

# Phenotypic Study of Virulence Factors in Escherichia Coli Isolated From Antenatal Cases, Catheterized Patients, and Faecal Flora

SHRUTHI N, RAVIKUMAR, RAVISHKUMAR

## ABSTRACT

**Introduction and Objective:** The bacterial strains that cause symptomatic urinary tract infections possess diverse distinctive properties that enable them to overcome the local host defenses. In Escherichia coli, virulence results from the cumulative impact of several virulence factors, which can vary according to the patient populations. Hence, a study was undertaken to assess the prevalence of the virulence factors in the E.coli isolates which were isolated from pregnant woman, catheterized patients and from the stool samples of healthy individuals.

**Methods:** A total of 93 E.coli isolates were obtained from symptomatic cases of urinary tract infections and 31 E.coli faecal isolates were obtained from apparently healthy individuals and they were tested for haemolysin production, mannose resistant haemagglutination to indicate P fimbriae, cell surface hydrophobic-

ity, and the gelatinase enzyme.

**Results:** Among the 93 E.coli isolates from the cases group, 39(41.9%) were haemolytic, 38(40.9%) were MRHA positive, 29(31.2%) were hydrophobic and 18(19.4%) were positive for gelatinase. Among the 31 controls, 01(3.2%) were haemolytic, 02(6.5%) were MRHA positive and 03(9.7%) were hydrophobic and none of the isolates were positive for gelatinase. The difference between the cases and the control group was significant ( $P<0.001$ ). Multiple virulence factors were observed in 10% of the isolates.

**Interpretation and Conclusions:** The present study showed that the expression of the virulence factors was more in the urinary isolates of the antenatal cases and in the catheterized patients as compared to the faecal isolates.

**Key Words:** *Escherichia coli*, Virulence factors, Antimicrobial susceptibility, Drug resistance

## INTRODUCTION

Urinary Tract Infections (UTIs) are one of the most prevalent bacterial infections [1] E.coli accounts for 50% - 90% of all the uncomplicated urinary tract infections [2]. These E.coli are primarily derived from the faecal flora, which can colonize the periurethral area, overcome the local host defenses and enter and multiply within the urinary tract. These E. coli strains are designed as Uropathogenic E.Coli (UPEC) which possess distinctive traits that confer an enhanced extraintestinal virulence potential [3-5].

UTI is predominantly a disease of the females, because of the anatomy of the female urethra. The incidence of bacteruria increases during pregnancy, due to the anatomical and the hormonal changes. In most of the hospitalized patients, nearly all the UTIs are preceded by the instrumentation of the urinary tract, mainly urinary catheterization and it is a frequent cause of significant morbidity, sepsis and death [4].

Most of the strains of E.coli which cause UTIs belong to a restricted range of serotypes which are different from the distribution in the faecal isolates. However, the serotypes alone cannot explain the uropathogenicity of E. coli and various other factors are necessary for its virulence [6,7].

The Virulence Factors (VFs) serve in distinguishing the potential pathogens from the harmless intestinal strains [8]. The VFs like toxins, adhesions and the surface hydrophobicity and the presence of the gelatinase (protease) enzyme have an influence on

the pathogenicity of the organisms [9,10]. The virulence of the individual strains in a given infection is determined by the presence and the actual expression of the virulence genes which are present in them and also by the environmental conditions in the host [10]. These VFs are responsible initially for the colonization of the organisms and subsequently for the tissue damage. The pathogenic strains adhere to the urinary tract epithelial cells and the survival of the complement action appears to contribute to the urinary tract virulence, which are not as frequently observed among the normal faecal flora. These markers of (UPEC) are expressed with different frequencies in different disease states, which range from asymptomatic bacteriuria to chronic pyelonephritis [1, 11]. Most of the UPEC strains with virulence factors belong to the phylogenetic group B2 and to a lesser extent to group D, while the commensal strains belong mainly to the groups A and B1 [12].

## MATERIALS AND METHODS

This study was conducted in the Kempegowda Institute of Medical Sciences, Bangalore, for a period of 1 year and 6 months from January 2009 to June 2010. A total number of 124 E.coli isolates were obtained from 93 urine samples(cases) and 31 stool samples (controls). Among the cases, 48 urinary isolates were obtained from antenatal cases and 45 urinary isolates were obtained from catheterized patients. An ethical clearance was obtained from the institution.

The inclusion criteria was E.coli which was isolated from the urine of pregnant woman who had significant bacteriuria, from catheterized patients (48-72 hrs after the catheterization) and from the stool samples of healthy individuals.

The patients who were on a current antibiotic therapy and those with a past history of recurrent UTIs and urologic abnormalities were excluded from study.

#### A.) Sample collection:

Mid-stream clean catch urine, catheterized urine and stool samples were collected in sterile containers which were labelled with the patients' details. The specimens were transported to the laboratory in leak proof boxes and they were processed as soon as possible. When the processing was delayed, they were stored at 4°C.

#### B.) Specimen processing:

The urine samples were observed macroscopically for their colour and turbidity. Wet mounts of the samples were prepared and examined for the presence of pus cells and organisms.

Semiquantitative cultures were done by inoculating thoroughly the mixed urine onto a 5% sheep blood agar plate and on a Mac Conkey's agar plate with a calibrated loop. The inoculated plates were incubated at 37°C overnight.

The identification of the isolates was done on the basis of the colony morphology, gram staining and the standard biochemical tests. All the E.coli isolates which were obtained, were screened for the presence of virulence markers.

#### Virulence tests:

A standard uropathogenic E.coli MTCC strain was used as a positive control and an E.coli ATCC strain 25922 was used a negative control.

##### 1. Haemolysin:

The bacteria was inoculated onto 5% sheep blood agar and incubated overnight at 37°C. Haemolysin production was detected by the presence of lysis, a zone of complete lysis of the erythrocytes around the colony and clearing of the medium.

##### 2. Mannose resistant fimbrial haemagglutination (MRHA)

The E.coli which were grown on the Mac Conkey's agar plates were inoculated into 5ml of Muller Hinton broth to give a turbid suspension [ $2.4 \times 10^9$  CFU/ml] which equalled the tube 8 of the standard McFarland's solution. The tubes were incubated at 37°C for 5 days to get fimbriae enriched E.coli. The pellicle which was formed on the surface was noted and it was subcultured onto Colonization Factor Antigen (CFA) agar and incubated overnight at 37°C. The group A positive venous blood was added to equal amounts of the Alsever's solution, this was washed three times and a 3% erythrocyte suspension was made in PBS, pH 7.4

Forty µl of this erythrocyte suspension was added to 40µl of PBS 7.4 and 40µl of 3% D-mannose in a different well of the same VDRL slide. The colonies from the CFA agar plates were mixed in both the wells. The slides were placed on a VDRL rotator for 4 minutes and the haemagglutination reactions were recorded after 2, 5, 10 and 15 minutes.

The haemagglutination was considered to be mannose resistant when it occurred in the presence of D-mannose and it was considered to be mannose sensitive when it was inhibited by D-mannose.

### 3. Cell surface hydrophobicity:

Salt aggregation tests: The E.coli which was grown on the Mac Conkey's agar plates were inoculated into 1 ml of phosphate buffer, pH 6.8 and the turbidity was matched with the Mcfarland's standard 6 to get a colony count of  $5 \times 10^9$  colonies/ml.

Ammonium sulphate solutions of molar concentrations 1M, 1.4M and 2M were prepared. Forty µl of the E.coli suspension was mixed with an equal volume of the ammonium sulphate solution of different molarities on a VDRL slide. The slides were placed on a VDRL rotator for 4 minutes and the clumps which were formed in different concentrations of the ammonium sulphate were observed.

The E.coli strains were considered as hydrophobic if they aggregated in the ammonium sulphate solution of concentration, <1.4M.

### 4. The gelatinase test:

The gelatinase production was tested by using gelatine agar. The plate was inoculated with the organism and it was incubated at 37°C for 24hrs. The plate was then flooded with a mercuric chloride solution. The development of an opacity in the medium and a zone of clearing around the colonies were considered to be positive for gelatinase.

## STATISTICAL METHODS

The data which was collected was analyzed by computing the descriptive statistics, namely the mean, standard deviation and the range. Any significant differences between the mean values of the study groups and the control groups was tested. The Chi-square/ Fisher Exact test was used to find the significance of the study parameters on a categorical scale between two or more groups. The one proportion Z- test was used to find the significance of the proportion of the virulence factors according to the age and the gender of the subjects.

## RESULTS

A total of 93 E.coli isolates were obtained from the symptomatic cases of UTI, 48 from the antenatal cases and 45 from the catheterized patients. 31 E.coli isolates were obtained from the stool samples of the apparently healthy individuals for the control group. A majority (39%) of the patients in the case group were in the age

Age & Gender	Cases		Controls	
	No	%	No	%
<b>Age in years</b>				
1-10	2	2.2	13	41.9
11-20	8	8.6	5	16.1
21-30	36	38.7	3	9.7
31-40	6	6.5	2	6.5
41-50	5	5.4	4	12.9
51-60	9	9.7	2	6.5
61-70	12	12.9	2	6.5
71-80	12	12.9	0	0.0
>80	3	3.2	0	0.0
<b>Gender</b>				
Male	19	20.4	18	58.1
Female	74	79.6	13	41.9
<b>Total</b>	<b>93</b>	<b>100.0</b>	<b>31</b>	<b>100.0</b>

[Table/Fig-1]: Comparison of age and gender between cases and controls

Virulence factors	Antenatal cases (n=48)	Catheterized patients (n=45)	Control (n=31)	P value	
	No	No(%)	No(%)	EMMCC-CS	All
<b>β Hemolysis</b>					
Positive	15(31.3%)	24(53.3)	1(3.2)	0.037*	<0.001**
Negative	33(68.8%)	21(46.7)	30(96.7)		
<b>MRHA</b>					
Positive	18(37.5%)	20(44.4)	2(6.5)	0.532	<0.001**
Negative	30(62.5%)	25(55.6)	29(93.5)		
<b>CSH</b>					
Positive	14(29.2%)	15(33.3)	3(9.7)	0.823	<0.001**
Negative	34(70.8%)	30(66.7)	28(90.3)		
<b>Gelatinase</b>					
Positive	8(16.7%)	10(22.2)	0(0.0)	0.602	<0.001**
Negative	40(83.3%)	35(77.7)	31(100)		

**[Table/Fig-2]:** Comparison of Virulence factors in cases and control groups

group of the 21-30 years, followed by those who were in the age group of 61-80 years, with a female preponderance, as has been seen in [Table/Fig-1].

Haemolysis and MRHA was the most common virulence factors which were present. CSH was second most common virulence factor. Gelatinase was present in only 18(14.5%) isolates. There was a significant difference between the presence of the virulence factors in the cases and the control groups, as has been shown in [Table/Fig-2]. In the antenatal case group, MRHA was the predominant virulence factor which was present and in the catheterized patients group, haemolysin was the predominant virulence factor which was present. In the control group, only a small number of isolates were positive for haemolysis, MRHA and CSH and none were positive for gelatinase.

## DISCUSSION

E.coli accounts for 50- 90% of all the uncomplicated urinary tract infections [2]. The uropathogenic E.coli express several surface structures and they secrete a protein which is peculiar to the strains of E.coli which cause UTIs. Various chromosomally encoded factors have been identified and designated as the candidate virulence markers which are expressed in different frequencies in different disease states. The VFs serve in distinguishing the potential pathogens from the harmless intestinal strains [8]. Apart from the bacterial factors, the host defense must also be considered, since certain individuals contract UTIs more frequently than others and as certain individuals are predisposed to a particular type of condition [13].

### a. Sex distribution:

In the present study, among the 93 E.coli urinary isolates, 74 were from females and 19 from males. This observation was comparable with that of the previous studies which were done by Anja Siitonen [14] as well as Risto et al., [15], where UTIs were more common among females than among males. As per Foxman and Brown, women have a higher incidence of UTI than men, with an annual incidence of 12% as compared to the 3% incidence among men [16]. The higher incidence among the females is attributed to the difference in the anatomy, the moist periurethral area in women and the shorter distance between the anus and the urethral opening and between the urethral opening and the bladder.

### b. Age distribution:

In the present study, UTIs were found to occur more commonly among all the age groups, with a peak incidence in the 20-30 years age group, as has been seen in [Table/Fig-1]. This was in agreement with the findings of the research which was done by Foxman and Brown, where UTIs were found to occur more frequently in people of all age groups, with a peak incidence in women of the age group of 20-24 years. Among men, UTIs were found to occur in the oldest age group of 85 years with a 7.3% incidence [16].

However, in a previous study which was done by Braumer et al. on 104 patients with UTIs, 54 were found to have a median age of 64 years and 50 were found to have a median age of 63 years (ranges of 12-93 years and 4-84 years respectively) [17].

A study which was done by Anja Siitonen showed that the mean age of both the women and the men was 45 years (ranges of 15-81 and 16-70 years respectively) [14].

### c. The virulence markers of E.coli

#### i. Haemolysin:

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 E. coli strains is known as alpha haemolysin. The 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 [8].

In the present study, the difference between the cases and the controls for the production of haemolysin was highly significant ( $p < 0.001$ ). However, no significant difference was noted between the urinary isolates from the antenatal cases and the catheterized patients [31.3% and 53.3% respectively]. This observation was similar to the findings of the study which was conducted by Raksha et al, where haemolysin was found to be produced by 41% of the urinary and 12% of the faecal isolates [12]. The prevalence of the virulence factors in other studies which were reported from India was similar to that in our data [2,10,11,12,18]. It has been suggested that the colonization with the haemolytic strains of E. coli is more likely to cause urinary tract infections. Haemolysis, though it is not essential for the establishment of acute pyelo-

nephritis, may contribute to tissue injuries, to the survival of the organisms in the renal parenchyma and to their entry into the blood stream.

## ii. Haemagglutination:

The haemagglutination and the adherence are mediated by fimbriae [19]. The MRHA can be mediated by P fimbriae and also by X, FIC and Dr fimbriae. These adhere to the fibronectin on the uroepithelial cells, thus contributing to the persistence. In the present study, MRHA was positive in 38(40.9%) isolates in the cases group with the group A+ve erythrocytes and in 2(6.5%) isolates in the control group. Hence, there was a significant difference in the MRHA positivity between the cases and the controls ( $p < 0.001$ ). This was similar to the findings of the study which was carried out by Siegfried et al, where 42% E.coli isolates were MRHA positive with the group A+ve erythrocytes [20]. However, there was no significant difference among the E.coli isolates in the antenatal cases and among those in the catheterized patients.

The expression of the type 1 fimbriae is indicated by MSHA. The MSHA were more in the faecal strains than in the urinary isolates in our study [9].

A good proportion of the E. coli isolates which cause UTIs in pregnancy are P fimbriated. These UTIs, if not treated, can progress to pyelonephritis in about 30-50 per cent of the cases. Therefore, the E. coli which are isolated from asymptomatic bacteriuria in pregnant women should be tested for its virulence factors to identify the risk of developing pyelonephritis [1].

The presence of both P fimbriae and haemolysin which was reported in other studies was 15% [9], and in our study, about 5% of the isolates possessed both the virulence factors. One of the probable reasons for this difference in our study could be that the other blood groups like guinea pig and bovine erythrocytes were not studied and that other adhesins like X, FIC, S and Dr fimbriae could have been involved and were not characterized.

## iii. Cell surface Hydrophobicity.

The surface hydrophobicity is another important virulence factor of E. coli that causes extraintestinal infections. Hydrophobicity was a recently described as a virulence mechanism of E.coli. The bacterial surface structures are of considerable interest, because they have a key role to play in the interaction with the surrounding cell surface. The crystalline surface layers (S layers) which are present on both the gram positive and the gram negative organisms play an important role in this interaction. The high hydrophobicity of the bacterial cell surface promotes the adherence of the bacteria to various surfaces like the mucosal epithelial cells.

In the present study, there was a significant difference for CSH between the cases and the controls ( $p < 0.001$ ). This was consistent with the results of previous studies, where 26.36% [12], 33.4% [10], 46% [20] and 55% [21] of the isolates were hydrophobic respectively.

## Gelatinase

Gelatinase is a less important virulence factor which has been demonstrated in E.coli. In the present study, 18(19.4%) E.coli isolates from the cases group and none from the controls produced the gelatinase enzyme. In the present study, there was a significant difference for the production of the gelatinase enzyme between the cases and the controls ( $p < 0.001$ ). This was high, as

compared to the results of the previous studies, where 7% of the isolates were gelatinase positive [9]. Not many studies on the gelatinase test have been done in India.

## d. Multiple virulence factors

The results of our study showed that 59 out of the 124 urinary isolates had more than one virulence factor. In the present study, 10% of the haemolysin producing isolates were hydrophobic and MRHA positive. This was consistent with the findings of a previous study, where a combination of all the three virulence factors, such as haemolysin, surface hydrophobicity and MRHA positivity, was present in 11.2% of the isolates [10,12,22].

A previous study indicated that 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 [9], and the compromising host conditions decrease the need for multiple virulence factors in the strains which cause serious infections [12].

In this case- control study, we conclude that the E.coli strains are definitely associated with the aetio-pathogenesis of UTIs. The E.coli strains with virulence factors were significantly more in the urinary isolates than in the controls.

## CONCLUSION

E. coli has the capacity to adapt and survive at extra intestinal sites like the urinary tract, by producing various virulent factors. Thus, the observations which we made in this study indicate that the pathogenic E.coli express more MRHA, they are more haemolytic and that they have a higher cell surface hydrophobicity which may help in the initiation of infections. Haemolysin and adherence through P fimbriae are an important properties of the uropathogenic E.coli.

A good proportion of the E. coli which cause UTIs in pregnancy are P fimbriated, as was seen in this study also. This has led to the recommendation that the E. coli which is isolated from asymptomatic bacteriuria in pregnant women, be tested for virulence factors to identify the pregnant women who are at a risk of developing pyelonephritis.

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**AUTHOR(S):**

1. Dr. Shruthi N
2. Dr. Ravi kumar
3. Dr. Ravish kumar

**PARTICULARS OF CONTRIBUTORS:**

1. Consultant, Department of Microbiology, Anand Diagnostics Laboratory, Bangalore, India.
2. Professor & HOD, Department of Microbiology, KIMS, Bangalore, India.
3. Assistant Professor, Department of Microbiology, Basaveshwara Medical College, Chitradurga, India.

**NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:**

Dr. Shruthi N,  
Consultant Microbiologist,  
Phone: 9880525530  
E-mail: drshruthi15@gmail.com

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