

STUDY OF VIRULENCE FACTORS IN UROPATHOGENIC ESCHERICHIA COLI

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ABSTRACT: Uropathogenic E. coli (UPEC) is a causative agent in the vast majority of Urinary Tract infections (UTIs), including Cystitis, Pyelonephritis which may result in renal failure in healthy individuals and in renal transplant patients. UPEC express a multitude of virulence factors to break the inertia of the mucosal barrier. **PURPOSE:** To study the virulence factors and antimicrobial susceptibility pattern. **METHODS:** 200 E.coli strains from symptomatic cases of UTI and 50 E.coli strains from other clinical samples were taken as controls. Samples were screened for virulence factors like Haemolysin, Mannose resistant, Mannose sensitive Haemagglutination (MRHA, MSHA), Serum resistance and Cell surface hydrophobicity (CSH) by recommended methods. Antimicrobial susceptibility testing was done by Kirby-Bauer disc diffusion method as per CLSI guidelines. **RESULTS:** Among 200 E.coli strains 50(25%) were haemolytic, 60(30%) showed MRHA, 76(38%) showed MSHA, 98(49%) were serum resistant and 86(43%) were hydrophobic. Among 50 controls 8 (16%) were haemolytic, 2(4%) showed MRHA, 4(8%) showed MSHA, 16(32%) were serum resistant and 3(6%) were hydrophobic. The difference between cases and controls for MRHA, Serum resistance, Cell surface hydrophobicity production were significant ($p < 0.001$, $p < 0.05$, $p < 0.01$ respectively). Least percentage of resistance was seen to Imipenem (1%), Amikacin (2%) and Nitrofurantoin (5%). High percentage of resistance was found to Penicillins and Fluoroquinolones. **CONCLUSION:** The present study revealed that UPEC exhibited one or the other virulence factors. Identifying virulence markers will definitely be of help to the treating clinicians as timely treatment of such patients will prevent unwarranted complications like chronicity, persistence and extension of infection to kidney.

KEYWORDS: Uropathogenic Escherichia coli, virulence factors, haemolysin, haemagglutination, serum resistance, cell surface hydrophobicity, antimicrobial susceptibility.

INTRODUCTION: Urinary tract infections are one of the most common infections encountered in clinical practice mainly being associated with different members of the family Enterobacteriaceae and among them Escherichia coli (E.coli) is the most prominent pathogen.¹ The pathogenic E.coli are broadly classified as either Enteric/Diarrhoeagenic E. coli or Extraintestinal E. coli (ExPEC). Two types of Ex PEC are Neonatal meningitis E. coli (NMEC) and Uropathogenic E.coli (UPEC). Enteric/Diarrhoeagenic E. coli give rise to enteritis but rarely cause disease outside the intestinal tract. On the other hand Ex PEC strains maintain the ability to exist in the gut without consequences but have the capacity to disseminate and colonize other host niches including Blood, the Central nervous system and the Urinary tract resulting in disease.

Certain strains of E. coli like CFT073, J96 & 536 are consistently associated with uropathogenicity and are designated as UPEC. UPEC clones are selected subsets of fecal flora that possess different virulence factors that enable them to colonize the urinary tract. UPEC account for

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90% of all UTIs among ambulatory patients and up to 50% of all nosocomial UTIs. Since 1970s, an array of virulence factors have been proposed as virulence markers for UPEC. These include K antigen, Somatic O antigen, Pili/Fimbriae (Type-1 fimbriae, P fimbriae, S fimbriae, PAP), Afimbrial adhesins, Haemagglutination of erythrocytes, Haemolysin, Resistance to the bactericidal activity of Serum, Cell Surface Hydrophobicity, expression of Siderophore Aerobactin, production of Colicin V and Cytotoxic Necrotizing Factor.² These virulence markers are expressed with different frequencies in different disease states ranging from asymptomatic bacteriuria to urethritis, cystitis, pyelonephritis, bacteremia and septic shock.

The study was undertaken to determine the prevalence of virulence factors (haemolysin, haemagglutination of human erythrocytes and effect of D-mannose on haemagglutination, serum resistance and cell surface hydrophobicity) in urinary and other isolates of E.coli obtained from clinical samples in GGH Kurnool.

HAEMOLYSIN: UPEC produces two types of haemolysin - Beta haemolysin (cell bound) and Alpha haemolysin (cell free factor). Lysis of RBCs may result in making iron and other nutrients available for the growth of bacteria. It may contribute to tissue injury and survival in the renal parenchyma.

P - FIMBRIAE: Phenotypic expression of P-fimbriae can be detected by MRHA of human erythrocytes from individuals with the common blood group "P", encoded by "pap G" gene.³

TYPE-1 FIMBRIAE: More important in bladder colonization than P-fimbriae, encoded by "fim" gene cluster. Phenotypic expression of Type-1 fimbriae can be detected by MSHA.⁴

SERUM RESISTANCE: Resistance to bactericidal activity of serum results from individual or combined effects of capsular polysaccharide, O antigen and surface proteins.

CELL SURFACE HYDROPHOBICITY: It's a novel mechanism which mediates bacterial adherence to mammalian cells. Crystalline surface layer "S" layer on the surface of Gram negative bacteria plays a role in this mechanism.

MATERIALS AND METHODS: The study was conducted in Government General Hospital (GGH), Kurnool for a period of 1 year from September 2013 to August 2014. A total of 200 E.coli isolates from symptomatic cases of UTI attending OPD of GGH, Kurnool were studied for detection of virulence markers of E.coli in the department of Microbiology. 50 strains of E.coli identified from samples other than urine like pus, faeces, sputum etc. were taken as controls. Urine samples were collected and processed as per standard protocol. Lactose fermenting colonies on MacConkey agar showing significant bacteriuria were processed and identified as E.coli with standard biochemical tests.

DETECTION OF VIRULENCE FACTORS⁵:

- 1. HAEMOLYSIN:** E.coli isolates were inoculated on 5% sheep Blood Agar and observed for a zone of lysis around the colony after overnight incubation.

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- SERUM RESISTANCE:** Overnight cultures of *E. coli* grown at 37°C on Mueller Hinton agar (MHA) were harvested and the cells were suspended in Hank's balanced salt solution (HBSS). 0.05 ml each of bacterial suspension and serum were added to each well of microtitre plate. Control wells contained 0.05 ml of HBSS only. The plate was placed in water bath at 37°C for 3hrs. 10µl of each sample and control was withdrawn and spread on MHA plate and incubated for 18-24hrs at 37°C and viable count was determined. Strains were termed serum sensitive if the viable count dropped to 1% of initial value and resistant if 90% of organism survived after 3hrs. of incubation period.
- HAEMAGGLUTINATION:** *E. coli* grown on Mac Conkey agar plate were inoculated into 5ml of phosphate buffered saline (PBS) and incubated for 5 days at 37°C to get fimbriae enriched *E. coli*. The pellicle formed on the surface was noted and the tubes were centrifuged at 1500rpm for 5min. The supernatant was discarded leaving behind 1ml of sediment. 5ml of citrated group a positive fresh venous blood was taken and plasma separated by centrifugation. Erythrocytes were washed 3 times with normal saline and a 3% erythrocyte suspension was made with PBS. A 2% mannose sugar solution was prepared. On a VDRL slide 20µl each of *E. coli* suspension, 3% erythrocyte suspension and 2% mannose were added to a cavity. Controls: For MSHA - ATCC 2922.

For MRHA - In house control.

INTERPRETATION: Haemagglutination was considered to be Mannose resistant when it occurred in presence of D-mannose and Mannose sensitive when it was inhibited by D-mannose.

- CELL SURFACE HYDROPHOBICITY:** Salt aggregation test-*E. coli* grown on Nutrient agar slants were inoculated into 1 ml of PBS and turbidity was matched with Mac Farland tubes 6 & 7 to get a colony count of 5×10^9 /ml. Different molar concentrations of Ammonium sulphate namely 1M, 1.4M & 2M were prepared. 20µl of PBS was taken in the first column of VDRL slide. 20µl each of 1M, 1.4M & 2M concentrations of Ammonium sulphate were taken in each well of other columns of VDRL slide. 20µl of *E. coli* suspension was added to each of these wells. The clumps formed in different molar concentrations of Ammonium sulphate were observed microscopically at 20x magnification.

INTERPRETATION: Strains were considered hydrophobic if they aggregated at concentration of 1.4M and above.^{5,6}

ANTIBIOTIC SUSCEPTIBILITY TEST (AST): The AST was done by Kirby - Bauer disc diffusion method as per CLSI guidelines. The results were interpreted as Sensitive and Resistant as per the zones of inhibition supplied by the manufacturer. *E. coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC27853) were used as controls.⁷

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RESULTS: Out of 200 E. coli isolates 132 (66%) were from females and 68(34%) from males.

Sex	No. of Isolates	%
Female	132	66%
Male	68	34%

Table 1: SEX DISTRIBUTION OF TEST SAMPLES

Maximum number of E.coli isolates were obtained from samples in the age group 21-30(48%) followed by the age group 31-40 (31.6%). In the age groups > 40, 16-20 and 5-15, the percentage of isolates was 25%, 20% and 16.7% respectively.

Age range	No. of isolates	%
5 -15	1	16.7%
16 -20	2	20%
21 -30	70	48%
31- 40	24	31.6%
>40	3	25%

Table 2: AGE WISE DISTRIBUTION OF TEST SAMPLES

Among 200 E. coli strains 50(25%) were haemolytic, 60(30%) showed MRHA, 76(38%) showed MSHA, 98(49%) were serum resistant and 86(43%) were hydrophobic. Among 50 controls 8(16%) were haemolytic, 2(4%) showed MRHA, 4(8%) showed MSHA, 16(32%) were serum resistant and 3(6%) were hydrophobic. The difference between cases and controls for MRHA, Serum resistance, Cell surface hydrophobicity production were significant ($p<0.001$, $p<0.05$, $p<0.01$ respectively).

SL. No.	VIRULENCE MARKERS	CASES N=200	CONTROLS N=50	P VALUE
1	Haemolysin production	50 (25%)	8 (16%)	Not significant
2	Haemagglutination activity a. MRHA b. MSHA c. No HA	60 (30%) 76 (38%) 64 (32%)	2 (4%) 4 (8%) 44 (88%)	Highly significant ($p<0.001$)
3	a. Serum resistance b. Serum sensitive	98 (49%) 102 (51%)	16 (32%) 34 (68%)	Significant ($p<0.05$)
4	Cell surface hydrophobicity	86 (43%)	3 (6%)	Significant ($p<0.01$)

Table 3: VIRULENCE MARKERS OF UPEC OBTAINED FROM CASES AND CONTROLS

The test strains expressed high resistance to Ampicillin (83%), Norfloxacin (62%), Ofloxacin (61%), Trimethoprim/Sulphamethoxazole (58%), Ciprofloxacin (56%), Piperacillin (54%). The lowest percentage of resistance was for Imipenem (1%), Amikacin (2%), Nitrofurantoin (5%), Cefepime (8%), Ceftazidime (9%), Ceftriaxone (11%) and Cefuroxime (14%).

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SL. NO.	ANTIBIOTIC	STRENGTH OF THE DISC IN µg/ml	RESISTANCE	
			NO.	%
1	Amikacin	30	4	2
2	Ampicillin	10	164	83
3	Amoxyclav	30	40	20
4	Cephazolin	30	68	34
5	Cefipime	30	16	8
6	Ceftazidime	30	18	9
7	Ceftriaxone	30	22	11
8	Cefuroxime	30	28	14
9	Ciprofloxacin	5	112	56
10	Gentamycin	10	52	26
11	Imepenem	10	2	1
12	Nalidixic acid	30	114	57
13	Nitrofurantoin	300	10	5
14	Norfloxacin	10	124	62
15	Ofloxacin	5	122	61
16	Piperacillin	100	108	54
17	TMP/SMX	25/475	11	58
18	Tobramycin	10	62	31

Table 4: PREVALENCE OF RESISTANCE AMONG UPEC ISOLATES

Thirty-six (18%) isolates were resistant to at least three classes of the tested antimicrobial agents: Penicillins, Penicillins plus Clavulanic acid, Fluoroquinolones, Trimethoprim/Sulfamethoxazole, Aminoglycosides and Cephalosporins and were designated as Multi-drug resistant (MDR).⁸

DISCUSSION: E. coli accounts for 70-90% of UTIs, about 44% of them recurring over 12 months. These strains causing recurrent UTI are called Uropathogenic strains and have been provided with various virulence factors encoded by pathogenicity islands.⁹

SEX DISTRIBUTION OF E. COLI ISOLATES: In this study, it has been found that the prevalence of UTI was more in females. It is well documented that women are more prone to suffer from UTI than men. There was no history of catheterization and hence these isolates could be considered as community isolates.¹⁰

AGE DISTRIBUTION OF E.COLI ISOLATES: In the present study, maximum percentage of isolates (70%) belonged to age group 21-30 years. UTIs are common in the sexually active age group which in a developing country like ours, starts from a very early age after puberty. The inaccessibility of a qualified physician in the rural set up, more so if the victim is a woman, illiteracy, early marriages, poverty, ignorance and negligence of personal hygiene, all contribute to development of UTIs in the sexually active age group.¹¹

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VIRULENCE MARKERS OF E. COLI ISOLATES:^{10,11,12} In the present study, 25% of E. coli isolates were positive for haemolysin. Raksha et al (2003) have reported 41.36% of strains to be haemolytic. Mandal et al (2001) reported 45.5% of strains to be haemolytic. In the study of Rebecca et al (2005), 40.7% were haemolytic. Compared to the other Indian studies mentioned above, the percentage of haemolytic strains in the present study is much less.

In this study 120 (60%) strains were MRHA positive and 152 (76%) MSHA positive. MRHA of human RBCs is the phenotypic expression of P-fimbriae on E. coli. MSHA is the phenotypic expression of Type 1-fimbriae which mediate adherence and are more important in bladder colonisation than P-fimbriae.

Sl. No.	Year	Author	Place	No. of strains	MRHA		MSHA	
					No.	%	No.	%
1.	2001	Mandal et al	New Delhi	170	75	44	80	47
2.	2003	Raksha et al	Bangalore	191	68	36	ND*	
3.	2005	Rebecca et al	CMC Vellore	163	48	30	51	32
4.	2006	Manjula et al	Lucknow	160	40	25	55	34
	2009	Yasmeen Kausar	Bijapur	200	60	30	72	36
5.	2012	N.Fatima	Aligarh	120	75	30	45	18
6.	2014	Present Study	Kurnool	200	120	60	152	76

Table 5: Comparative Study of Haemagglutination in UPEC

*ND – Not Done

43% are CSH positive at 1.4M and 2M concentrations of Ammonium Sulphate. Raksha et al (2003) observed that 58 (30.36%) of strains were cell surface hydrophobic. In their study there was 18% positivity of CSH among the controls. However the percentage of CSH positive isolates in the present study is significantly more among the test group than the control group. Thus CSH in the present study can be considered as a virulence marker

Measuring a phenotype in vitro does not always correlate with in vivo expression and very often underestimates the presence of a virulence factor in vivo (Kaper et al 2004). Identifying a genotype does not mean that it is always expressed in the body. The distribution of virulence properties can also vary depending upon host characters, type of infection and predisposing factors which determine the host parasite interaction in vivo which can culminate in an active infection and this may or may not recur^{13,14}. The in vitro study of the phenotypic characters of uropathogenicity as studied above can only be of presumption to a possible recurrent UTI.

In the present study, Imepenem, Amikacin, Nitrofurantoin are observed to be the antibiotics of choice. In the present day of increasing bacterial resistance there is a constant variability of antimicrobial susceptibility pattern among clinical isolates. Thus it is felt that each isolate has to be individually studied for antimicrobial susceptibility and the choice of antibiotic decided.

CONCLUSION: UPEC E. coli is now a worldwide pathogen causing potentially severe antimicrobial resistant infections. Periodic review and formulation of antibiotic policy are needed for control of acquisition of drug resistance. The infections of urinary tract can affect the quality of life. UPEC

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endowed with multiple virulence factors is the main culprit¹⁵. The methods of detection of the virulence markers are reasonably easy and screening them in a clinical Microbiology laboratory is a worthwhile exercise. Identifying virulence markers will definitely be of help to the treating clinicians as timely treatment of such patients will prevent unwarranted complications like chronicity and persistence and extension of infection to kidney.

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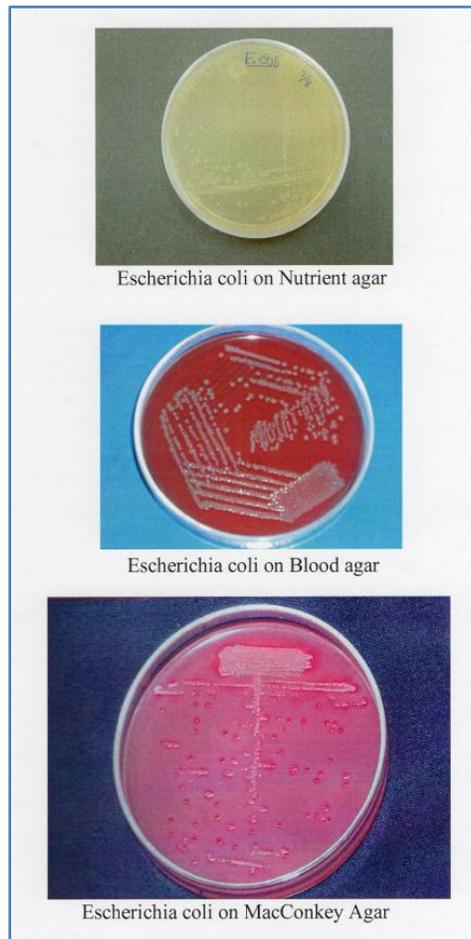


Figure 1

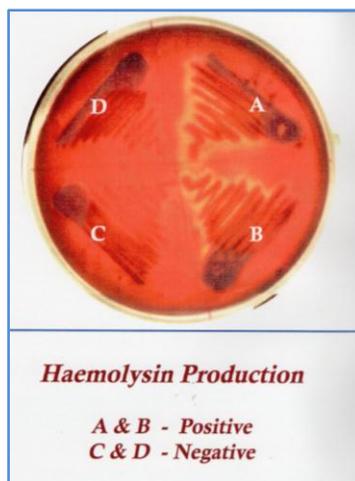


Figure 2

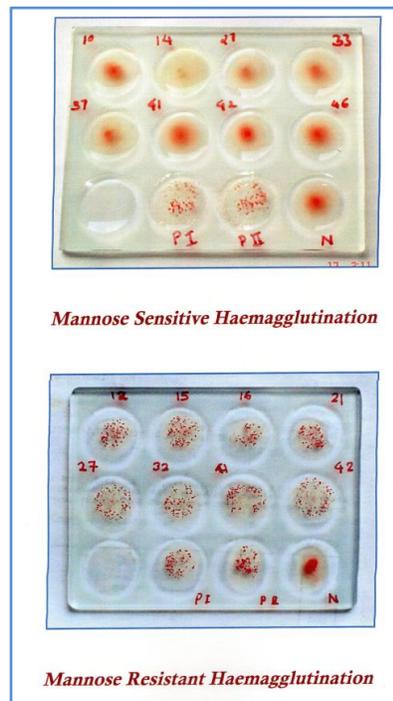


Figure 3

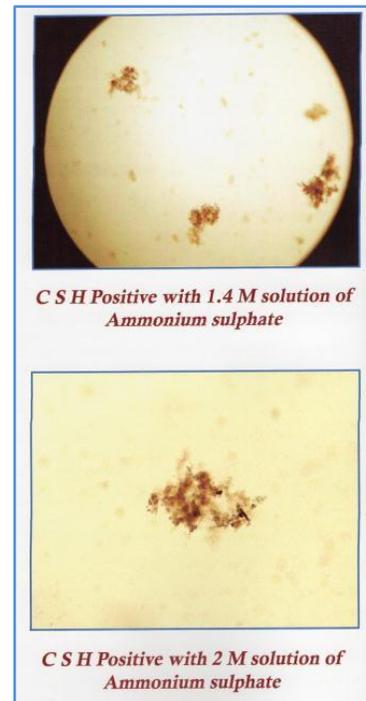


Figure 4

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