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**Manikant Tripathi**

Centre of Excellence, DST-FIST  
Supported Department of  
Microbiology, Dr. Ram Manohar  
Lohia Avadh University,  
Faizabad, (U.P.), India

**Mohammed Haroon**

Centre of Excellence, DST-FIST  
Supported Department of  
Microbiology, Dr. Ram Manohar  
Lohia Avadh University,  
Faizabad, (U.P.), India

**Shailendra Kumar**

Centre of Excellence, DST-FIST  
Supported Department of  
Microbiology, Dr. Ram Manohar  
Lohia Avadh University,  
Faizabad, (U.P.), India

## Antibiotic resistance in *Escherichia coli* isolates associated with child diarrhoea cases

**Manikant Tripathi, Mohammed Haroon and Shailendra Kumar**

**Abstract**

Uttar Pradesh is the largest state in the country. Diarrhoea is one of the major cause for death of young children below the age of 5 years. The aim of the present study was to study bacterial isolates from the diarrhoeal stool samples of pediatric patients and their antibiotic sensitivity assay. A total of 30 patients were screened for the symptoms of disease and sampling was performed. The sampling and isolation resulted in 18 Gram-negative rod shaped isolates and other Gram-positive cocci. The Gram-negative bacteria were subjected to biochemical identification and antibiotic sensitivity assay followed by plasmid DNA isolation and characterization. The isolates were identified biochemically as *Escherichia coli*. Further, the antibiotic sensitivity assay showed great resistance among the isolates for the commonly used antibiotics against diarrhoeal diseases. The plasmid study showed that the trait of antibiotic resistance is located on the plasmid as the plasmid curing study led to the development of sensitivity of selected isolates towards the antibiotics.

**Keywords:** Antibiotic resistance; Diarrhea; *Escherichia coli*; Plasmid curing

**1. Introduction**

Diarrhoea is characterized as a problem of frequent bowel movements with at least three or more loose/liquid faecal matter each day (W.H.O., 2013). The bowel movements can lead to decreased urination, loss of skin color, an acute heart attack, and a decrease in responsiveness with increasing severity. It has been a leading cause of malnutrition in young children of developing countries, as reported by WHO (2013). There are three types of diarrhea based on clinical symptoms such as acute watery diarrhea, short period bloody diarrhea, and persistent diarrhea (WHO 2013).

The major cause of diarrhea includes contaminated water with human and animal faeces, poor personal and food hygiene, etc. (Prado *et al.* 1998) The causative agents of diarrhoea are bacteria, virus and parasites, as well as food intolerance, reaction to medicines, and other physiological anomaly (Abrami *et al.* 1998). Diarrhoea due to infection is endemic and epidemic throughout developing countries (WHO 2013). WHO reported rotavirus and *E.coli*, are the most common etiological agents of moderate to severe diarrhoea in low-income countries. The low infectious dose and high virulence of *E. coli* O157:H7 make human infections severe and life threatening (Griffin *et al.* 1994; Tilden *et al.* 1996). It is the leading cause of morbidity and mortality among children in developing countries (Hill 1996). The key measures to prevent diarrhea are access to safe drinking water, use of improved sanitation, hand-washing with soap, good personal and food hygiene, health education and rotavirus vaccination (WHO).

The enteric pathogens are identified microbiologically, biochemically, serologically and genetically. The intestinal infections by the members of enterobacteriaceae are now being reported resistant to commonly used antibiotics leading to life threatening condition of the patients. Antibiotic resistance rates in *E. coli* are rapidly rising and they are readily acquired through food and water (Collignon 2009). The antibiotic resistance is due to the presence of resistance trait on plasmid DNA, which is transferable from resistant organism to sensitive strain of pathogen (Allen *et al.* 2010).

Keeping the above in view, the present study was aimed to characterize bacteria by rectal sampling of patients, microbiological and biochemical identification of bacterial isolates, their antibiotic sensitivity assay and analysis of plasmid borne resistance trait.

**Materials and Methods****Sample collection**

The samples were collected from 30 children suffering from diarrhea aged below 5 years. The presterilized swab impregnated with normal saline was used to collect stool sample from

**Correspondence****Shailendra Kumar**

Centre of Excellence, DST-FIST  
Supported Department of  
Microbiology, Dr. Ram Manohar  
Lohia Avadh University,  
Faizabad, (U.P.), India

patients and transferred into the 5 ml of Cary-Blair transport broth medium in sterile screw capped culture tube and incubated at 37 °C for 24 h. The bacterial isolation was carried out on MacConkey's agar medium as described previously (Kumar 2010).

Morphologically different colonies growing on plates were selected and streaked on MacConkey's agar medium for single isolated colony. The pure cultures of bacterial isolates were stored on nutrient agar slant at 4°C for further use.

### Bacteria Identification

The bacterial isolates were identified by morphological and biochemical characterization following standard Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994).

### Antibiotic sensitivity assay

Sensitivity assay was performed against the bacterial isolates using 14 antibiotics of different groups (aminoglycosides, beta lactam antibiotics, cephalosporins class I & II, quinolone, and fluoroquinolone) as described by Bauer *et al.* (1966). The sensitivity assay was performed as described previously by Kumar (2010). The inhibition zone was measured following standard method using zone scale. On the basis of the diameter of zone of inhibition the isolates were designated as sensitive, intermediate or resistant in accordance with NCCLS.

### Plasmid curing

The MIC of acridine orange for bacterial isolates was determined by inoculating nutrient broth added with varying concentrations of acridine orange. The plasmids of the two bacterial isolates were cured in medium amended with MIC level of acridine orange.

Following incubation, 0.1 ml of the exponential phase culture of both the bacterial isolates was spread aseptically to seed Mueller Hinton agar plates. After seeding the medium the antibiotic were placed using. Further, these Petri dishes were incubated in upright direction at 37°C for overnight. The zone of inhibition was measured as per standard protocol using zone scale and colonies were tested for loss of resistance on Mueller Hinton agar plates incorporated with the appropriate antibiotics.

### Determination of MIC for Chloramphenicol

The exponential phase bacterial cultures were inoculated into 5 ml nutrient broth containing variable concentration of chloramphenicol antibiotic (2-12 µg/ml), incubated at 37°C for 24 h. The concentration of antibiotic which inhibited the complete bacterial growth was considered the MIC of that isolate.

### Isolation of plasmid DNA

In this experiment, the HiPurA™ Plasmid DNA Miniprep Purification Kit supplied by Himedia Labs was used for plasmid DNA isolation. The isolates grown in culture tubes containing 5 ml LB medium amended with maximum

tolerable concentration of chloramphenicol. Overnight grown bacterial culture (2 ml) was centrifuged at 13000 rpm for 1 minute. The supernatant of culture medium was discarded. The bacterial pellets were re-suspended in 250 µl of Resuspension solution (HP1) and mixed by gently by pipetting till no cell clumps were visible. The lysis solution (HP2) (250 µl) was mixed thoroughly by gently inverting the tubes 4-6 times. In the next step, 350 µl of neutralization solution (HN3) was added and immediately mixed thoroughly by gently inverting the tubes 4-6 times. The mixture was centrifuged at approximately 13000 rpm for 10 minutes to obtain compact white pellets. If supernatant was not clear, transfer the supernatant to a fresh tube and spin for an additional minute at 13000 rpm to remove the interfering salts/precipitates completely. Further, carefully removed the supernatant, transferred into capped spin column tubes and centrifuged at 13000 rpm for 1 minute and discarded the flow through liquid. The column was washed by adding 700 µl of diluted wash solution (HPE) and centrifuged at 13000 rpm for 1 minute. Then, discarded the flow through liquid and centrifuged the empty tubes with the column for an additional 1 minute to remove any traces of wash solution. After this step, transferred the column to a clean 2.0 ml uncapped collection tube and added 50 µl of the elution buffer (ET) and allowed to stand for 1 minute at RT, followed by centrifugation for 1 minute at 13000 rpm.

### Agarose gel electrophoresis

The electrophoresis of isolated plasmid DNA was carried out in sub marine horizontal agarose slab gel apparatus as described by Sambrook *et al.* (1989). After gel electrophoresis the gel was examined on UVitec gel documentation system.

## Results and Discussion

### Isolation and identification of bacteria from stool samples

Forty bacterial cultures were isolated from the stool samples of patients suffering from diarrhea. Out of 40, 18 isolates were Gram negative and rod shaped. The isolates were subjected to microbiological and biochemical tests for their identification.

The isolates have been identified as *Escherichia coli* on the basis of microbiological and biochemical tests. The isolates have been reported responsible for gastrointestinal infections in children as well as adults. *E. coli* is a coliform, which is normal inhabitant of human and animal intestine. The bacteria have been isolated from water sources (Tewari 2002; Kumar *et al.* 2010).

### Antibiotic Susceptibility Test

The antibiotic susceptibility test results revealed amikacin as the most effective antibiotic against *E. coli* isolates. Nitrofurantoin and Gentamycin were not effective against the isolates as all the isolates showed resistance towards these antibiotics. Other antibiotics were effective against 72-89 per cent isolates (not shown).

**Table 1:** Antibiotic sensitivity test of 18 *E. coli* isolates

Bacterial strains	Antibiotic (Concentration mcg/discs)													
	AK 30	AT 30	CI 5	OF 5	FIX 5	NOR 10	CRO 30	CTX 30	XM 30	CAZ 30	CD 5	NA 30	NI 300	GM 10
1.	S	S	S	S	S	S	S	S	I	S	S	S	R	R
2.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
3.	S	R	R	R	R	R	I	I	R	R	R	R	R	R
4.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
5.	I	S	R	R	R	R	S	S	I	I	R	R	R	R
6.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
7.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
8.	I	R	R	R	R	R	R	R	R	R	R	R	R	R

9.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
10.	S	S	S	S	S	S	S	S	I	S	S	S	R	R
11.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
12.	S	R	R	R	R	R	R	I	R	R	R	R	R	R
13.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
14.	I	S	R	R	R	R	S	S	I	I	R	R	R	R
15.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
16.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
17.	I	R	R	R	R	R	R	R	R	R	R	R	R	R
18.	I	R	R	R	R	R	R	R	R	R	R	R	R	R

R- Resistance, S- Sensitive, I- Intermediate

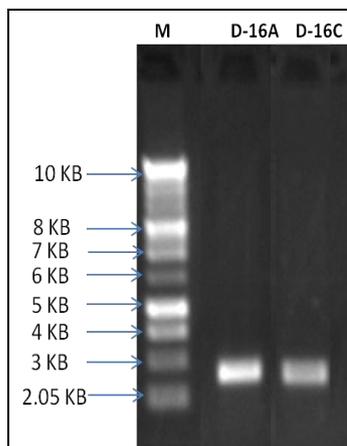
The *Enterobacteriaceae* members, viz., *E. coli* and species of *Enterobacter*, *Klebsiella*, *Salmonella*, *Shigella* have worldwide distribution, and are significant causes of gastrointestinal diseases. Antibiotic resistance among them has been regularly monitored in the hospitals (Andres *et al.* 2005). Antibiotic resistant *E. coli* have been reported throughout the world. Amikacin was the most effective and third generation cephalosporins were effective variably to different isolates (Kumar 2010). While, second generation cephalosporins, fluoroquinolones and quinolones were less effective relatively as they were sensitive only for about 10-30% of isolates.

#### Determination of MIC for Chloramphenicol

The minimum inhibitory concentration of chloramphenicol for nine bacterial isolates were determined in nutrient broth to increase plasmid copy number. The results were obtained as, the isolates D-16 A, D-16 C showed maximum MIC (12 µg/ml) for chloramphenicol. Ahmad *et al.* (2007) reported that the amplification of plasmid DNA in the presence of antibiotics in some isolated bacterial species. They have reported enormous increase in plasmid DNA concentrations in amplified cultures of *Klebsiella oxytoca*, *Salmonella* and *E. coli* isolates after treatment with 150 µg/ml chloramphenicol. The concentration of plasmids reached to more than six times the concentration in normal cultures.

#### Plasmid DNA Characteristics

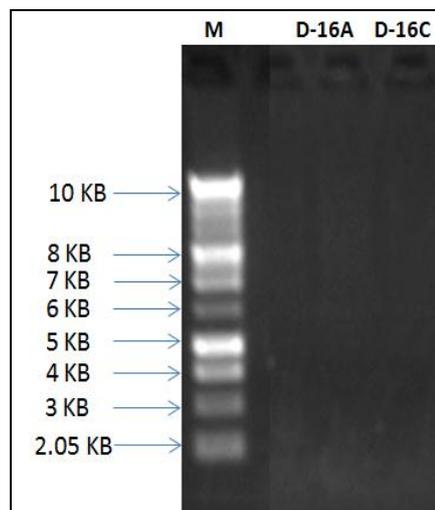
The electrophoresis of plasmid DNA isolated from the bacterial isolates showed the presence of a plasmid in the gel. Two bacterial cultures were used to screen for the presence of plasmid DNA was electrophoresed on a 1% agarose gel amended with ethidium bromide at 70 V for 60 minute and visualized on gel doc system. Lane 1: supercoiled plasmid DNA ladder, markers range from 2050 to 10,000 kb; Lanes 2–3: containing plasmid of two bacteria. The results are depicted in Fig. 2. Our results revealed the size of plasmid of the isolates was ~3 kb



**Fig 2:** Electrophoresis of plasmid DNA isolated from selected bacterial cultures (D-16A and D-16C) in agarose gel electrophoresis

#### Plasmid Curing

The plasmid was cured in two isolates D-16A and D-16C. The sensitivity of isolates increased after exposure to acridine orange. The plasmid was also not visible in the electrophorogram (Fig. 3). The results reveal that the antibiotic resistance property of bacteria was plasmid borne. Many researchers also reported similar findings.



**Fig 3:** The electrophorogram showing no plasmid DNA in gel.

#### Conclusions

The biochemical characterization showed that the isolates from children diarrhea samples mainly belonged to *Escherichia coli*. The isolates were also resistance to several commonly used antibiotics. The plasmid study showed that the trait of antibiotic resistance is located mainly on the plasmid as revealed by plasmid curing experiment. The development of antibiotic resistance among the enterobacterial pathogen is cause of concern.

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