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## *Contents*



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*Original Article*

# Plasmid detection in uropathogenic Escherichia coli of urine of suspected urinary tract infection cases and its association with drug resistance

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## Abstract

**Background:** *Escherichia coli* is reported to be the major cause of urinary tract infection (UTI). Treatment of UTI is becoming difficult due to increasing trend of antibiotics resistance and this may necessitate up to date knowledge of resistance pattern and also to know the causes of such resistance. **Objective:** The aim of the study is to explore the possibility of developing MDR and plasmid(extrachromosomal element) in bacterial urine culture *Escherichia coli* isolates to analyze their susceptibility profile to commonly used antibiotics. **Materials & Methods:** This study has been carried out in the Department of Microbiology of Rajshahi Medical College & Department of Genetics and Biotechnology of Rajshahi University in the period from july 2014 to 2015. Midstream urine were processed for microscopy and culture. The pathogens were identified by standard methods. Antimicrobial sensitivity was carried out by Kirby-Bauer disc diffusion method according to clinical and laboratory standards institute guidelines. Plasmid DNA was extracted from the MDR *Escherichia coli* strains following the protocol of Brinboim and Doly (Alkaline lysis method). Agarose gel electrophoresis of the extracted DNA was done in horizontal gel apparatus and photograph was taken. **Results:** A total of 96 uropathogens were isolated from the indoor and outdoor samples and among them *Escherichia coli* was the most prevalent isolate 66(68.75%). Drug resistance pattern of these isolates were investigated and *Escherichia coli* showed variable pattern of susceptibility. Among 66 *Escherichia coli*, 38 were multidrug resistant (>3 groups of drugs). A total 26 plasmid detected among 38 MDR strains through alkaline lysis method. Plasmid band ranged in size from 3-10 kb, forming a unique banding pattern. Middle ranged plasmid 3 kb&4 kb was found to be present in 54% &10 kb was 46% *Escherichia coli* isolates. Almost all MDR *Escherichia coli* isolates contained plasmids and many of them share some common plasmids too. However, their number (plasmid copies) also plays a critical role in imparting various characteristics to the pathogen, such as resistance towards different antibiotics. **Conclusion:** These results showed the drug resistance pattern & the presence of transferrable elements in these MDR *Escherichia coli* isolates from urine samples and also find out the probable link between them.

**Key words**: LVH, ECG, SBP, HTN

## Introduction

Members of the enterobacteriacae especially *Escherichia coli* is the important opportunistic pathogen that has shown antimicrobial resistance to most antibiotics. *Escherichia coli* is the normal flora of the human intestine and therefore easily colonizing the urinary tract, leading cause of urinaty tract infection (UTI). In the United States about 8 million physician visits and more than 1 lac hospital admissions per year are due to UTI[1]. Epidemiological studies showed marked variations in the prevalence of bacteriuria among different population.Four major risk groups of community acquired UTI are school aged girls,sexually active young women and in male with prostatic obstruction.In generally, UTI are more common in female than male in ratio about 2:1 with exceptions of the neonatal period when sexes are

equally affected[2]. Urinary tract infections are mostly caused by *Escherichia coli* accounting for more than 70% of uncomplicated cases both inpatients & outpatient[3].

In recent years, bacterial resistance to different antibiotics has raised dramatically leaving patients with few therapeutic options. Methicillin Resistant Staphylococcus aureus (MRSA), Extended Spectrum Beta Lactamase (ESBL), Vancomycin Resistant Enterococci (VRE) have become common hospital problems. Since resistance pattern differ from region to region, it is useful to avail of information of antibiotic resistance among the common urinary pathogens. However, monitoring of resistance pattern is necessary to improve guidelines for empirical antibiotic therapy. There is a large reservoir of resistant genes, in bacterial genomes and in extrachromosomal pieces of DNA (plasmid) that encode different mechanisms of drug resistance. The drug resistance character is most often encoded in plasmids and transmit to other bacterium via various gene transfer mechanisms[4]. A plasmid is an extrachromosomal, circular, double standard DNA molecule and can replicate independently within the suitable host. Plasmid size vary from 1 to 1000 kb pairs (kbp) under some cirumstances. It may appear as circular, relaxed circular, linear or supercoiled. It may be conjugative and non conjugative by their ability to transfer among bacteria. Conjugative plasmid from gram negative bacteria can transfer among bacteria of some of different genuses through sex pili which are formed by the encoded" tra" genes.Some bacteria produce enzymes such as beta lactamase under the influence of plasmid genes that cause drug resistance[5]. Antibiotic resistance of bacteria can be achieved through intrinsic or acquired mechanisms. Intrinsic mechanism involves naturally occurring genes in the bacterial chromosomes such as AmpC beta lactamases gene for gram ngative bacteria and many MDR efflux gene both gram positive and gram negative bacteria. Acquired mechanism involve mutation in genes targeted for antibiotics and the transfer of resistance determinants present on the plasmids, transposons & other mobile genetic materials of bacteria and bacteriophages. In general, these exchange is achieved through the processes of transduction, conjugation and transformation[6]. Nearly 60% to 90% of drug resistance are mediated by plasmid gene and rest are by mutation of chrosome genes.

Antibiotic selection against UTI is done by susceptibility test. But sometimes bacteria develop resistance to multiple antibiotics and creat a major problem.In Pakistan, E. coli was 65.5% resistant to 3 or more antibiotics[7]. Whereas in Bangladesh E. coli 87% resistant to more than 3 drug and 80% of them herboured plasmids[8]. A study in Nigeria revealed that 38.5% of MDR uropathogens carried plasmids of various size mostly at above 2.1 kb[9]. In Jordan, E.coli were 59.9% resistant to multiple drugs[10]. So,the present study has designed to isolate uropathogens and its susceptibility to find out the resistant pathogens and also detect plasmid which is responsible for drug resistance and find out the correlation of plasmid profile and MDR. This study will not help the individual patients but also will provide early warning of the emergency of resistant isolates to clinicians for better management and will prevent spread MDR uropathogens in community & in the hospitals.

It will also encourage other researchers to do molecular study of plasmid genes responsible for MDR.

**Objective of the study**

**1. To detected plasmid DNA of uropathogenic** *Escherichia coli* and its association with antibiotic resistance.

## Materials & Methods

**Place of study:** This study had been conducted in two institutions. The urine samples were collected from inpatient and outpatient of Medicine, Gynae and Obstetrics, Nephrology and Urology Departments of Rajshahi Medical College and Hospital. The laboratory work was done at Microbiology Department of Rajshahi Medical College and Plasmid detection was done at Genetics and Biotechnology Department of Rajshahi University. Permission for the study was taken from the concerned authorities of the respective departments and institutions.

**Period of study:** July 2014 to June 2015.

**Study design:** Descriptive type of cross sectional study.

**Stydy population:** The sample size was calculated according to the following formula - and the sample size was 254 cases.

**Specimen collection:** After taking proper informed written consent from the patient in a predesigned consent sheet. The early morning mid stream urine was collected in a sterile, wide mouth, leak proof, detergent free container labelled with patients identification number and date.

**Isolation of organisms:**A total of 254 urine samples were collected from the same number of clinically suspected patients of UTI. Semiquantitative urine culture using a calibrated loop was performed.Samples were transferred to the laboratory immediately and inoculated on Blood agar, MacConkey's agar and Highchrome agar media and incubated aerobically at $37^0$C for 24 hours for bacterial growth.

**Cultural & biochemical findings of E.coli:** After 24 hours incubation morphological characteristics (size, shape, colour, surface, texture, elevation, opacity) were carefully observed and recorded.

***Escherichia coli***

If smooth rosy pink coloured colonies were found on MacConkey's agar media and large rounded, grey coloured colonies, with or without haemolysis on Blood agar media and pink colonies on Hichrome agar media ----then it was *Escherichia coli.*

**Motility test** : Motile

**Biochemical tests**

- Oxidase test : Negative
- Indole test: Negative
- Methyl red reaction: Positive
- Citrate utilization test: Negative(both butt and slant are green)
- Urease test: Negative
- Triple sugar iron agar: Butt-yellow(acid),Slant-yellow(acid),Gas-produced,H2S-not produced.
- Sugar fermentation test : Positive with production of gas, Lactose-positive, Manitol-positive, Maltose- positive,Salicin-positive.

**Antimicrobial susceptibility test:** Antimicrobial susceptibility test of the identified isolates were performed by Kirby-Bauer disc diffusion technique using Muller-Hinton agar media and commercially available antimicrobial discs. Antibiotic discs used for gram negative bacilli were cephradine, ceftazidime, amoxyclav, cotrimoxazol, azithromycin, ciprofloxacin, imipenem, amikacin and nirofurantoin. The inoculating plates were examined and the diameter of zone of inhibition was measured and compaired with the zone of inhibition of the supplied inhibitory zone chart of respective antibiotic. The zone of inhibition was equal to or larger than or less than that of zone chart was treated as sensitive and resistant. Zone of inhibition was measured in millimeter by using a ruler over the surface of the plate with the lid open.

**Preservation of isolated bacteria:** The isolates which showed resistance more than three antibiotics were treated as multi drug resistant bacteria and were preserved for plasmid analysis and future use. The MDR strains were subcultured on nutrient agar slope in a six inches screw capped test tubes and incubated at $37^0$C for 24 hours.When visible growth appeared then sterile liquid paraffin were poured to prevent contamination and dehydration and kept in a refrigerator at $-20^0$C.

**Procedure of plasmid isolation:** Multidrug resistant strains were studied for plasmid profiles which was carried out by using the alkaline lysis method[11]. The MDR isolates cultured in 5 ml of Luria broth in a test tube and incubated at 37°C in shaking condition 140 rpm for 24 hours. Then 1.5 ml shaked culture broth was taken in an Eppendorf tube and centrifuged at 13,500 rpm for 7 minutes to collect the bacterial growth and supernatant was discarded. The pellets were suspended in 100 µl of solution I and was kept at room temperature for 5 minutes. Two hundred microlitre of solution II was added and mixed gently by inverting the tubes for 6 times. Then 150 µl of icecold solution III was added to neutralized the solution and mixed vigorously by vortexing for a few seconds. The tube was kept on ice for 5 minutes and centrifuged at 13,500 rpm for 10 minutes at 4°C.

Approximately 400 µl of clear supernatant was taken to a fresh Eppendorf tube and deposit contain chromosomal DNA was discarded. Then appx. 800 µl cold 100% ethanol was added and vortexed for a few seconds and centrifuged at 13,500 rpm for 10 minutes. The supernatant was discarded. Then 100 µl of 70% cold ethyl-alcohol (–20$^0$C) was added to the pellet and inverted 3 times and centrifuged at 13,500 rmp for 10 min at 25$^0$C. The supernatant was discarded and the pellet were dried in a drier at 45°C for 45 minutes. Finally the dried DNA was dissolved in 50 µl Tris EDTA buffer and run gel electrophoresis.

**Procedure of Agarose gel electrophoresis of plasmid DNA**

Agarose gel electrophoresis was carried out in a horizontal gel apparatus. One gram agarose was dissolved in 100ml TAE buffer by boiling in a micro-oven for five minutes. Then it was allowed to cool to 55-60$^0$C and 10 µl ethedium bromide was added in it, mixing well. To make multiple wells a comb containing various number of teeth was placed in the plastic chamber and agarose gel was poured and allowed to solidified for half an hour at room temperature. Then extracted 8 µl of plasmid DNA and 2 µl of loading buffer mixed carefully with the help of a micropipette and loaded accordingly to the mentioned well. A known standard plasmid DNA (1 kb plus DNA ladder) was loaded to as control. Similarly other wells were loaded with different test plasmid DNAs. Wells contain agarose gel was placed in the electrophoresis tank and 1x TAE buffer solution was poured so that the gel was under buffer solution and run electrophoresis at room temperature at 100 Volts (500 mA) for 1 hour until the loading dye moved from anode end to cathode end.

**Documentation of the plasmid DNA amplified product:** Plasmid DNAs were moved according to molecular weight and different bands were produced which were visualized by UV transillumination and photograph was taken.

## Results

A total of 254 urine samples were collected from different age groups of people and were cultured on appropriate bacteriological culture media. Isolated *Escherichia coli* resistant to more than three groups of antibiotic were obtained by antibiotic sensitivity test and plasmid were detected to see the correlation between plasmid and MDR carriage of the isolates.

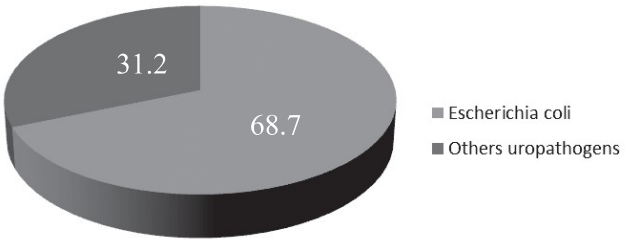
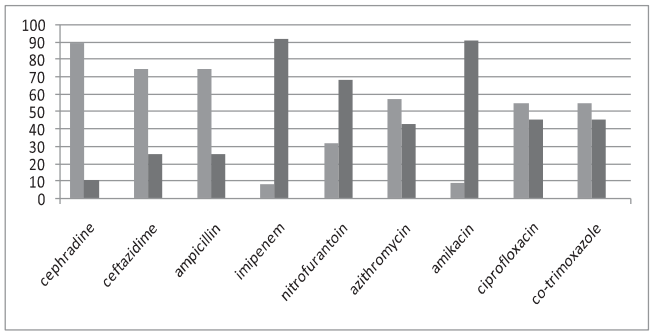



**Figure 1: Culture yielded growth of uropathogens from urine culture (N=254)**

Out of 254 urine samples 96 (37.79%) showed variable growth. Among 96 uropathogens or culture positive isolates, Escherichia coli was predominating pathogens 66 (68.75%). And rest were others gram negative bacilli and gram positive cocci. (Figure-1)




Note: Blue- Resistant, Red- Sensitive

**Figure 2: Antimicrobial susceptibility pattern of identified Escherichia coli.**

Commonly used drugs are namely ampicillin, cephradine, ceftazidime, co-trimoxazole, ciprofloxacin, azithromycin, nitrofurantoin, imipenem, and amikacin. Among 66 Escherichia coli, 59 (89.39%) are resistant to cephradine, 49(74.24%) to both ampicillin and ceftazidime , 36 (54.54%) to both ciprofloxacin & co-trimoxazole, 38(57%) to azithromycin, 21(32%) to nitrofurantoin and remaining are insignificant. Lower resistance was noted to imepenem (8%), amikacin (9.09%) and nitrofurantoin (32%) .

**Table-I: Resistance pattern of multidrug resistant Escherichia coli.**

| Groups of antibiotics | Number of resistant *Escherichia coli* |
|---|---|
| 4 groups of antibiotics | 00(00) |
| 5 gro ups of antibiotics | 13(34.21) |
| 6 groups of antibiotics | 20(52.63) |
| 7 groups of antibiotics | 5(13.15) |
| 8 groups of antibiotics | 00(00) |
| Number of total MDR *E.coli* isolates N(%) | 38(57.57) |

Note:
N=number
Figures within parenthesis indicate percentages.
4 = In combination of 4 groups of antibiotics (A, B, D & E)
5 = In combination of 5 groups of antibiotics (A, B, E, G & H)
6 = In combination of 6 groups of antibiotics (A, B, C, E, G & H)
7 = In combination of 7 groups of antibiotics (A, B, C, D, E, G & H)
8 = In combination of 8 groups of antibiotics (A, B, C, D, E, F, G & H)

A-Cephalosporin group
B-Penicillin group
C-Carbapenem group
D-Furanes group
E-Macrolid group
F-Aminoglycoside group
G-Quinolone group
H-Anti folate antagonist

Bacteria considered to be MDR when it shows resistance to more than three groups of drugs. Out of 66 Escherichia coli, multidrug resistant E.coli were 38 (57.57%). Among 38 (57.57%) MDR E.coli isolates, 13(34.21%) were resistant to 5 antibiotics, 20 (52.60%) were to 6 antibiotics and 5 (13.15%) were to 7 antibiotics.

**Table-II: Correlation of multidrug resistant Escherichia coli strains with detected plasmid.**

| Identified Isolates | Multidru g resistant strains | | | Detected plasmid | |
|---|---|---|---|---|---|
| | 5 | 6 | 7 | | |
| *Escherichia coli* N=38 | 13(34.21) | 20(52.63) | 5(13.15) | 8<br>14<br>4 | 26(68.42%) |

*Note:* N=number
Figures within parenthesis indicate percentages

Table–II shows, Out of 38 MDR *Escherichia coli* isolates, plasmid detected on 26 (68.42) isolates. Among 26 plasmids containing *E.coli*, 8 were resistant to 5 antibiotics, 14 were to 6 antibiotics and 4 were to 7 antibiotics.This multiple drug resistance pattern strogly suggest that the resistance was mediated by R-factors.

## Discussion

Multiple drug resistance of gram negative bacteria have been a long and well recognized problem especially in urinary tract infection. This study had been carried out to investigate the drug resistance pattern and the relationship between antibiotic resistance and plasmid carriage of the MDR isolates in UTI patients. In developing countries antibiotics are available freely without prescription and this widespread inappropriate antibiotic uses exert a selective pressure that acts as a driving force in the development of antibiotic resistance. When bacteria develops resistant to "first-line" antibiotics, therapy with second-line or third-line antibiotics will increased the chance to develop of resistance to the new class of antibiotics[12].

Among 96 significant bacteria, *E.coli* was 66(66.75%). This finding is almost similar to the finding of Rahman *et al.*(2008)[13] in Bangladesh, Mirzarazi *et al.(* 2013)[14] in Iran and their isolation were 66.92%and 68% respectively. Antibiotic resistance pattern of uropathogens isolated from confirmed UTI cases were also studied. In our study, *E.coli* were highly resistant to commonly used antibiotics like- ampicillin (74.24%), ceftazidime (74.24%), cephradine (89.39%). This study is agreement with Rahman *et al.*(2008)[13] in Bangladesh.They showed 86.09% and 73.04% *E.coli* were resistant to ampicillin and ceftazidime. Similar finding was reported by Sharma *et al.*(2013)[15] in Nepal, Sood *et al.*(2012)[16] in India and Alzohairy *et al.*(2011)[17] in Saudi Arabia where *E.coli* showed 81.7%, 81.2% and 75.4% resistance to ampicillin respectively. In our study, *E.coli* was 54.54% resistance to both ciprofloxacin and cotrimoxazole which is matched with Dash *et*

*al.*(2013)[18] and Shaifali *et a.l* (2012)[19] in India showed 53.4% and 60.9% resistant to ciprofloxacin. Regarding cotrimoxazole our findings is similar to the studies conducted by Sharma *et al.*(2013)[15] in Nepal, Moniri *et al.* (2003)[20] in Iran and Dash *et al.*(2013)[18] in India where *E.coli* showed 54.01%, 52.8% and 52.9% resistance to cotrimoxazole respectively. Resistance to cotrimoxazole is generally associated with resistance to additional antibiotics often to penicillin, cephalosporins, quinolones and multidrugs resistance may be transferred on a single plasmid. Lower resistance was noted to imepenem (8%), amikacin (9.09%) and nitrofurantoin (32%) against *E.coli* which is agreement with other studies in Bangladesh conducted by Rahman *et al.*(2009)[13], Shahnaz (2008)[21] and Noor *et al.*(2013)[22]. Amikacin being injectable and nitrofurantoin can not be recommended for serious upper UTI or for those patients with systemic involvement. So, its restricted and limited use in the clinical practice is the reason for high sensitivity. Imipenem has only recently been introduced in Bangladesh and is very expensive which has further restricted its widespread use. In our study, 38 (57.57%) *E.coli* was resistant to multiple antibiotics which is nearly similar with the studies of Azra *et al.* (2007)[23] in New Delhi, Jan *et al.* (2009)[24] in Nagpur, India, and Al mardeni *et al.* (2009)[10] in Jordan where 52.9%, 50%& 59.9% respectively. Prolonged misuse of common antibiotics against in this locality may be the reason for high MDR *E.coli.* Our study showed, *E.coli* isolates 13(34.2%) were resistant to 5 antibiotics, which is similar to Mirzarazi *et al.*(2013)[14] in Iran (42.42%) ; 20 (52.63%) were resistant to 6 antibiotics similar to Khadgi *et al.*(2013)[25] in Nepal (50%) and Mirzarazi *et al.* (2013)[10] Iran (60.60%) ; 5(13.15%) were resistant to 7 antibiotics which nearly similar to Khadgi *et al.* (2013)[25] in Nepal(8%).

In many under developed or developing countries including Bangladesh treatment of UTI often occured without any professional consultation or without any bacteriological investigation. The improper and indiscriminate use and easy availability of antibiotics are major risk factors for the development of antimicrobial resistance.

Among 38 MDR isolates, plasmid detected in 26 (68.42%) *E.coli* isolates which is nearly similar to the finding of Jan *et al.* (2009)[24] in Nagpur, India, Laz *et al.* (2001)[26] and Quashem (1987)[27] in Bangladesh which were 52.65%, 71.4% and 71.42% respectively. But our study is dissimilar to the findings of Lina *et al.* (2007)[8] in Bangladesh, and Khadgi *et al.* (2013)[25] in Nepal which were 86% and 100%. Among 26 plasmid bearing *E.coli* isolates, 8 were resistant to 5 antibiotics, 14 were to 6 antibiotics and 4 were to 7 antibiotics. This multiple drug resistance patterns strongly suggest that the resistance was mediated by R-factors.

During the last two decades bacterial resistance mediated by plasmids. However all the isolates containing plasmid were resistant to 5-8 antibiotics indicating the importance of plasmid in multidrug resistance isolates. Furthermore, these extra-chromosomal elements have greater mobility when compared with chromosomal genes[28] . Multiple plasmid can exist within a single bacterium.In our study,On the basis of gel electrophresis, the plasmid copies were found to vary between 1 to 3. The maximum three plasmid bands were detected from a total of 12 isolates, Two bands form 09 isolates and single band from 05 isolates and plasmid bands ranged in size from 3-10 kb. Same molecular size of the plasmid band also indicate that multiple samples contained same plasmid.

In our study, no plasmid detected in 12(31.57%) isolates which is similar to Quashem (1987)[27] in Bangladesh which was 30.59%. Plasmid bands were not demonstrated possibly because plasmid were lost due to MDR samples were stored in–20$^0$C for about 6 months before isolation of plasmid DNA. Loss of plasmid due to storage has also been reported by many workers[29]. The failure of plasmid detection were also might be the cause of that the resistance determinants were either carried by chromosomes or by small molecular weight plasmids. Therefore it can be concluded that although plasmid bearer play an important role in antibiotic resistance, the multidrug resistance is not always associated with increased plasmid carriage or even there may be absence of plasmid DNA occasionally.

## Conclusion

From this study we conclude that *Escherichia coli* are still now prominent uropathogen in this locality. Regarding antibiotic resistance pattern *E.coli* is resistant to commonly used antibiotics such as ceftazidime, ampicillin & cephradine which hearlds the coming therapeutic problem in the treatment of infections caused by this MDR microorganisms. The plasmids in the *Escherichia coli* studied were randomly distributed in different strains. The genes for resistance to various antibiotics were probably distributed among plasmids of various sizes as well as the chromosome. Large number of isolate lost their plasmids may be due to the lack of selection pressure and host factor.

### Ethical issues

Informed written consent was taken from all study populations. The study was not involved any additional investigative procedures and significant risk as well as economic burden to the patient.

### Conflict of Interest Statement

No potential conflicts of interest were disclosed.

### Limitation

No relation could be detected between the antibiotic resistance pattern and the plasmid profile analysis in the present study due to technical and financial limitations.But further study on the isolates by resistance transfer testing and plasmid curing to assign resistance genes to plasmid DNA may confirm the plasmid origin of MDR pattern.

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