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Virulence Factors and Antibiogram of Uropathogenic *Escherichia coli* isolated at Tertiary Care Hospital.

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ABSTRACT

Urinary tract infections (UTIs) are one of the most common infectious diseases encountered in the clinical practice, mainlybeing associated with different members of the family *Enterobacteriaceae*. *Escherichia coli* (*E. coli*) is the most common cause of urinary tract infections (UTIs). Most of these uropathogenic strains of *E. coli* are believed to display a variety of virulence properties that help them colonize host mucosal surfaces and avoid host defenses to allow invasion of the normally sterile urinary tract. Even colony count of $\leq 10^3$ CFU/mL uropathogenic strains could be associated with UTI and its complications. Among the 81 *Escherichia coli* strains, 54 (66.6%) were found to be uropathogenic since they possessed one or more virulence markers. Of the 54 uropathogenic *Escherichia coli,* 30 (55.5%) isolates were found to be positive for hemolysis, 32 (59.2%) isolates were positive for haemagglutination and 22 (40.7%) isolates were positive for cell surface hydrophobicity. Antibiotic sensitivity pattern of uropathogenic Escherichia showed maximum sensitivity to amikacin (87%) followed bynitrofurantoin (83.3%). Drugs showing the least prevalence of susceptibility were ampicillin, norfloxacin, cefuroxime showing 14.8%, 18.5% and 25.9% of susceptibility respectively. Among 54 uropathogenic *Escherichiacoli*, 21 (38.8%) were extended spectrum beta lactamase (ESBL) producing organisms.

Keywords: uropathogenic *Escherichia coli,* urinary tract infections, virulence factors, antibiogram.

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INTRODUCTION

Urinary tract infection (UTI) can be defined as a spectrum of diseases caused by microbial invasion of the genitourinary tract that extends from the renal cortex of the kidney to the urethral meatus.It is the most common infection experienced by human after respiratory and gastro-intestinal infections, and also the most common cause of both community-acquired and nosocomial infections for patients admitted to hospitals [1]. Among all the organisms causing urinary tract infection, *Escherichia coli* is the single most common causative agent that has been isolated from 80- 90% of the patients suffering from urinary tract infections. Other common organisms causing urinary tract infection are *Klebsiellapneumoniae*, *Pseudomonas aeruginosa*, *Citrobacterfreundii*, *Enterococcus* species, *Enterobacter* species, *Proteus* species, *Providencia* and *Morganella* species [2].

It has been traditionally described that certain serotypes of *Escherichia coli* were constantly associated with uropathogenecity and were designated as Uropathogenic *Escherichia coli* (UPEC) [3]. Cell morphology and molecular biology have revealed that these organisms express several surface structures and secrete protein molecules, peculiar to the strains of *Escherichia coli* causing urinary tract infections. Usually these organisms are thought to emerge from the faecal flora and even from the skin of the perineum causing infection by ascending up the urinary tract.

In the late 1970s it was recognized for the first time that *Escherichia coli* strains causing urinary tract infection typically agglutinate human erythrocytes despite the presence of mannose and this was mediated mainly by P fimbriae that provides the ability to adhere to the uroepithelium, thereby resisting elimination by flow of urine. Subsequently an array of virulence factors namely, adhesins (X adhesins, P associated fimbriae, other mannose resistant fimbriae, type 1 fimbriae, Dr fimbriae etc.), hemolysin production, hydrophobic cell surface, siderophore production, have been proposed as virulence markers for uropathogenic isolates of *Escherichia coli* [4].

Under normal circumstances, the urinary tract possesses effective protective mechanisms to prevent colonization of any organism. However, some bacteria like Uropathogenic *Escherichia coli* (UPEC) evade these mechanisms for better firm adherence, colonization and hence cause a persistent infection to the urinary tract, by virtue of their virulence factors that are encoded chromosomally.

It is said that the number of virulence factors the organism expresses is directly proportional to the severity of the infection it causes [5]. A possible correlation exists between the presence of bacterial adhesins and the difficulty in eradicating organisms with short course antibiotic therapy. In addition even a small bacterial load of the uropathogen can cause the disease [4].

Uropathogenic *Escherichia coli* can not only cause complications such as persistent, relapsing infections by evading host immunity but also they can ascend up the urinary tract up to the kidney to cause cystitis, pyelonephritis and so on if unnoticed [6]. Hence identification of Uropathogenic *Escherichia coli* is important in preventing the complications by proper management. Considering the high degree of morbidity and mortality of urinary tract infections, the subject of uropathogenic *Escherichia coli* is receiving increasing attention.

The present study is to check the prevalence, demonstrate the virulence factors and study the antibiotic susceptibility pattern of uropathogenic *Escherichia coli* in our clinical setting.

MATERIALS AND METHODS

This study was done during a period of six months, from June 2012 to November 2012 in Saveetha Medical College and Hospital, which is located at Thandalam in the Kancheepuram district, India. This study was conducted in the hospital after obtaining the ethical committee's clearance and the university scientific review board's approval. Both inpatient and outpatient department patients were included in study who fulfil the inclusion criteria. All suspected cases of urinary tract infection, irrespective of age and all samples that yield *Escherichiacoli* are included in the study.All samples that show the growth of gram positive organisms and that show the growth of gram negative organisms except *Escherichiacoli* were excluded.

Urine samples from clinicallysuspected patients were collected, by standard sterile techniques. Proper instructions were given to the patients for collecting midstream clean catch urine in case of non-catheterized patients and in case of catheterized patients, aspiration was done from the port of urinary catheter, using sterile syringe. All the samples were collected in a universal sterile container and were transported immediately to the Microbiology laboratory without delay. In case of a delay, the samples were refrigerated until they were processed.Samples received in the Clinical Microbiology laboratory were subjected to gram staining and culturing on basic media such as Blood agar and MacConkey's agar [7]. The suspected lactose and non-lactose fermenters, mucoid colony on MacConkey's agar were processed further for their species level identification using biochemical tests.All the identified organism are subjected for tests for detecting various virulence factors such as haemolysin production, haemagglutination (HA) and salt aggregation test.

Tests for haemolysin production [8]

The blood agar for detection of haemolysis was prepared by incorporation of washed red blood cells into nutrient agar in a ratio of 1:5. The erythrocytes to be used in the medium were washed thrice in phosphate buffered saline. The subcultured *Escherichia coli* isolate to be tested were streaked into blood agar plates with sterile inoculation loop and incubated overnight at 37°C. The plates were observed for haemolysin production, as indicated by a clear zone of lysis surrounding the colonies of growth.

Tests for haemagglutination (HA) [9]

This test was carried out as per the direct bacterial haemagglutination test. It is a slide test that can detect mannose sensitive and mannose resistant haemagglutination.Human erythrocytes were used for HA testing. The erythrocytes were washed thrice and suspended to a 4% concentration in fresh saline. The bacteria were inoculated into 1% nutrient broth and incubated at 37°C for 48 hours for full fimbriation. The slide test was done in a clean microscopic slide. One drop of RBC suspension was added to a drop of broth culture and the slide was rocked to and fro for 5 minutes. Presence of clumping will be taken as positive for haemagglutination.Mannose sensitive haemagglutination (MSHA) was detected by the absence of haemagglutination in a parallel set of test in which a drop of 2% D- mannose was added to the mixture containing a drop of RBC and broth culture. The expression of type 1 fimbriae was indicated by Mannose sensitive haemagglutination (MSHA).Mannose resistant haemagglutination (MRHA) was detected by the presence of haemagglutination of human RBC in the presence of 2% D- mannose. Mannose resistant haemagglutination (MRHA) positive indicated most likely the presence of P fimbriae though X, FIC and DR fimbriae can mediate MRHAControls used were ATCC *Escherichia coli* 25992 for MSHA, UPEC serotypes O6 and O11 as MRHA positive controls.

Cell Surface Hydrophobicity

Salt aggregation test [10]

Escherichia coli grown on MacConkey agar plates were inoculated into 1 mL of phosphate buffered saline (pH- 6.8) and turbidity was matched with McFarland's tubes 6 and 7. Different molar concentrations of Ammonium sulphate namely 1M, 1.4M and 2 M concentrations were prepared.40 µl of 0.2M phosphate buffered saline (pH- 6.8) was taken in first column of a slide. 40 µl of 1M, 1.4M, 2M concentrations of Ammonium sulphate was taken in each well of other columns of the slide. 40 µl of *Escherichia coli* suspension was added to each of these wells.The clumps that result in different molar concentrations of Ammonium sulphate were observed macroscopically.

Strains were considered hydrophobic if they aggregated in concentrations of 1.4M. Isolates which give doubtful results were retested.A strain of *Escherichia coli* which was hemolytic, Mannose resistant haemagglutination positive and consistently positive for cell surface hydrophobicity was used as positive control.A strain which was non- hemolytic, negative for mannose resistant haemagglutination and consistently negative for cell surface hydrophobicity was used as negative control.

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Antibiotic Sensitivity Testing

In vitro antimicrobial susceptibility test was done by Kirby Bauer disc diffusion method using Mueller Hinton agar and Central Laboratory Standards Institute (CLSI) 2010 guidelines. [11] Antibiotics used were Ampicillin (10µg), Gentamicin (10µg), Cefuroxime (30µg), Cefotaxime (30µg), Norfloxacin (5µg), Amikacin (30µg), Nitrofurantoin (300µ g), Ceftazidime/clavulinic acid (30µg), Ceftazidime (30µg) and Co-trimoxazole (1.25/23.75mcg).*Escherichia coli*ATCC 25922 was used for quality control.

Extended Spectrum β– Lactamases (ESBL) Detection

All isolates showing resistance to Ceftazidime were evaluated for ESBL production by phenotypic confirmatory test [11]. Briefly, a suspension of each isolate was spread onto a Mueller- Hinton agar plate and Ceftazidime (30µg) and Ceftazidime / clavulanic acid (30µg/ 10µg) discs were placed aseptically on the agar plate. The distance between the two discs was kept at about 10 mm (edge to edge) and the cultures were incubated at 37^{o} C overnight. The observation of a 5mm increase in zone diameter for the antimicrobial agent tested in combination with clavulanic acid versus its zone diameter when tested alone confirmed the presence of ESBL production by that organism. The increase in zone diameter was due to inhibition of the β- lactamase by clavulanic acid.

RESULTS

During the period of six months from June 2012 to November 2012, 412 urine samples were collected from urinary tract infection suspected cases of in-patient and out-patient department of Saveetha Medical College and Hospital.

Out of 81 urine samples from which *Escherichia coli* was isolated, majority of the samples 27 (33.33%) were from the age group 21- 40 followed by 23,17 and 12 from the age group 41-60, <20 and 61-80 respectively. The least number of samples were from the age group >81 the percentage of which was 2.47%.Out of 81 samples yielding *Escherichia coli,* 54 (66.6%) were from female patients and 27 (33.4%) were from male patients. Among the 81 *Escherichia coli* strains, 54 (66.6%) were found to be uropathogenic since they possessed one or more virulence markers. Of the 81 samples positive for *Escherichia coli*, 68 (83.9%) were from adults and 13 (16.1%) were from children. In case of the 54 samples yielding uropathogenic *Escherichia coli* (UPEC), 44 (81.5%) were from adults and 10 (18.5%) were from children. Among the uropathogenic *Escherichia coli* also, majority were from the age group 21-40 from which 19 samples (35.18%) were obtained. The least number of samples i.e. 2 samples (3.7%) were from the age group >81. Even among uropathogenic *Escherichia coli*, 37 (69%) samples were from female patients and 17 (31%) were from male patients.

Of the 54 uropathogenic *Escherichia coli,* 30 (55.5%) isolates were found to be positive for hemolysis, 32 (59.2%) isolates were positive for haemagglutination and 22 (40.7%) isolates were positive for cell surface hydrophobicity.

Among the uropathogenic *Escherichia coli*, 28 strains had just one marker, 22 had two markers and 4 had all the three markers. The distribution of virulence marker among the number of uropathogenic strains is as shown in Table-1.

Table 1: Distribution of virulence markers among the uropathogenic *Escherichia coli*

Number of virulence markers	Virulence markers	Number of strains	Total number of strains
One marker	HL	6	**28**
	HA	13	
	CSH	9	
Two markers	HL+ HA	13	**22**
	HL+ CSH	7	
	HA+ CSH	2	
Three markers	HL+ HA+ CSH	4	**4**

HL- Haemolysis; HA- Haemagglutination; CSH- Cell surface hydrophobicity

Of the 32 strains that were positive for haemagglutination, 13 strains showed mannose sensitive haemagglutination and 19 showed mannose resistant haemagglutination. Among the 81 samples positive for *Escherichia coli*, 3 samples were found to be from patients showing recurrence of the disease. They were found to be presumptively recurrent strains, since they had the same profile of virulence markers and the same sensitivity pattern. Of these 3 strains, one strain was hemolytic and the remaining two strains were positive for mannose resistant haemagglutination and haemolysin. The virulence markers, colony counts and the susceptibility pattern of the recurrent strains are shown in Table-2.

Table 2: Virulence markers, colony counts and the susceptibility pattern of the recurrent strains.

Recurrent strain	Number of times it recurred	Profile of virulence markers	Significant colony count		Susceptibility pattern
			On primary isolation	On recurrence	
Strain 1	Once	Hemolysin	10^4-10^5	10^4-10^5	S: Ak, Nit
Strain 2	Twice	Hemolysin and Haemagglutination (MR)	10^5	10^4	S: Ctx,CoT, Nit
Strain 3	Once	Hemolysin and Haemagglutination (MR)	10^5	10^3	S: Ak, Gen, CoT, Nit

MR- Mannose Resistant; S- Sensitive; Ak- Amikacin; Nit- Nitrofuratoin; Ctx- Cefotaxime; CoT- Cotrimoxazole; Cxm- Cefuroxime; Cac- Ceftazidimeclavulinic acid; Gen- Gentamicin; ESBL- Extended spectrum beta lactamase

Of the 81 *Escherichia coli* isolates, 55 (67.9%) were from significant bacteriuria with colony counts ≥ 10^5 CFU/mL, 19 (23.4%) were from a 'probably significant bateriuria' with counts 10^4-10^5 CFU/mL and 7 (8.6%) were from 'insignificant bacteriuria' with counts of 10^3 CFU/mL.A detailed account of the uropathogenic strains with 1 or 2 or all 3 markers with respect to their colony counts is given in the Table-3.

Table 3: Virulence markers and colony counts among uropathogenic *Escherichia coli*

Number of virulence markers	Significant colony counts (CFU/ mL)		
	10^5	10^4- 10^5	10^3
1	21	7	-
2	15	5	2
3	1	1	2

CFU/mL- Colony forming units/ mL

Nine strains of *Escherichia coli* isolated from the samples showed mucoid appearance in agar plates, of which 4 were uropathogenic. Three other strains among the *Escherichia coli* isolates were non-lactose-fermenting, anaerogenic *Escherichia coli.* All 3 of these *Escherichia coli* (Alkalescensdispar) were found to be uropathogenic.

In the case of 54 uropathogenic isolates, amikacin was found to be the most successful drug since 47 of the isolates (87%) were susceptible to the drug followed by Nitrofurantoin which is 83.3%. Drugs showing the least prevalence of susceptibility were ampicillin, norfloxacin, cefuroxime showing 14.8%, 18.5% and 25.9% of susceptibility respectively.

Out of eighty one *Escherichiacoli*, 30 were extended spectrum beta lactamase (ESBL) producing strains. Among the 30 isolates, 21 (38.8%) were from the 54 uropathogenic *Escherichiacoli* isolates while 9 (33.3%) were from the 27 non uropathogenic strains.

DISCUSSION

Urinary tract infection (UTI) is among the most common infectious diseases of humans and is the most common nosocomial infection in the developed world. They cause significant morbidity and mortality, with approximately 150 million cases globally per year. It is estimated that 40-50% of women and 5% of men will develop a UTI in their lifetime [12]. Uropathogenic *E. coli* (UPEC) is the primary cause of UTI.

In the present study, *Escherichia* coli were isolated from 81 out of the 412 samples. Out of the 81 samples, 27 samples (33.3%) were from the age group 21-40, followed by the age group 41-60 from which 23 samples (28.42%) were obtained. Out of the 81 samples, 67% were from female patients and 33% were from male patients almost similar observation was made by a study [13] where 62.47% were females and 37.67% were males. This predilection can be attributed to various anatomic and physical factors in females that make them more vulnerable to the disease [4]. Sex wise distribution was similarly observed among the uropathogenic strains in which, females contributed 69% and males 31%. Among the elderly age groups most of them were males indicating that their vulnerability is due to development of complications like prostate enlargement or neurogenic bladder with advancing age [5].

Among the 81 *Esherichia coli* strains, 54 (66.6%) were found to be demonstrating character of uropathogenic. Much higher percentage was noted by other study where the percentage was about68.6% [10] respectively.

Among the *Escherichia* coli positive samples yielding 10^5, $10^{4-}10^5$ and 10^3 colony counts, 67.3%, 68.4% and 57.2% of strains were found to be uropathogenic strains, respectively. Thus the uropathogenic strains are distributed more or less equally in all the colony counts. Hence even in insignificant bacteriuria with colony counts of $<10^3$ CFU/mL, uropathogenic strains can be seen, insisting that even insignificant colony counts should not be ignored. This correlates well with the recent practice guidelines according to which cystitis can be defined even with colony counts of $\geq 10^2$ CFU/ml and pyelonephritis with colony counts of $\geq 10^3$ CFU/mL for an uropathogen [4].

Nine strains of *Escherichia coli* were found to have mucoid appearance in culture plates, of which 4 (44.4%) were found to be uropathogenic. Out of these four strains, one each was positive for hemolysis and mannose resistant haemagglutination respectively. Mucoid nature of colonies is due to the presence of capsule that confers serum and phagocyte resistance to some *Escherichia coli* strains. This resistance can be attributed to the content of sialic acid which reduces ability of bacterial surface to activate complement by alternative pathway [5]. Higher percentage of mucoid strains showing these properties was noted in other study[10]in which 16.6% of mucoid strains were hemolytic and 11.1% of strains were positive for mannose resistant haemagglutination (MRHA).

Three strains (3.7%) of *Escherichia coli* were found to be anaerogenic, non-lactose fermenting atypical strains (Alkalescensdispar) and all were uropathogenic. Haemolysis and haemagglutination were observed in 33.3% of strains and cell surface hydrophobicity was positive for 66.6% of strains. In a study [14]done on atypical *Escherichia coli* in urinary tract infections, 12% were hemolytic out of 26. Even in their study all the atypical strains were uropathogenic possessing one or more virulence factors. Altered phenotype is due to an altered genetic makeup. Since atypical strains are consistently associated with virulence factors, this atypical phenotype probably contributes to their virulence [10].

The cell bound form of the cytolytic protein toxin is known as beta haemolysin and the cytolytic protein toxin which is secreted by most of the haemolytic*Escherichia coli* strains is known as alpha haemolysin. This haemolysin is strongly pro-inflammatory, leading to the secretion of IL - 6 and chemotaxins, which sets the pace for the pathogenesis of renal diseases, especially the more severe forms of the infection. [5]Among the 54 uropathogenic *Escherichia coli*, 30 (55.5%) were haemolytic.

Presence of fimbriae which is the organ of adhesion is detected by haemagglutination. Mannose sensitive haemagglutination (MSHA) is brought about by type 1 fimbriae. Mannose resistant haemagglutination (MRHA) indicates most likely the presence of P fimbriae though X, FIC and Dr fimbriae can mediate MRHA. P fimbriae are found to be consistently associated with pyelonephritis. [9]A good proportion of the *Escherichia coli* which cause UTIs in pregnancy are P fimbriated. These UTIs, if not treated, can progress to pyelonephritis in about 30-50% of the cases [15]. Therefore, the *Escherichia coli* which are isolated from asymptomatic bacteriuria in pregnant women should be tested for its virulence factors to identify the risk of developing pyelonephritis. In our study totally 32 (59.25%) out of 54 strains were haemagglutinating, but in other study done in North India [16] 48%of strains were positive for haemagglutination. Of the total uropathogenic strains, 13 (24%) strains showed MRHA and 19 (35.18%) showed MSHA.

Cell surface hydrophobicity (CSH) is a recently described novel virulence mechanism by *Escherichia coli.* The authors in a study [17]indicated their role in mediating bacterial adherence to mammalian cells. Crystalline surface layer "S" present on both gram positive and gram negative organisms play a role in this. In our study, 22 (40.7%) out of 54 uropathogenic *Escherichia coli* showed positivity for cell surface hydrophobicity (CSH).

In 48.1% of strains more than one virulence factor were present. Positivity for all the 3 virulence factors (Haemolysin, haemagglutination and cell surface hydrophobicity) was observed in 7.4% of strains. Occurrence of *Escherichia coli* with both hemolysin and MRHA was more in number compared to other factors that occurred together. These findings emphasize the occurrence of hemolysin and P fimbriae together in many of the UPEC strains. Although the virulence of an organism cannot be accurately predicted on the basis of its measurable virulence factor phenotype, the presence of multiple virulence factors increases the virulence of the organisms. The compromising host condition decrease the need for multiple virulence factors in the strains which cause serious infections. In general the more virulence factors a strain expresses, the more severe an infection it is able to cause [6].

In the present study 3 samples were found to occur recurrently from the same patients. The virulence profiles of 66.6% of these organisms were positive for mannose resistant haemagglutination (MRHA), indicating the presence of fimbriae. One of the main reasons for recurrence is the uropathogenic virulence factors of the invading bacteria that can aid the organism in adhering and persisting in the urinary tract forming an 'infective reservoir' of organisms that results in recurrences. [18]It is also suggested that if treatment of UTI does not result in the elimination of the virulent strains from the body, then these strains tend to cause recurrences for a long time [19].

In our study while determining the antibiotic susceptibility pattern for the 54 uropathogenic strains, highest susceptibility was found for amikacin (87%) and nitrofurantoin (83.3%). Percentage of sensitivity to amikacin ranging from 85 to 99% was recorded by other studies [20,21]and for nitrofurantoin the sensitivity was 90.9% , indicating amikacin and nitrofurantoin being the primary drug in the treatment.The percentage of extended spectrum beta lactamase producing organisms in our study was 55.55%, among which 38.8% were UPEC and 33.3% were non UPEC whereas other studies [22,23]observed higher percentage of ESBL producing UPEC ranging from 43%to 53% suggesting ESBL producing organisms are common among UPEC.

CONCLUSIONS

Screening for uropathogenic strains should be taken up as a routine procedure in laboratory so as to avoid recurrence in patients and also decrease the complications that the patients could develop due to the disease. Especially in cases of pregnant women where if P fimbriated UPEC strains are left unnoticed, even asymptomatic bacteriuria can develop to pyelonephritis. Colony counts of $\leq 10^3$ CFU/mL should not be ignored since uropathogenic strains can be seen even in lower colony counts. Atypical strains of *Escherichia coli* are constantly associated with virulence factors and they are uropathogenic too. So identification of UPEC is important in preventing the complications by proper management. Moreover changing trends in antibiotic resistance necessitates the periodic generation of antibiogram data to help health authorities revise treatment strategies for urinary tract infections caused by *E. coli*.

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