

Original Article

**Resistance profiles of Azithromycin in *Escherichia coli* isolated from human urine**

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**ABSTRACT:** Bacterial drug resistance is now a main hindrance to use antibiotics in treating infectious diseases and global public health concern contributing to increased mortality in low-income countries. The plasmid mediated drug resistance which confers resistant to a number of antibiotics. Here, we studied the azithromycin susceptibility by paper disc plate technique and agar diffusion assay in bacterial strains collected from urine at the department of Microbiology Rajshahi Medical College Hospital, Rajshahi. We found that azithromycin at certain concentration is resistant to *Escherichia coli* (*E. coli*) but not *Klebsiella*, *Proteus*, *Streptococcus* and *Pseudomonas* strains. Furthermore, *E. coli* was also resistant to number of commonly used antibiotics like Ampicillin, Amoxicillin, Tetracycline, Doxycycline, Ciprofloxacin, Cephadrine, PenicillinG and Chloramphenicol. In present study plasmid DNA was isolated from azithromycin resistant *E. coli*. Together, our primary results suggest that *E. coli* is multidrug resistant due to possibly the presence of approximately 4kb in size of resistant plasmid in Rajshahi area.

**Keywords:** Resistance profile, Azithromycin, *E.coli*, Urine sample, Resistant plasmid, Rajshahi area.

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**INTRODUCTION**

Bacterial drug resistance is now being considered as an important growing global problem and public health concern contributing to increased morbidity and mortality particularly in low-income countries.<sup>1,2</sup> Moreover, antibiotic resistant pathogenic strains has alarmingly increased over time. Two types of genetic mechanisms of bacterial drug resistance have been

reported: one is chromosomal resistance and second is extra-chromosomal DNA resistance that plasmid mediated.<sup>3,4,5</sup> Most of the antibiotic resistance is mediated by the resistant (R) plasmid or the 'R' factors, which confers resistant to a number of antibiotics.

Each form of resistance is due to a gene product which acts as an enzyme that destroys a specific antibiotic. Most plasmid DNA produces enzymes that modify the antibiotic by the addition of chemical group causing acetylating, phosphorylation or nucleotidylation of the drug.<sup>6</sup> Plasmid study is very important in medicine because of its carrying gene may resistant to naturally occurring antibiotics in a competitive environmental niche, or its gene product protein may act as toxins under similar circumstances.<sup>7,8,9,10</sup>

Remarkly, many bacteria have shown poor therapeutic problems with antibiotics resistant and geographic location at different times.<sup>11</sup> *Escherichia coli* (*E. coli*) is one of the serious bacterial pathogens that can cause tremendous therapeutic problems by developing acquired resistant against antibiotics in the community.<sup>12,13,14,15</sup> Accordingly, the incidence of urinary tract infections (UTIs) caused by *E. coli*, *Klebsiella*, *Proteus*, *Streptococcus* and *Pseudomonas* are one of the most common diseases in the world particularly in the developing countries.<sup>16,17,18,19</sup> This is also known as a major public health problem and cause of high death rate especially among young children and adult.<sup>16</sup> Remarkly, UTIs is much more prevalent in Bangladesh and United States of America, where it is thought that the end stage of UTI is kidney disease which is the serious health problem.<sup>16,20,21</sup> Notably, *E. coli* is also responsible in sexually transmitted diseases and diarrhoeal disease which are the major public health problem in Bangladesh.<sup>22,23</sup> In summary, reports suggest that the development of drug resistant in *E. coli* compare to other bacteria has become a serious problem in treating its infections worldwide.<sup>24,25</sup>

Azithromycin is a broad spectrum antibiotic active against many Gram-positive and Gram-negative bacteria.<sup>26</sup> This drug has been reported as alternative medicine in outbreaks caused by multiple resistant *E. coli* strains. But in many cases, *E. coli* is resistant to azithromycin. Thus, it is very important to study the *E. coli* resistant plasmid to azithromycin that will help to design and identify alternative or more effective medicine. The main aim of this work was to study the state of UTI by observing *E. coli* resistant pattern of different antibiotics and to isolate the drug resistant plasmid. This may help us to take precaution to solve the problem of resistant against this deadly microorganism *E. coli*.

## MATERIALS AND METHODS

### Source of bacterial strains

*E. coli*, *Klebsiella*, *Proteus*, *Streptococcus* and *Pseudomonas* bacterial strains were isolated from urine samples (n=160) at the pathology laboratory of the Department of Microbiology, Rajshahi Medical College and Hospital, Rajshahi. Urine samples were collected from male and female patients (infected 80

with *E. coli*, 20 with *E. coli* and *Klebsiella*, 20 with *Proteus*, 20 with *Streptococcus* and 20 with *Pseudomonas*) coming with symptoms of UTI admitted at the Hospital during the period 2008-2017. Bacterial strains were identified at the pathology laboratory of the Department of Microbiology, Rajshahi Medical College and Hospital, Rajshahi on the basis of cultural characteristics, morphological study and biochemical tests.

### Bacteriological media and azithromycin plate preparation

Luria agar (LA) and MacConkey agar media were prepared by following standard protocol recommended by the manufacture company. Media were sterilized by autoclaving at 15 lbs/sq. inch and 121° C for 15 minutes (min) and stored at 4°C. Azithromycin discs and plates were prepared at four different concentrations 10µg/ml, 20µg/ml, 30µg/ml and 40µg/ml from azithromycin stock solution (5mg/ml in methanol) that was prepared from azithromycin authentic material (Incepta Pharmaceuticals Ltd. Bangladesh).

### Isolation of drug resistant bacteria

Azithromycin resistant strain was isolated from the collected strains using the disc diffusion method.<sup>27</sup> Azithromycin discs were placed on the plate and kept the plate at 4°C for 4 hour (hr) so that antibiotic can diffuse on the agar media. Plates were then incubated at 37°C for 12-16 hr and the growth of the bacteria was observed. Azithromycin sensitivity was determined by measuring the diameter of clear zone produced around the respective antibiotic discs. Slide caliper was used to measure the zone of inhibition (ZOI). The presence of a clear zone around the disc and presence of some colonies within the clear zone indicate that the collected strains are resistant. The azithromycin resistant bacteria isolated by disc diffusion method were again confirmed by spread plate method using azithromycin conc. of 10µg/ml, 20µg/ml, 30µg/ml and 40µg/ml in agar media. Furthermore, isolated azithromycin resistant strain was also tested against the following antibiotics (Mast Lab. Ltd. UK): Ampicillin (AP, 10µg), Amoxicillin (A, 10µg), Cloxacillin (CX, 5µg), Tetracycline (T, 30µg), Doxycycline (DO, 30µg), Ciprofloxacin (CIP, 15µg), Nalidixic acid (NA, 30µg), Cephadrine (CE, 30µg), PenicillinG (PG, 10µg), and Chloramphenicol (C, 30µg).

### Isolation and purification of azithromycin resistant *E. coli* plasmid DNA

This study used mini-scale isolation of plasmid DNA by boiling method to isolate *E. coli* plasmid DNA.<sup>28</sup> About 1.4 ml of bacterial culture was taken in an eppendorf tube and centrifuged at 14000 rpm for 1 minute at 4°C, and repeated this step 3 times. The supernatant was discarded carefully and the DNA precipitate was washed with 70% ethanol. Finally the

DNA pellet in each tube was dissolved in 20  $\mu$ l TE buffer (pH 8.0) and mixed well by vortex. Purification of plasmid DNA was carried out by precipitation with polyethylene glycol.<sup>29</sup> For the quantitative estimation of DNA content, the absorption of DNA was taken at wavelength of 260 nm and 280 nm by UV-Spectrophotometer (Shimadzu, Model UV-1200, Japan). The plasmid DNA was stored at -20°C for later use.

#### Detection of *E. coli* plasmid DNA by Agarose gel electrophoresis

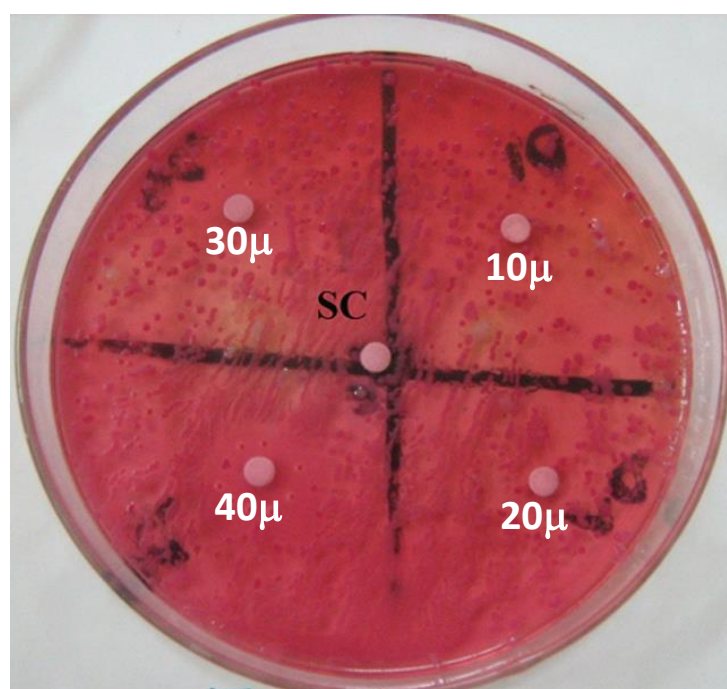
0.8% agarose gel electrophoresis was carried out on a horizontal slab gel apparatus (Mupid electrophoresis apparatus, Japan). The A/C power source (Model, DESATRONIC 500/500, Germany) was supplied with regulated current. Electrophoresis was carried out at 70 volts for 3 to 4 hour. The gel was then stained in ethidium bromide solution and photographed by a

Digital Camera (Sony, Cyber-Shot DSCW-W30, 6 Mega Pixel, Japan). The plasmid profile of *E. coli* was compared with pBR322 marker DNA.

## RESULTS

### *E. coli* is resistant to azithromycin

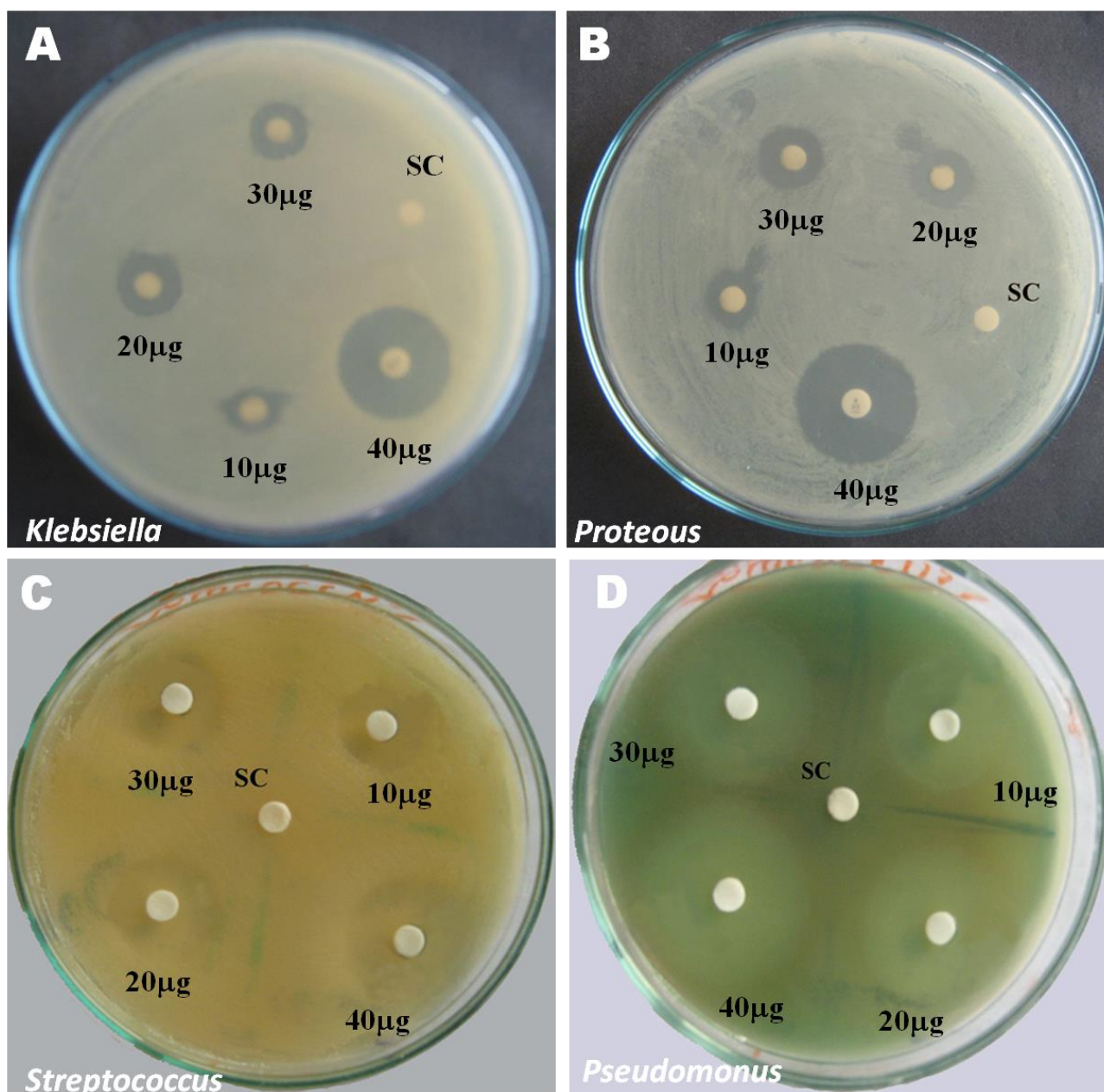
Collected bacterial strains were subjected to azithromycin susceptibility test by disc diffusion and spread plate methods. Four different concentrations of azithromycin 10  $\mu$ g/disc, 20 $\mu$ g/disc, 30 $\mu$ g/disc and 40 $\mu$ g/disc were used on MacConkey agar plate in our study.<sup>30</sup> The MacConkey agar is commonly used for bacterial pure culture. The presence of colonies around disc indicates that the strain is resistant to that concentration. Our results showed that *E. coli* is resistant at all concentrations of azithromycin compared to standard control (SC) (Fig.1).



**Figure1.** *E. coli* is resistant to azithromycin. Four different concentrations 10 $\mu$ g, 20 $\mu$ g, 30 $\mu$ g and 40  $\mu$ g of azithromycin are used to test susceptibility against *E. coli*. The level of resistant of *E. coli* is gradually decreased with 10 $\mu$ g, 20 $\mu$ g, 30 $\mu$ g and 40 $\mu$ g of azithromycin compared to standard control (SC).

However, *Klebsiella* and *Proteus* are intermediate sensitive at 10 $\mu$ g, 20 $\mu$ g and 30 $\mu$ g, and sensitive at 40 $\mu$ g of azithromycin compared to SC. *Streptococcus* is intermediate sensitive at 10 $\mu$ g and sensitive at 20 $\mu$ g,

30 $\mu$ g and 40 $\mu$ g of azithromycin compared to SC. *Pseudomonas* is sensitive at all concentrations of azithromycin compared to SC (Fig.2).



**Figure2.** *Klebsiella*, *Proteus*, *Streptococcus* and *Pseudomonus* are sensitive to azithromycin. Four different concentrations 10μg, 20μg, 30μg and 40μg of azithromycin are used to test the susceptibility. *Klebsiella* and *Proteus* are intermediate sensitive (Zone of inhibition (ZOI) < 18mm) at 10μg, 20μg and 30μg, and sensitive (ZOI > 18mm) at 40μg of azithromycin compared to SC. *Streptococcus* is intermediate sensitive at 10μg and sensitive at 20μg, 30μg and 40μg of azithromycin compared to SC. *Pseudomonus* is sensitive at all concentrations of azithromycin compared to SC.

Moreover, the azithromycin resistant bacteria isolated by disc diffusion method were again confirmed by spread plate method using 10μg, 20μg, 30μg and 40μg of azithromycin solution in media (Table-I).

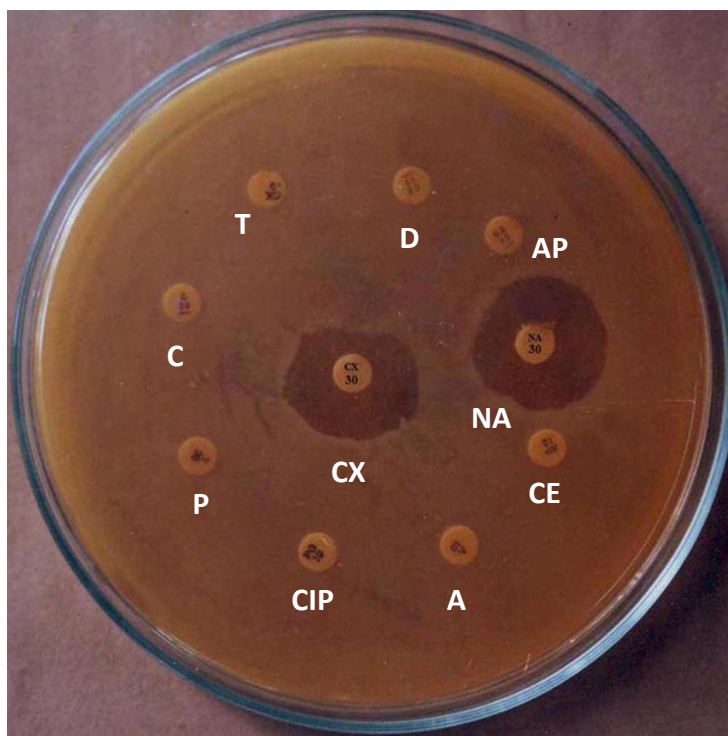
**Table 1.** *E.coli* samples are resistant to azithromycin. Azithromycin susceptibility test in *E.coli* by spread plate method for four different concentrations 10µg, 20µg, 30µg and 40µg. Four *E.coli* samples tested by this method in this study are resistant to azithromycin at all concentrations of azithromycin.

<i>E.coli</i> Sample No.	Concentration of azithromycin (µg/ml)				Comments
	10	20	30	40	
	No. of bacterial colonies appeared on plate				
1	61	55	45	36	Resistant
2	55	51	48	45	Resistant
3	42	40	35	29	Resistant
4	40	38	35	30	Resistant

***E. coli* is also resistant to number of commonly used antibiotics**

Azithromycin resistant *E. coli* was also subjected to susceptibility test for Ampicillin, Amoxycillin, Cloxacillin, Tetracycline, Doxycycline, Ciprofloxacin, Nalidixic acid, Cephadrine, PenicillinG and

Chloramphenicol by the disc diffusion method and the spread plate method. We observed that *E. coli* is resistant to Ampicillin, Amoxycillin, Tetracycline, Doxycycline, Ciprofloxacin, Cephadrine, PenicillinG and Chloramphenicol. However, *E.coli* is only sensitive to Nalidixic acid and Cloxacillin (Fig.3).

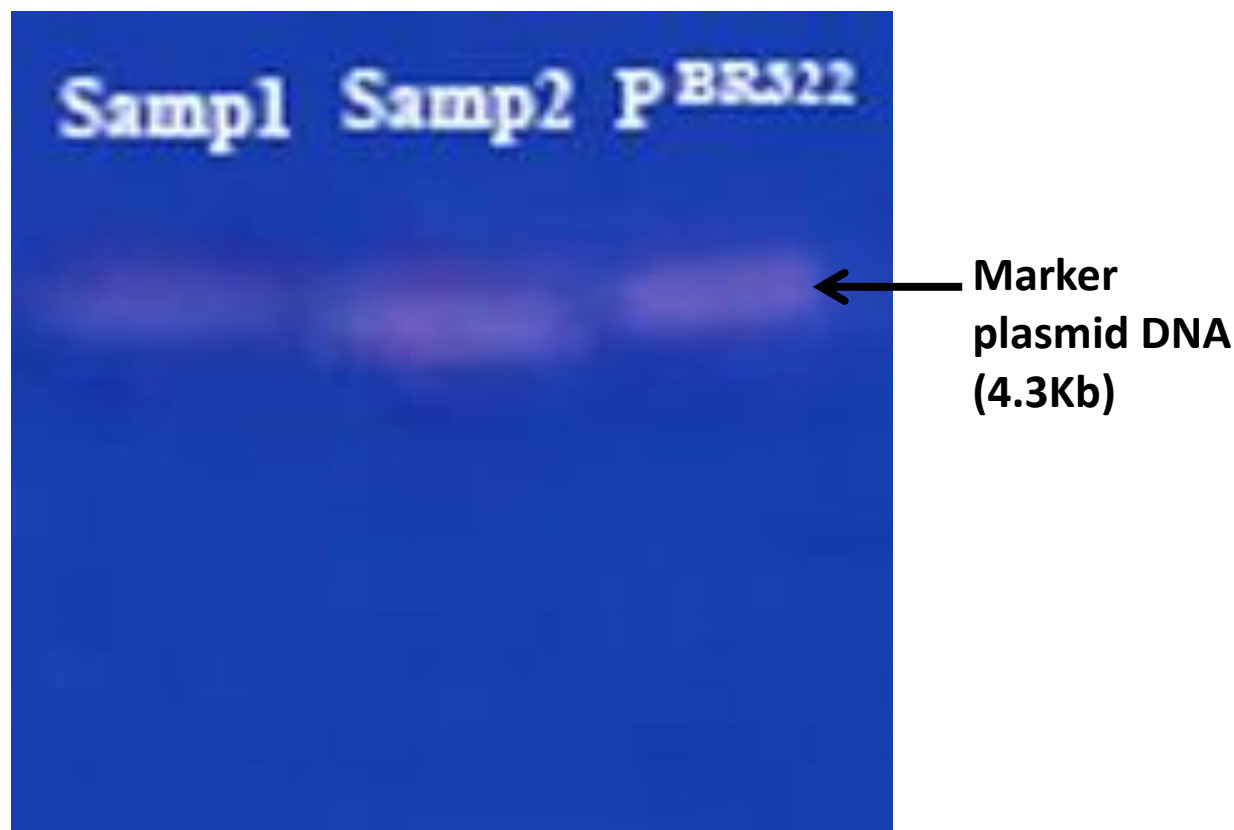


**Figure 3.** Multiple antibiotics susceptibility test of azithromycin resistant *E.coli*. Ten different antibiotics Ampicillin (AP, 10µg), Amoxycillin (A, 10µg), Cloxacillin (CX, 5µg), Tetracycline (T, 30µg), Doxycycline (DO, 30µg), Ciprofloxacin (CIP, 15µg), Nalidixic acid (NA, 30µg), Cephadrine (CE, 30µg), PenicillinG (PG, 10µg), and Chloramphenicol (C, 30µg) are used to test the susceptibility against *E.coli*. Cloxacillin and Nalidixic acid are only sensitive whereas rest antibiotics are resistant to *E.coli*.

### Size determination of azithromycin resistant *E. coli* plasmid

Plasmid DNA was isolated from multiple-drug resistant *E. coli* according to the procedure described.<sup>28</sup> For a pure plasmid DNA, the ratio of the absorption of 260 nm and 280 nm ( $OD_{260}/OD_{280}$ ) should be 1.8. The absorption ratio of our sample DNA was 1.9 which is nearly 1.8. Thus, it can be

assumed that the isolated plasmid DNA is approximately pure. Isolated pure plasmid DNA was subjected to agarose gel electrophoresis. Gel was then stained in ethidium bromide solution and photographed by a Digital Camera. Our result shows that, the *E. coli* strain contains a plasmid of approximately 4kb in size compared with marker plasmid DNA pBR322 (4.3Kb) (Fig.4).



### DISCUSSION

Antibiotics have become one of the most important medical interventions for the therapeutic treatments. The abundant use of antibiotics particularly in hospitalized patients leads the suppression of gut flora and favors the persistence of drug resistant bacteria including *E.coli*, *Salmonella*, *Shigella*, *Enterobacter*, *Klebsiella* and *Proteus*.<sup>31</sup> Among them antimicrobial resistance in *E. coli* has been reported worldwide and treatment for its infection has been increasingly complicated by the emergence of resistant to most antimicrobial agents.<sup>24,32,33,34,35,36</sup> Thus, different kind of infections caused by *E. coli* has limited the treatment facility and restricted the choice of appropriate alternative medicine for immediate prevention of various diseases.<sup>37</sup> Consequently, failure to recognize the multi-drug resistant strains may result in higher mortality of infectious diseases.<sup>1,2</sup> Indeed, multiple antibiotic-resistant bacteria have been recognized as a serious threat for global public health worldwide.<sup>38,39,40,41</sup> Thus, advantage of antibiotics use is greatly hampered and remarkably increased in developing countries.<sup>42</sup> To solve this problem it is

important to investigate susceptibility of new antibiotic against multiple antibiotic-resistant bacterial strains.

This study aimed to isolate the azithromycin resistant plasmid in *E.coli* collected from patient's urine sample. In the preliminary step of this study, our results confirmed that the *E.coli* was resistant to azithromycin (Fig.1). Azithromycin resistant together with multi-drug resistant of *E.coli* suggests that azithromycin alone could not be effective in treatment of urinary tract infection (Fig.1 and Fig.3). Notably, antimicrobial drug resistance in *E. coli* has been reported worldwide.<sup>43</sup> Thus, treatment for *E. coli* infection has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents.<sup>24</sup> Nevertheless, results of this study suggest that Nalidixic acid and Cloxacillin could be effective in the treatment of severe urinary tract infections caused by multi-drug resistant *E. coli* (Fig.3). Moreover, our study results also suggest that azithromycin resistance observed in *E. coli* in Rajshahi area is possibly due to the presence of a drug resistant plasmid of approximately 4kb in size (Fig.4). Plasmid

mediated drug resistance has been found in *E.coli* isolated from chronic periodontitis patients.<sup>44</sup> Remarkly, size dependent plasmid mediated multi-drug resistant has been found in drinking water.<sup>45</sup> Thus, multidrug resistant strains of *E. coli* are a matter of concern as resistant genes are easily transferable to other strains. It should be mentioned that geographical variation of antibiotic resistance is existed.<sup>46</sup> Therefore, our next aim is to study multidrug susceptibility in *E.coli* collected from other regions/divisions of Bangladesh. Moreover, several study findings suggest that inadequate selection and abuse of antibiotics may lead resistance in various bacteria.<sup>43</sup> Study is so far needed to confirm the exact mechanism of azithromycin resistance arises in *E.coli*. All together, our preliminary findings may help in selecting proper antibiotic and/or medication of *E. coli* infected diseases.

## CONCLUSION

This study highlights that *E.coli* isolated from UTI patient's urine in Rajshahi city is resistant to azithromycin. This study also finds *Klebsiella*, *Proteus*, *Pseudomonas* and *Streptococcus* are sensitive to azithromycin. This trend in antibiotic resistance in *E. coli* is possibly the presence of resistant plasmid approximately 4 Kb in size. Notably, this study suggests that Nalidixic acid and Cloxacillin are effective in treating severe urinary tract infections caused by multi-drug resistant *E. coli*.

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