

Original Research Article

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Haemagglutination and Resistance to the Bactericidal Activity of Serum as the Urovirulence Markers of Uropathogenic *Escherichia coli*

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ABSTRACT

Bacterial strains causing extra intestinal infections harbor virulence factors that enhance the ability to cause systemic infection. *Uropathogenic Escherichia coli* (UPEC) responsible for UTI express a multitude of virulence factors to break the inertia of the mucosal barrier. To study the haemolysin, haemagglutination and resistance to the bactericidal activity of the serum property as urovirulence factors of *E.coli* and antimicrobial susceptibility pattern. 100 *E.coli* strains isolated from urine samples out of 400 symptomatic cases were screened for virulence factors like Haemolysin, Mannose resistant, Mannose sensitive Haemagglutination (MRHA, MSHA) and Serum resistance by phenotypic methods. Antimicrobial susceptibility testing was done by Kirby-Bauer disc diffusion method as per CLSI guidelines. Among 100 *E.coli* strains 33% were hemolytic, 9% showed MRHA, 14% showed MSHA, there was no haemagglutination in 73% strains and 76% were serum resistant. Least percentage of resistance was seen to Imipenem (1%), Piperacillin/tazobactam (1%) and Nitrofurantoin (19%). High percentage of resistance was found to Penicillins and Norfloxacin (79%). The present study revealed that UPEC exhibited one or more virulence factors. Identifying virulence markers will definitely prevent the complications like recurrent, chronic and persistent urinary tract infections.

Keywords

Uropathogenic
Escherichia coli,
antimicrobial
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haemagglutination,
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Introduction

Escherichia coli classified as the pathotype Uropathogenic *Escherichia coli* (UPEC) causing urinary tract infections usually occurs due to the movement of the strains from the intestinal tract or in some cases from the vagina into the urinary tract. It is considered as one of the most important opportunistic pathogen associated with UTI (Blanco *et al.*, 1996) when the intestinal commensal turns to a pathogen moves out

from its habitat, colonize host mucosal surfaces and circumvent host defenses to allow invasion of the normal sterile urinary tract (Moblely *et al.*, 2000).

UPEC exhibits a wide variety of virulence properties, includes capsular K antigen, somatic O antigen, adherence, haemagglutination of erythrocytes, haemolysin, resistance to the bactericidal

activity of serum, phagocytosis, cell surface hydrophobicity, expression of siderophore aerobactin, production of colicin V and cytotoxic necrotizing factor. These UPEC isolates express chromosomally encoded virulence markers and are expressed with different frequencies in different disease states ranging from asymptomatic bacteriuria to chronic pyelonephritis.

Adhesion is often mediated by fimbriae (pili) proteinaceous, hairlike extensions from the bacterial cell surface that may recognise specific receptor structures like carbohydrates, on the epithelial cell membrane. These fimbriae have been studied mostly in *E. coli* in relation to gastroenteritis and UTI consisting of P fimbriae, type 1, afimbrial adhesins like S, Dr, afa.

Bacterial adhesion is divided into mannose sensitive (adhesion inhibited by mannose) type 1 fimbriae and mannose resistant (adhesion not inhibited by mannose) P fimbriae which helps the bacteria to resist the flow of urine by attaching to the uroepithelium and to the kidney cells with glycosphingolipids receptors.

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 contributing 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 "papG" gene (De Ree *et al.*, 1987).

Type-1 Fimbriae

More important in bladder colonization than P-fimbriae, encoded by 'fimH' gene cluster. Phenotypic expression of Type-1 fimbriae can be detected by MSHA (Duguid *et al.*, 1979).

Serum Resistance

Resistance to bactericidal activity of serum results from individual or combined effects of capsular polysaccharide, O antigen and surface proteins (Vijayalakshmi *et al.*, 2015).

This study was undertaken to determine the prevalence of virulence factors - haemolysin, serum resistance, haemagglutination of human erythrocyte and effect of D-mannose on haemagglutination in urinary isolates of *E. coli* obtained from a tertiary hospital.

Materials and Methods

The prospective study was conducted at the Department of Microbiology in a tertiary care hospital from October 2015 to March 2016. A total of 400 urine samples collected from the patients was labelled appropriately and transported immediately to the Microbiology laboratory for processing. The samples were cultured by bacteriological standards and confirmed as *Escherichia coli* by biochemical identification. The prevalence of virulence factors haemolysin, Haemagglutination, resistance to the bactericidal activity of serum. Antibiotic susceptibility pattern was carried out using Kirby-Bauer disc diffusion method.

Antibiotic Susceptibility testing pattern

Inoculum was prepared by inoculating the 2 to 3 colonies from culture media in nutrient broth and standardized with 0.5 MC Farland

standard and swabbed onto a 90 mm Muller-Hinton agar plate using Kirby Bauer disc diffusion method and incubated at 37° C overnight and zone of inhibition was measured as per CLSI guidelines. Antibiotic discs - Ampicillin 10µg, Amoxycylav 30µg, Cefuroxime 30µg, Cefepime 30µg, Cefpodoxime 30µg, Ceftazidime 30µg, Ceftriaxone 30µg, Gentamicin 10µg, Norfloxacin 10µg, Nitrofurantoin 300µg, Nalidixic acid 30µg, Co-trimoxazole 25µg, Imipenem 10µg, Piperacillin/ Tazobactam 100/10µg were used.

Haemolysin production

The plate hemolysis test was done for the detection of β-hemolysis produced by the *E. coli*. The bacteria were inoculated into 5% sheep blood agar and incubated overnight at 35°C. Hemolysin production was detected by the presence of a zone of complete lysis of the erythrocytes around the colony and clearing of the medium (Raksha *et al.*, 2003).

Haemagglutination of Human group O erythrocytes

The strains of *E. coli* was inoculated into 1% nutrient broth and incubated at 37°C for 48 h for full fimbriation. Blood group "O" red blood cells were then washed thrice in normal saline and made up to a 3% suspension in fresh saline. They were used immediately or within a week when stored at 3-5°C. The test was carried out on Venereal Disease Research Laboratory (VDRL) slides. One drop (100µl) of bacterial suspension was mixed with onedrop of erythrocytes and one drop of phosphate-buffered saline (PBS) with and without 3% mannose on a VDRL slide. The slide was rotated for five minutes at room temperature and the presence or absence of macroscopic haemagglutination was noted.

Haemagglutination was considered to be mannose resistant (MRHA) when it occurred in the presence of D-mannose indicating P fimbriae and mannose sensitive (MSHA), when it was inhibited by the presence of D-mannose representing type 1 fimbriae (Siegfried *et al.*, 1994). Controls: For MSHA - ATCC 2922. For MRHA - In house control.

Serum bactericidal activity

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). 50µl each of bacterial suspension and serum were added to each well of microtitre plate. Control wells contained 50µl of HBSS only. 10µl of each sample and control was withdrawn and spread on MHA plate at 0hr and at 3 hrs after incubation in water bath at 37°C for 3hrs, 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 the initial value and resistant if 90% of organism survived after 3hrs incubation period.

Results and Discussion

Out of 400 urine samples 100 were *E. coli* isolates in which 66% were females and 34% of males. Maximum *E. coli* were isolated from the age group of 11 – 20 yrs (19%), 21 – 30 yrs and 41 – 50 yrs each accounting 15%, followed by 61 – 70 yrs which is of 14% (Table 1).

Antibiotic susceptibility pattern showed 100% sensitivity to imipenem, piperacillin/tazobactam 99%, followed by nitrofurantoin 81%, gentamicin 62%, co-trimoxazole 59% and the resistance pattern was high in nalidixic acid 94%, ampicillin 84%, amoxycylav 84%, norfloxacin 79%,

cefepime 73%, cefuroxime 72%, ceftazidime 71% (Table 2).

Among 100 *E.coli* isolates a) Haemolysin production: 33% had hemolytic property and 77% were non-hemolytic. b) Haemagglutination: 9% showed P fimbriae (MRHA), 14% were Type 1 fimbriae (MSHA) and there is no haemagglutination in 73 isolates. c) Serum bactericidal activity: Higher resistance 76% to serum were noted in this study and the serum bactericidal action was present in only 24% isolates (Table 3).

Innate bacteria often stick to surfaces like stones, leaves, and roots. Epithelial cell surfaces make no exception. Bacteria belonging to the 'normal' flora as well as pathogens may adhere by hydrophobic or electrostatic bonds, or both, of an 'unspecific' nature, or through a specific interaction between bacterial adhesins and epithelial cell receptors (Gibbons *et al.*, 1971; Savage *et al.*, 1980; Colleen *et al.*, 1982). The ability of pathogens to adhere to mucous membranes is recognized as a potential factor in virulence.

In this study, females were infected with high percentage of *E.coli* 66%. This may be an expression of a more general biological abnormality, since buccal cells of women are prone to UTI showed an increased

adhesive capacity (Duguid *et al.*, 1955). In the present study, maximum percentage of isolates belonged to age group 11-20 years and 21-30 years in females. The inaccessibility of a qualified physician in the rural set up a woman of illiteracy, early marriages, poverty, ignorance and negligence of personal hygiene, all contribute to the development of UTIs in the sexually active age group in a developing country like ours, starts from a very early age after puberty (Rebecca *et al.*, 2005).

Urovirulence markers of *E.coli* isolates

Haemolysin

In the present study, 33% 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 hemolytic, (Vijyalakshmi *et al.*, 2014) showed 25% hemolytic.

Haemagglutination

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 colonization than P-fimbriae.

Percentage of Haemagglutination			
Previous studies	No. of isolates	% MRHA (P fimbriae)	% MSHA (Type 1 fimbriae)
Mandal <i>et al.</i> , 2001	170	44	47
Rebecca <i>et al.</i> , 2005	163	48	32
Manjula <i>et al.</i> , 2006	160	40	34
N.Fatima <i>et al.</i> , 2012	120	75	18
Vijyalakshmi <i>et al.</i> , 2014	200	60	76

Percentage of Serum bactericidal assay			
Previous studies	No. of isolates	% Serum Resistant	% Serum Sensitive
Prabhat <i>et al.</i> , 2010	220	32.72	67.28
Vijayalakshmi <i>et al.</i> , 2014	200	49	51
Yasmeen Kausar <i>et al.</i> , 2009	200	49.5	51.5
Present study	100	76	24

The present study showed 9% P fimbriae (MRHA), 14% were Type 1 fimbriae (MSHA) and there was no haemagglutination in 73% isolates and showed very less percentage of MRHA and MSHA type of haemagglutination when compared to the previous studies. The reason behind it may be that, the phenotype expression was absent in vivo, masked by some mutation, can also vary depending upon host characters, type of infection and predisposing factors determining the host parasite interaction in vivo which can

culminate in an active infection and this may or may not recur (Johnson *et al.*, 1991; Shrikhande *et al.*, 1999).

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). Also identifying a genotype does not mean that it is always expressed in the body. The in vitro study of the phenotypic characters of uropathogenicity is only a presumption of a possible recurrent UTI.

Table.1 Age and Gender distribution of *E.coli* isolates among symptomatic UTI patients

Age	No. of <i>E.coli</i>		Total
	Male	Female	
≤1-10	1	9	10
11-20	8	11	19
21-30	5	10	15
31-40	1	6	7
41-50	4	9	13
51-60	5	10	15
61-70	6	8	14
71-80	3	3	6
81-90	1	0	1
91-100	0	0	0
Total	34	66	100

Table.2 Antibiotic Susceptibility pattern of *E.coli* from urine samples.

Antibiotics	Sensitive %	Resistant %
Ampicillin	16	84
Amoxyclav	16	84
Ceftriaxone	26	74
Cefuroxime	28	72
Norfloxacin	21	79
Nalidixic acid	6	94
Nitrofurantoin	81	19
Cefepime	27	73
Cefpodoxime	25	75
Ceftazidime	29	71
Gentamicin	62	38
Co-trimoxazole	59	41
Imipenem	100	0
Piperacillin/Tazobactam	99	1

Table.3 Virulence markers of UPEC

S.No.	Virulence markers	Percentage
1.	Haemolysin production <ul style="list-style-type: none"> • Hemolytic • Non-hemolytic 	33% 77%
2.	Haemagglutination <ul style="list-style-type: none"> • MRHA • MSHA • NO HA 	9% 14% 73%
3.	Serum bactericidal assay <ul style="list-style-type: none"> • Serum resistance • Serum sensitive 	76% 24%

Fig.1 β -hemolysin production

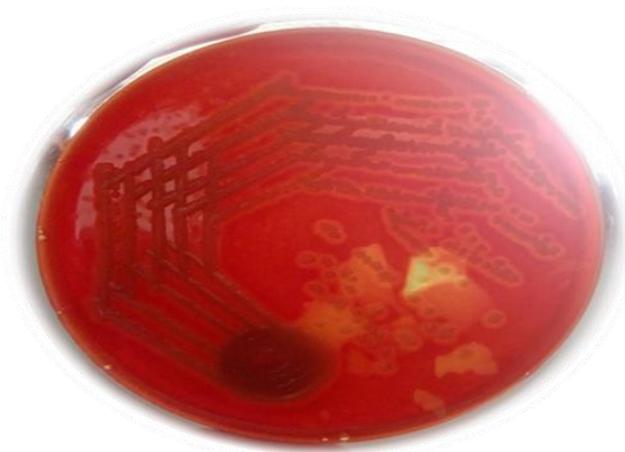


Fig.2a Haemagglutination – Macroscopic appearance

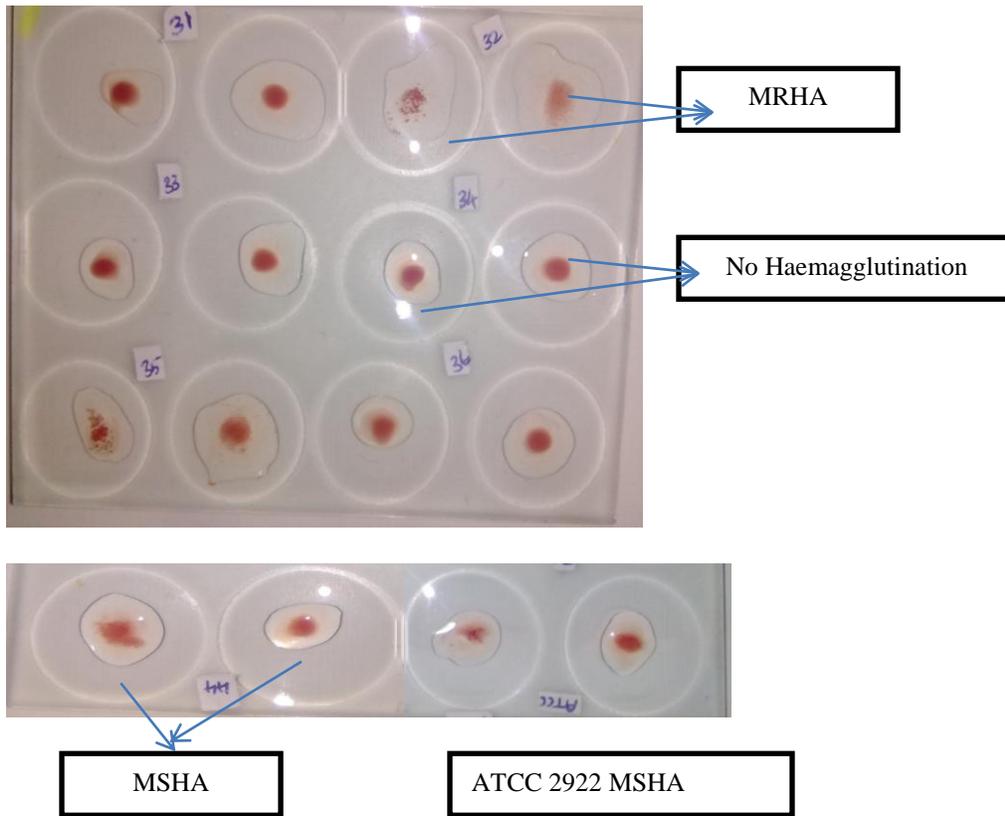


Fig.2b Haemagglutination – Microscopic appearance under low power (10x) objective.

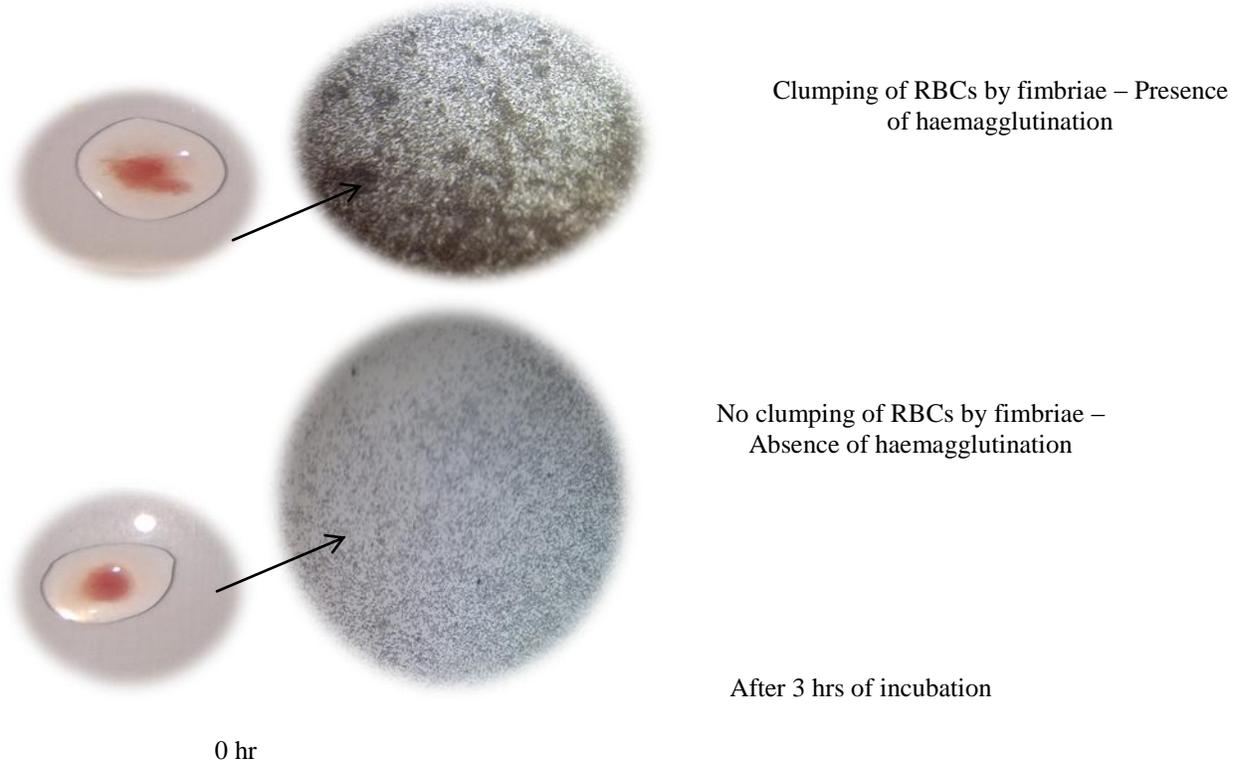
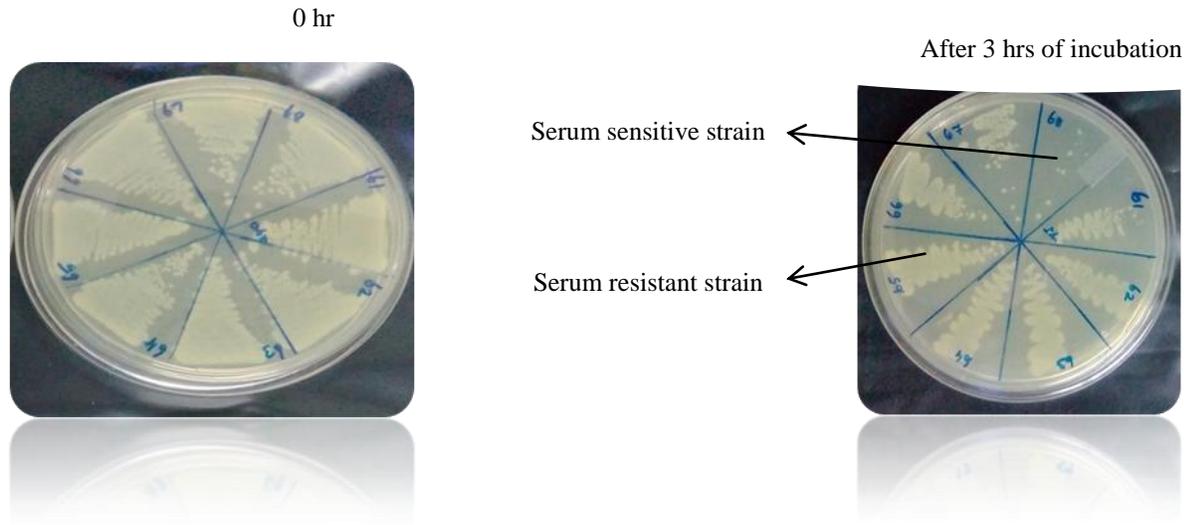


Fig.3 Serum bactericidal activity



Serum Bactericidal activity

The capsule/K antigen on the cell surface may confer serum and phagocyte resistance to some *E.coli* strains (Warren *et al.*, 1999). Comparing to other studies, this study showed that a higher percentage of resistance towards serum. This property was attributed to the content of sialic acid, which reduced the ability of the bacterial surface to activate and complement by an alternative pathway (Altwegg *et al.*, 1998).

In the present study, Imipenem, Piperacillin/tazobactam, Nitrofurantoin, are observed to be the antibiotics of choice. Empirical antimicrobial treatment is initiated in almost all UTIs before the laboratory results of the urine culture are available (Dash *et al.*, 2013). Misuse and self-medication in many countries, including India may be considered a major problem as antibiotics could be purchased without any prescription. Up to 95% of UTI cases are treated without bacteriological investigations (Warren *et al.*, 1999). Many reports suggested that the resistance of *E.coli* strains to commonly used antimicrobial agents have been rising rapidly.

In conclusion, there is a need to define the strategies better to prevent the emergence and more studies in this area are clearly required. Periodic review and formulation of antibiotic policy are needed for control of acquisition of drug resistance. Further studies on better understanding of the interaction of different virulence factors at the molecular level are necessary as most urovirulent strain expresses multiple virulence factors simultaneously.

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