Virulence factors in uropathogenic *escherichia coli* (upec) causing urinary tract infections.

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Abstract:

Introduction: Escherichia. coli is the most frequent pathogen causing urinary tract infections. This study was conducted to determine the prevalence of virulence factors and antibiotic resistance in Escherichia coli (Esch coli) strains causing complicated and uncomplicated urinary tract infection (UTI).

Materials and methods: A total of 100 Esch coli isolates from patient suffering from UTI and *50* Esch coli isolates (controls) from stool samples of healthy volunteers were included. These isolates were screened for virulence factors :α-haemolysin, mannose resistance and mannose sensitive haemagglutination(MRHA:P-fimbriae, MSHA: type-1 fimbriae), cell surface hydrophobicity , and serum resistance by recommended methods.

Results: When 100 Esch. coli isolates from patients and 50 isolates from control (stool samples) were compared for presence of virulence factors it was seen that there was significant difference between cases and controls for P-fimbriae showing MRHA (36% vs 8%) and α-hemolysin(54% vs 40%) (P = 0.0002, P=0.001 respectively). 52% isolates of Esch. coli from the cases had three or more virulence factors as against just 2% isolates of Esch.coli from the control.

Conclusion: Multiple virulence factors were more frequent in isolates from patients with UTI as compared to controls. Isolates from uncomplicated and upper UTI were more virulent than those from complicated and lower UTI.

Key words: Uropathogenic, Escherichia coli, fimbriae, haemolysin, mannose resistant haemagglutination, Mannose sensitive haemagglutination

Introduction: Urinary tract infection is an most important causes of morbidity and mortality in patients being treated in an hospital. Escherichia coli (Esch. coli) is the most frequent urinary pathogen isolated from 50-90% of all urinary tract infection (UTI)[1]. These often originate from faecal and perineal flora which act as reservoir for initiation of UTI[2,3].It is know recognized that there is a subset of faecal Esch.coli which can colonise the periurethral area , enter urinary tract and cause disease. These are currently designated as Uropathogenic Escherichia coli (UPEC).These UPEC are endowed with chromosomally encoded virulence factors. These include ability to adhere to uroepithelial cells, presence of some specific O and K antigens[4,5], resistance to phagocytosis and also to the bactericidal action of normal serum.[6,7] Other factors known to contribute to the virulence of Escherichia coli include the production of alpha-hemolysin[8,9], colicins[10,11], aerob-actins[12,13], cytotoxic necrotising factors[14,15], and presence of cell surface hydrophobicity[16]

Manifestations of UTI depend on both host and bacterial factors, hence they vary from asymptomatic bacteriuria to symptomatic cystitis, pyelonephritis or blood stream infection[2,3]. UTI occurring in patients with intact urinary tract is called uncomplicated UTI. In these patients infect-

ions like pyelonephritis are caused by strains that possess most of the above mentioned virulence factors. Only a few uropathogenic clones cause such infections. UTI are also seen in patients having functional and structural abnormalities of the urinary tract and in those associated with chronic illnesses like diabetes mellitus. Such infections are called complicated UTI. These are usually caused by *Esch. coli* with lower virulence. Such strains also show greater drug resistance as compared to those isolated from uncomplicated UTI[17]

There have been very few studies on detecting the prevalence of these virulence factors in *Esch.coli* causing different types of UTI. This can help us in better understanding of the pathogenesis of UTI which can eventually improve the ability to identify the patients at risk and prevent or minimise complications.[18]The present study was designed to determine prevalence of virulence factors namely alpha-hemolysin, type 1 fimbriae, P-fimbriae, cell surface hydrophobicity and serum bactericidal assay, in patient isolates (uncomplicated and complicated UTI) vis a commensal/ gut isolates.

MATERIALS AND METHODS: The study was conducted in B.J. Medical College and Sassoon General Hospital for a period of two years from January 2006-December 2007. A total of 100 isolates of Esch.coli from urine samples of UTI patients attending the OPD of Medicine, Urology, Obstetrics were studied . Out of these100 isolates, 22 isolates were from complicated UTI (benign enlargement of prostrate, renal calculi) and 78 isolates were from uncomplicated UTI which included,19 from upper UTI, 40 from lower UTI, 11 from renal failure and 8 from UTI in pregnant women . These urine samples were processed by semi quantitative method and Esch.coli with significant count were selected. 50 Esch.coli isolates from stool samples of healthy volunteers were also included in this study. These Esch.coli were identified using biochemical reactions[19] .

The antibiotic susceptibility testing was carried out on Mueller Hinton agar by modified Kirby Bauer method , ESBL production was also detected [20] .

The antibiotics used were Ampicillin(10 µg), Amikacin(80µg) ,Cepho-taxime(30µg) ,Tetracycline(30 µg) , Gentamicin(10 µg),Norfloxacin(10 µg), Nitrofurantoin(300 µg), Cotrimoxazol (25 µg). *Esch. coli* isolated were maintained on nutrient agar slopes and kept in refrigerator at 4^{o} C These strains were screened for virulence markers namely alpha-hemolysin , type 1 fimbriae, P- fimbriae, Cell surface hydrophobicity, Serum bactericidal assay as per method described below.

Detection of virulence marker :

Haemolysin: The cytolytic protein toxin secreted by most haemolytic Esch.coli is alpha haemolysin. The method used for detection of alpha haemolysin was as described by Smith et al.[21]. Cultures were incubated in alkaline meat extract broth at 37^{o} C for 2 ½ hour , centrifuged (6000 rpm for 30 min.) and then supernatant was collected. An equal amount of 2% sheep RBCs, washed 3 times in normal saline, were added. Then this mixture was incubated at 37^{o} C for 2 hr with intermittent agitation and seen under high power microscope for signs of haemolysis.[21]

Detection of P-fimbriae and Type-1 fimbriae :

Fimbriated *Esch. coli* produces haemagglutination in the absence of antibodies and other biological factors. Hence fimbriated strains can be detected by demonstrating clumping of erythrocytes (A Rh+ve).The method followed was that of Seigfred et al. 5 ml of A +ve venous blood was collected using a disposable syringe from voluntary donors and added to an equal amount of Alsever solution . This was washed 3 times and 3% erythrocyte suspension was made with phosphate buffer saline(PBS) pH 7.4. *Esch. coli* grown on nutrient agar was inoculated into 5 ml phosphate buffered saline pH 7.4.This was incubated for 5 days to get a pellicle (which was presumed to contain the fimbriated

strains) on the surface. From the pellicle, bacteria were inoculated onto Colonisation Factor Antigen agar and incubated overnight at 37 ^{o}C.. The procedure was performed on VDRL slides . 40 µl of bacterial suspension was mixed with 40 µl of human A +ve blood and 40 µl of PBS with and without 3% D. mannose. The slide was then placed on VDRL rotator and rotated for four minutes and haemagglutination reaction with saline and mannose were recorded. Controls used with each test were ATCC Esch. coli 25922 for MSHA and a known strain of Esch coli repeatedly giving MRHA positive as a MRHA positive control[22] .

Haemagglutination inhibited in presence of D-mannose was labeled as Mannose Sensitive Haemagglutination (MSHA) indicating type 1 fimbriae, and if agglutination occured even in presence of D-mannose, it was called Mannose Resistant Haemagglutination (MRHA) indicating presence of P-fimbriae[22,23]

Cell Surface Hydrophobicity (CSH) : Bacteria were tested for their hydrophobic property by using different molar concentrations of ammonium sulphate. Those which aggregated with salt particles and formed clumps were considered hydrophobic.[24] *Esch. coli* on nutrient agar plate was inoculated into 1 ml of 0.2 M PBS, pH 6.8 (5 x 10^9 colonies/ml).40 µl of 1M, 1.4 M, 2 M ammonium sulphate stained with methylene blue was taken in three different wells of VDRL tile. 40 µl of bacterial suspension in PBS pH 6.8 was added in each of these three wells. Strains were considered hydrophobic, if they aggregated in the concentrations of ≤ 1.4 M. [24]

Serum Bactericidal Assay: The complement pathway has been shown to be important in killing of *Esch. coli* in serum. Bacterial strains which are resistant to this action of serum were termed as serum resistant isolates. The assay was done according to Siegfried et al with some modifications[116]. Over night cultures of *Esch. coli,* grown on MacConkey agar at 37^{o} C. were suspended in Hanks Balanced Salt Solution(HBSS) (bacterial count of 1.64 x 10^4) .25 µl of above suspension mixed with 75 µl of pooled serum, taken in a sterile test tube The mixture was kept in water bath at 37^{o} C with intermittent agitation. This was then inoculated on three plates of NA at 0 hr, 1 hr and 2 hr of incubation in water bath. The plates were incubated at 37^{o} C overnight. Viable count was determined. The plate inoculated at 0 hr was taken as control

Statistical anal. Esch. coli was considered sensitive if count dropped to 1% and was considered resistant if >90% of organisms survived after 2 hrs of incubation. Esch. coli isolate which was consistently serum resistant was taken as positive control and ATCC Esch. coli 25922 that was serum sensitive as negative control.***ysis:*** Chi square test was used to compare the occurrence of virulence factors in cases and controls.

RESULTS AND OBSERVATION:

Out of 100 Esch. coli isolates from the patients, 22 isolates were from complicated UTI and 78 isolates were from uncomplicated UTI. Whereas 26 isolates were from upper UTI and 52 isolates were from lower UTI.(Table.1)

Out of these 100 Esch. coli isolates, 30(30%) showed mannose sensitive haemagglutination (type 1 fimbriae), 36(36%) showed mannose resistant haemagglutination (P-fimbriae), 54(54%) isolates were haemolytic, 76(76%) had hydrophobic cell surface and 52(52%) isolates were resistant to serum bactericidal action. (Table 2)

Among 50 Esch. coli isolates from controls, 12(24%) showed MSHA, 4(8%) showed MRHA, 1(2%) was hemolytic, 20(40%) were serum resistant and 35 (70%) exhibited cell surface hydrophobicity. (Table 2)

Virulence markers like P-fimbriae (showing MRHA) and α-hemolysin (P = 0.0002, P=0.0001 respectively) were significantly present in Esch.coli isolates from cases as compared to isolates from control, as shown in Table.2. 52% Esch. coli isolates from the cases had three or more

virulence factors as against just 2% of isolates from the control (Table 3). Among 22 Esch. coli isolates from complicated UTI , 4(18.2%) were positive for MRHA, 5(22.7%) were positive for MSHA, 3(13.6%) were hemolytic, 7(31.8%) were serum resistant and 14(63.6%) had hydrophobic cell surface. And among 78 Esch. coli isolates from uncomplicated UTI, 32(41%) were positive for MRHA, 25(32%) were positive for MSHA, 51(65%) were hemolytic, 45(57%) were serum resistant and 62(79%) had hydrophobic cell surface. Hemolytic strains were significantly more in uncomplicated UTI as compared to complicated UTI.(P=0.0001). Whereas MRHA, Serum resistant strains were more in uncomplicated UTI but were not significantly more (P= 0.07 and P=0.051 respectively) (Table 4).

Among 26 *Esch. coli* isolates from upper UTI, 23 (88.5%) were positive for MRHA, 10(38.5%) were positive for MSHA, 25(96.1%) were hemolytic, 20 (76.9%) were serum resistant and 22(84.6%) had hydrophobic cell surface. And among 40 *Esch. coli* isolates from lower UTI 9(17.3%) were positive for MRHA, 15(28.9%) were positive for MSHA, 26(50%) were hemolytic, 25(48%) were serum resistant and 40(76.9%) had hydrophobic cell surface.Hemolytic, MRHA, Serum resistant strains were significantly associated with upper UTI as compared to lower UTI (0.0001, 0.0001 and 0.01 respectively) (Table 4) .55% of the total *Esch.coli* isolates were multidrug resistant (resistant to two or more drugs). No significant difference in susceptibility pattern was found between in the isolates from uncomplicated and complicated UTI.(Table 5)

DISCUSSION: The mere presence of organisms in urinary tract is not always enough to produce urinary tract infection. UTI occurs due to interplay of bacterial factors and host defense mechanisms, because of this certain individual contract the disease more frequently than others. These Uropathogenic *E.coli* express several surface structures and secrete protein molecules some of them cytotoxic, peculiar to the strains of *E.coli* causing UTI [1]. These virulence factors are expressed in different frequencies in different disease states[23]. Hence the present study was conducted to find out which virulence factors are expressed more frequently by UPEC isolated from different clinical categories like upper UTI, lower UTI, uncomplicated and complicated UTI.

Esch.coli produces three types of haemolysin namely α, β and γ. α-hemolysin is secreted in the broth culture and can be detected by various technique. The present study demonstrated highly significant difference for the production of α–hemolysin by the isolates from cases (54%) as compared to the control (2%) (P=0.0001). This was similar to the findings of Johnson et al, where haemolysin was produced by 38% of urinary isolates and 12% of faecal isolates[23]. Minshew et al also reported significant difference in α-haemolysin production between urinary (49%) and faecal (5%) isolates[26].

P- fimbriated strains can be detected by mannose resistant haemagglutination[23]. In the present study there was highly significant difference for MRHA between isolates from cases (36%) and control (8%) (P = 0.0002). Many studies had similar findings, Green and Thomas demonstrated MRHA in 56% of Esch. coli isolates from the cases and in only 17% of faecal isolates[27]. Johnson et al could show the presence of MRHA in 52% of UPEC and in only 19% of faecal Esch.coli[23].

Bacteria are killed by normal serum via the lytic activity of complement system. Bacterial resistance to serum results from individual and combined effects of capsular polysaccharide, O polysaccharide and surface proteins[28]. In the present study, serum resistant Esch. coli isolates from cases (52%) were more as compared to controls (40%). However there was no significant difference. Our findings were in agreement with those of Raksha et al who showed presence of serum resistance in 32.72% of UPEC as against

24% in faecal Esch. coli[29]. Other virulence factors like expression of type - 1 fimbriae, indicated by mannose sensitive haemagglutination (p=0.56), and cell surface hydrophobicity (p= 0.43). were not significantly present in isolates from cases as compared to controls in the present study. Johnson et al[23], Raksha et al[29] also demonstrated similar findings. Hence more work is required to assess role of MSHA and CSH in the pathogenecity of UTI.

Hence when prevalence of these virulence factors were compared, one can conclude that production of α-haemolysin , presence of P- fimbriae and serum resistance were important virulence factors for causing UTI, which were more frequently associated with *Esch coli* from cases as compared to the control. The presence of a block of genetically linked determinants for different virulence factors is responsible for multiple expression of virulence factors. This expression of multiple virulence factors is more common in isolates causing UTI than fecal or periurethral isolates[23] .Even in the present study 52% of *Esch.coli* isolates produced 3 or more than 3 virulence factors as against 2% of isolates from controls. These factors act synergistically to cause UTI, P-fimbriae helps to adhere to uroepithelial cells , alpha haemolysin which is also called as cytotoxic necrotizing factor and serum resistance helps in survival and invasion by evading the host defence mechanisms.

When prevalence of virulence factors in *Esch coli* causing upper and lower UTI was considered it was found that haemolysin production, MRHA(P-fimbriae) and serum resistance were significantly more predominant in isolates of upper UTI as compared to lower UTI .Seigfried et al also have showed similar findings for haemolysins (92% vs 64%) and MRHA (71% vs 46%)[22].P-fimbriae adhere to alpha-D-galactopyranosyl-(1,4) beta-D galactopyranose (Gal-Gal) [1]receptors, present on proximal and distal convoluted tubules, glomeruli and bowmans capsules , hence helps to localize the organism to upper urinary tract and it along with other factors stimulates inflammatory response causing upper UTI[23].

Relation of these virulence factors with the host factors was also studied in the present study by including Esch coli isolates from complicated and uncomplicated UTI. It was found that isolates from uncomplicated UTI were multi virulent. These isolates showed presence of haemolysin production significantly more than isolates from complicated UTI (P=0.0001, 65%). Also other virulence factors like P-fimbriae (41% vs 18.2%)and serum resistance (57% vs 31.8 %) were predominantly more in these isolates. Bhalla and Agarwal have reported a much higher percentage (80.3%) of hemolytic strains from uncomplicated UTI as compared to those from complicated UTI[30] .Johnson et al noted that 100% of isolates from uncomplicated UTI had P-fimbriae as compared to 57% of the isolates from complicated UTI and 60% isolates from uncomplicated UTI were resistant to serum bactericidal action as against only 49% strains from complicated UTI[31].This concludes that lower virulent strains can take the opportunity of defect in host defense mechanism and cause infection.

No significant difference was found in the resistance pattern of isolates from both complicated and uncomplicated UTI. Also ESBL production was demonstrated in 30% isolates from complicated UTI and 20 % isolates from uncomplicated UTI. However Johnson J R et al mentioned that antibiotic resistance was more commonly seen in the isolates from patients suffering from complicated UTI as compared to those suffering from uncomplicated UTI,[31] which was not found in our study.

Thus to summarize, the common virulence factors associated with UTI were presence of P-fimbriae (MRHA), haemolysin production and serum resistance. Whereas cell surface hydrophobicity and type-1fimbriae did not seems to play major role in causing UTI. Infections of urinary tract even if not always fatal can affect the quality of life of

the patient. Esch.coli endowed with multiple virulence factors is the main culprit. Considering the multidrug resistance pattern of more than 50% of isolates in the present study, one cannot exclude the possibility of antivirulence factor interventions being used in the prophylaxis and treatment of UTIs in the near future.

Table no. 1 : **Distribution of cases according to clinical diagnosis**

Clinical Diagnosis	**No. of cases (%)**
Complicated UTI	**22**
Upper UTI (pyelonephritis)	**26**
Lower UTI (Cystitis)	**52**

Table no 2 : Occurrence of virulence factors in *Esch coli* isolates from cases and controls

Virulence factors	**Number of isolates from Cases(%) Total cases=100**	**Number of isolates from Controls(%) Total controls=50**	**Fisher test P-value**
MRHA	**36(36)**	**4(8)**	**0.0002**
MSHA	30(30)	12(24)	0.56
Hemolysin	**54(54)**	**1(2)**	**0.0001**
SBA	52(52)	20(40)	0.22
CSH	76(76)	35(70)	0.43

Table No 3 : Presence of three and more virulence factors amongst *Esch. coli* isolates from cases and controls

Number	Virulence factors	Cases	Controls
5	MRHA+Hemolysin+CSH +SR+MSHA	**8**	**0**
4	MRHA+L+CSH+SR	**9**	**0**
4	MRHA+MSHA+L+CSH	**4**	**0**
4	MRHA+MSHA+L+SR	**1**	**0**
4	MRHA+MSHA+CSH+SR	**0**	**0**
4	MSHA+L+CSH+SR	**4**	**0**
3	MRHA+CSH+L	7	0
3	MRHA+L+SR	0	0
3	MRHA+CSH+SR	3	0
3	CSH+SR+L	8	0
3	MSHA+L+SR	0	0
3	MSHA+CSH+L	4	0
3	MSHA+CSH+SR	4	1
Total		52	1

Table no.4 : **Comparison of occurrence of virulence factors in complicated UTI and uncomplicated UTI**

Virulence Factors	Complicated UTI(%) N=22	Uncomplicated UTI(%) N=78	Fisher
MRHA	**4(18.2)**	**32(41)**	**0.07**
MSHA	5(22.7)	25(32)	0.44
Hemolysin	**3(13.6)**	**51(65)**	**0.0001**
SBA	**7(31.8)**	**45(57)**	**0.051**
CSH	14(63.6)	62(79)	0.15

Table No.5 : Comparison of occurrence of virulence factors between Upper UTI and Lower UTI

Virulent factors	Upper UTI(%) Total no. 26	Lower UTI(%) Total no. 52	Fisher test
MRHA	**23 (88.5)**	**9 (17.3)**	**0.0001**
MSHA	10 (38.5)	15(28.9)	0.44
Hemolysin	**25 (96.1)**	**26(50)**	**0.0001**
SBA	**20 (76.9)**	**25(48)**	**0.01**
CSH	22 (84.6)	40 (76.9)	0.55

Table 6 : Comparison of susceptibility pattern of *Esch coli* isolated from complicated and uncomplicated UTI

Drugs	Uncomplicated		Complicated	
	Resistant strains N=78	%	Resistant strains N=22	%
Amikacin	17	25.4	5.0	22.7
Ampicillin	44	65.7	15.0	68.1
Cefotaxime	38	56.8	10	45.4
Co-trimoxazole	46	68.6	13	59
Gentamicin	41	61.2	13	59
Tetracycline	41	61.2	15	68
Norfloxacin	44	65.6	15	68
Nitrofurantoin	15	22.4	4	18.1

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