2016 guidelines using Kirby-Bauer disk diffusion method (Clinical and Laboratory Standards Institute (CLSI) 2016).

Test for extended spectrum β-lactamase production

Screening by standard disk diffusion method: Screening for ESBL production was done according to criteria recommended by CLSI. Two discs, Ceftazidime (30 µg) and Cefotaxime (30 µg), testing by Kirby-Bauer disk diffusion method. Zone diameters were read using CLSI criteria. An inhibition zone of ≤ 21 mm for Ceftazidime and ≤ 26 mm for Cefotaxime indicated a probable ESBL producing strain requiring phenotypic confirmatory testing.

Phenotypic confirmatory methods: To confirm ESBL production by *E. coli* strains disk diffusion method is used. Ceftazidime (30 µg) vs. Ceftazidime/Clavulanic acid (30/10 µg) and Cefotaxime (30 µg) vs. Cefotaxime/Clavulanic acid (30/10 µg) placed onto Mueller Hinton agar plate lawned with the test organisms and incubated overnight at 35 °C. Regardless of zone diameters, a ≥ 5 mm increase in a zone diameter of an antimicrobial agent tested in combination with Clavulanic acid vs. its zone size when tested alone, indicated ESBL production (Clinical and Laboratory Standards Institute (CLSI) 2016).

Carbapenemase production

Carbapenemase-producing isolates usually test intermediate or resistant to one or more Carbapenems and usually test resistant to one or more agents in cephalosporin subclass III (eg, Cefoperazone, Cefotaxime, Ceftazidime, Ceftizoxime, and Ceftriaxone).

(1) A 0.5 McFarland standard suspension (using either direct colony suspension or growth method) of *E. coli* ATCC® 25922 (the indicator organism) in broth or saline, and dilute 1:10 in saline or broth. Inoculate an MHA plate as for the routine disk diffusion procedure. Allow the plate to dry 3 to 10 minutes. Place the appropriate number of Ertapenem or Meropenem disks on the plate.
(2) Using a 10-µL loop or swab, pick 3 to 5 colonies of test or QC organism grown overnight on a blood agar plate and inoculate in a straight line out from the edge of the disk. The streak should be at least 20 to 25 mm in length.

Following incubation, examine the MHA plate for enhanced growth around the test or QC organism streak at the intersection of the streak and the zone of inhibition Enhanced growth = positive for Carbapenemase production. Enhanced growth = negative for Carbapenemase production (Clinical and Laboratory Standards Institute (CLSI) 2016).

Metallo-β-lactamase (MBL) production

Isolates resistant to Imipenem or Meropenem is considered for confirmation of MBL production. A 0.5 McFarland standard suspension of the test organism was lawned on the Muller-Hinton agar recommended by the CLSI guidelines. Imipenem (10µg) and Imipenem/EDTA (10+750 µg) were placed on the Mueller Hinton agar at a distance of 20mm to each other. After the incubation period of 16-18 hours at 37^0 C, the increase in inhibition zone size ≥ 7mm around the IMP/EDTA compared to IMP alone was considered as MBL positive (Galini *et al*., 2008).

AmpC beta lactamase production

Isolates showing reduced susceptibility to Cefoxitin was considered for Amp C beta lactamase detection.

Method

A lawn of test organism was made on MHA plate and cefoxitin 30 mcg disk was placed, cefoxitin + phenylboronic acid 300mcg and cefoxitin + cloxacilln 200 mcg was placed in the same plate, after 24 hrs of incubation if there was enhanced zone of clearance around cefoxitin + phenylboronic acid disk of > 5mm and enhanced zone of > 3mm around cefoxitin+ cloxacillin was taken positive for AmpC beta lactamase production (Ingram *et al*., 2011).

RESULTS

75 UPEC isolates with significant colony count were included in the study. Among which 40 (53%) were from female patients and 35(47%) from male patients. 22(30%) were in the age group of 1-18 years, 40 (53%) were in the age group 19-59 years, 13 (17%) were≥60 years. 46 (61%) were from inpatients, 29 (39%) were from outpatients. Majority of the samples were from medicine department (35 (47%)) followed by paediatrics (22(30%)) OBG (12(16%)) and the rest were from urology and other departments. Antibiotic sensitivity of the UPEC isolates to Ampicillin was (2.6%), Amoxiclav (6%), Amikacin (80%), Gentamycin (61%), Ciprofloxacin (45%), Cotrimoxazole (40%), Ceftriaxone (21%), Cefotaxime (16%), Ceftazidime (17%), Cefoxitin (68%), Imipenem (92%), Norfloxacin (33%) and Nitrofurantoin (85%). Maximum sensitivity was seen with Imipenem, Nitrofurantoin and Amikacin, and least with Ampicillin and Amoxicillin-Clavulanate.

Table 1. Results of Antibiotic sensitivity of the UPEC isolates

Drugs	No. of sensitive isolates (n= 75)
Ampicillin	2 (2.6%)
Amoxiclav	5 (6%)
Amikacin	60 (80%)
Gentamycin	46 (61%)
Ciprofloxacin	34 (45%)
Cotrimoxazole	30(40%)
Ceftriaxone	16 (21%)
Cefotaxime	12 (16%)
Ceftazidime	13 (17%)
Cefoxitin	51 (68%)
Imipenem	69 (92%)
Norfloxacin	25 (33%)
Nitrofurantoin	64 (85%)

Hemolysis was seen in 32% of the isolates; Hemagglutination in 40% of the isolates, 24% of the isolates were MSHA and 16% of the isolates were MRHA; 47% of the isolates were showing serum resistance and 13% of the isolates produced gelatinase.45(60%) of 75 isolates had one or more virulence factors. 32(43%) of them had more than one virulence factors. 40 (53%) of the isolates were ESBL producers, among them 60% were from in patients and 40% from outpatients. 6 (8%) of the isolates produced AmpC beta lactamase, 83% from inpatients and 17% from outpatients. None of the 6(8%) isolates which were Imipenem resistant were Carbapenamace or MBL positive by phenotypic methods.

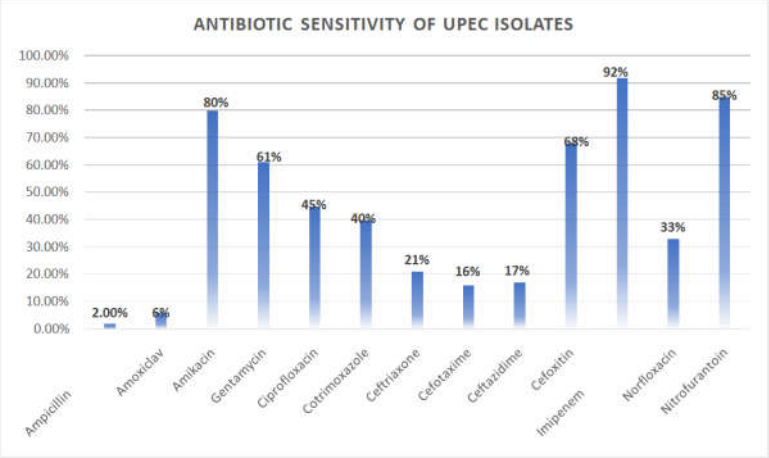

Figure 1. Antibiotic sensitivity of UPEC isolates

Table 2. Results of Virulence factors of UPEC

Virulence factors	No. of isolates n=75	ESBL	Carbapenamace	MBL	AmpC betalactamase
Hemolysis	24 (32%)	15 (63%)	0	0	1 (4%)
Hemagglutination	30 (40%)	17 (57%)	0	0	2 (7%)
MSHA	18 (24%)	9 (50%)	0	0	2 (11%)
MRHA	12 (16%)	8 (67%)	0	0	0
Serum resistance	35(47%)	18 (51%)	0	0	1 (3%)
Gelatinase	10 (13%)	5 (50%)	0	0	0

23 isolates among 63 Cefotaxime and Ceftazidime resistant isolates were non ESBL producers by phenotypic method. 63% and 1% of the isolates which showed hemolysis were ESBL and AmpC betalactamase producers respectively, similarly 57% and 7% of isolates which showed hemagglutination, 50% and 11% of MSHA, 67% and 0% of MRHA, 47% and 3% of isolates showing serum resistance; and 50% and 0% of gelatinase producers were ESBL and AmpC beta lactamase producers respectively.

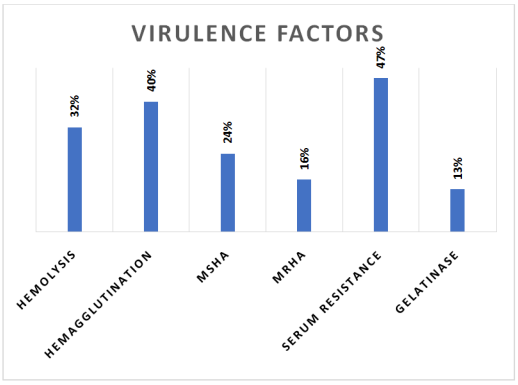

Figure 2. Virulence factors produced by UPEC isolates

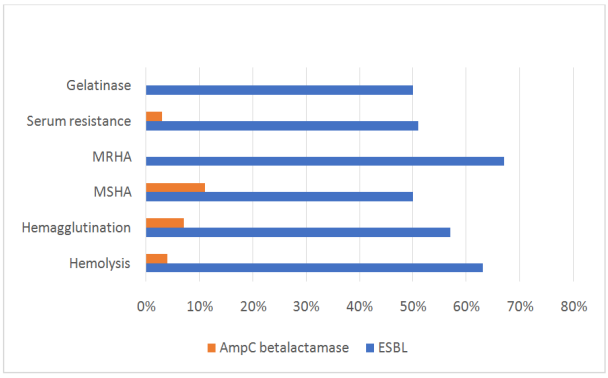

Figure 3. Percentage UPEC isolates with virulence factors producing beta lactamases

Table 3. Results of beta lactamase produced by The UPEC isolates

	IP	OP	Total
ESBL	24 (60%)	16 (40%)	40 (53%)
Carbapenamace	0	0	0
MBL	0	0	0
AmpC betalactamase	5 (83%)	1(17%)	6 (8%)

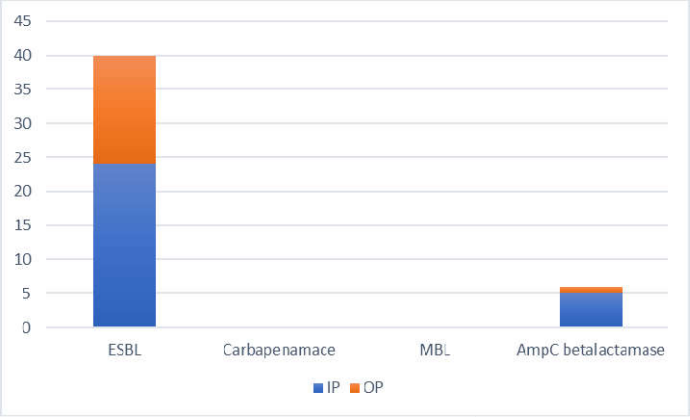

Figure 4. Beta lactamases produced by UPEC isolates

DISCUSSION

Urinary tract infection is the most common clinical presentation, especially in females due to anatomical and physiological factors (Agarwal *et al*., 2012). In the present study incidence of UTI was more in females (53%) compared to males (47%), which was seen in most of the studies conducted. Ghosh *et al*. (2016) in their study saw that out of 50 isolates 38 were from female patients and 12 from male patients, Jadhav *et al*. (2011) in their study the incidence of UTI was more in female patients (64%) and compared to 36% in male patients, Mittal *et al*. (2014) also saw that the incidence was more common in females in their study 53.3% compared to males 46.7%. In the present study 60% of the isolates were having one or more virulence factors and 43% of the isolates were having more than one virulence factors. 65% of the isolates having virulence factors were ESBL and/or AmpC beta lactamase producers. 32% of the isolates showed hemolysis, 40% of the isolates were hemagglutinating, 24% of the isolates were MSHA and 16% of the isolates were MRHA; 47% of the isolates were showing serum resistance and 13% of the isolates produced gelatinase. Various studies have been done on demonstrating virulence factors in UPEC. Mittal *et al*. (2014) in their study, hemolysin production was seen in 47.4%, hemagglutination in 74.8%, serum resistance in 59%, gelatinase in 67.5%, hemagglutination and gelatinase production is more compared to the present study. Raksha *et al*. (2003) in their study 91(41.36%) of the isolates were haemolytic, 68(30.9%) showed MRHA and 72(32.72%); were serum resistant which was in concordance with the present study. Kausar *et al*. (2009) in their study of 200 UPEC 42(21%) were haemolytic, 60(30%) showed MRHA and 72(36%) MSHA, 99(49.5%) were serum resistant. Vidhya *et al*., (2016) in their study had 50 urinary isolates, 10% were haemolytic, hemagglutinating property was seen in 20 % (MRHA 7 and MSHA 3 (n=10)) and 88 % of them had serum resistance, they had more isolates with serum resistance as virulence factor, and less of haemolysin and hemagglutination compared to the present study. Ranjan *et al*. (2010) in their study had 41.36% of the isolates showing hemolysis, 30% of the isolates showed hemagglutination and 32.72% of the isolates had serum resistance which is comparable to the present study. Fatima *et al*. (2012) 30% of the UPEC isolates showed haemolytic activity 48% isolates showed Haemagglutination (HA), 30.0% were MRHA positive and 18.0% were MSHA which is in concordance with the present study. In the present study 53% of the UPEC isolates were ESBL and 8% of the isolates produced AmpC beta lactamase. None of the isolates were Carbapenamace or MBL positive by phenotypic methods. Shariff *et al*. (2013) in their study found that 59% of their UPEC isolates were ESBL producers. Mittal *et al*. (2015) in their study found that 88% and 22% of their UPEC isolates which produced biofilm were ESBL and AmpC beta lactamase producer respectively and 6% of the isolates were MBL producers. Mukherjee *et al*. (2013) in their study found that 45% of their UPEC isolates were ESBL producers by phenotypic method. Ranjini *et al*. (2015) in their study 39.66% of their UPEC isolates were ESBL producers which is less compared to the present study. Dugal *et al*. (2013) in their study 70.9% of UPEC isolates were ESBL producers, 12.9% were carbapenemase producers and 3.2% were Amp C beta lactamase producers. So, the virulence factors studied in the present study and the ESBL production is found in similar numbers in various studies.

Conclusion

In the present study, virulence factors and beta lactamase production was found in more than 60 % of the UPEC isolates. This shows that these factors play an important role in the pathogenesis of *E.coli* in causing UTI. And by knowing the type of virulence factors produced and the mechanism of it, help in better understanding of pathogenesis and may help in treatment by developing therapeutics by targeting these factors, like drugs or vaccines in future. The isolates in this study were showing least sensitivity to penicillin group of drugs and third generation cephalosporins, moderate sensitivity to fluoroquinolones, and highest sensitivity to Nitrofurantoin, Amikacin and Imipenem.

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