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Original Article

A Comparative Study on *Escherichia coli* Isolates from Environmental and Clinical Samples

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Pathogenic *Escherichia coli* remain an important etiological agent of infantile diarrhea in Bangladesh. Present study focused on the features of *E. coli* strains isolated from clinical and environmental samples. A total of 6 surface water samples and 6 clinical samples were examined. Isolates were analyzed on the basis of colony morphology and biochemical traits. The antibiogram profile of the isolates was determined against 6 commonly used antibiotics. Ten isolates were found to be resistant against more than 1 antibiotic. Both types of isolates showed 50% resistance against trimethoprim-sulfamethoxazole. Clinical isolates showed 100% sensitivity against gentamicin whereas 2 environmental isolates were found to be resistant against the antibiotic. Eighty and sixty five percent of environmental strains were sensitive against ceptriaxone and ciprofloxacin, respectively; while 50% showed resistance against ampicillin and nalidixic acid. In case of clinical strains, 65% isolates were found to be sensitive against ceptriaxone whereas 65% showed resistance against ampicillin and nalidixic acid; and 50% isolates were found to be resistant against ciprofloxacin. The isolates (n=12) were also examined for the presence of plasmids conferring the antibiotic resistance. However, no such plasmid was observed.

Key words: E. coli, **clinical strain, environmental strain, antibiotic resistance plasmid.**

Introduction

Diarrhea is a world-wide health problem which ranges from a trivial nuisance for travelers to a fatal illness, particularly among children in underdeveloped countries.[1] It is one of the world's leading causes of morbidity and mortality, accounting for around two million deaths each year.[2] *Escherichia coli* strains belong to the coliform group of microorganisms which are a common part of the normal facultative anaerobic microflora of the intestinal tracts of most mammals including humans.[3] *E. coli,* belonging to the Enterobacteriaceae family, is a commensal bacterium in the intestinal tracts of warm-blooded animals; and some strains have acquired virulence causing intestinal or extra intestinal infections.[3]

Pathogenic forms of *E. coli* associated with human and animal diseases are remarkably diverse.[4] Six *E. coli* virotypes have been identified so far: enterohemorrhagic *E. coli* (EHEC) or verocytotoxigenic *E. coli* (VTEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC)[4]. Currently, antibiotic resistance is another vital issue in *E. coli* pathogenesis. *E. coli* is a prime candidate as a species in which new multi-drug resistant plasmids may evolve[5]. It has a very wide natural distribution[5] and a propensity for plasmid carriage[6]. Horizontal gene transfer is a recognized process that allows the rapid spread of antibiotic resistance genes inside and outside the hospitals, thereby impeding antimicrobial chemotherapy. Resistance determinants are readily acquired and disseminated within bacterial populations by conjugation, and hence resulting in a new phenotype[7]. R-plasmids are established to be promiscuously transferred among different enteric bacteria[5]. Typically, 10-20% of gut *E. coli* of healthy people (1960-1970) has R-plasmids and is found to be antibiotic resistant. Bacteria have been reported to produce colicins, lethal compounds that kill other bacteria which are specified by transmissible plasmids similar to F plasmid, for instance, ColE, ColV plasmids[5].

Water is another important factor for *E. coli* pathogenesis. Surface waters in trophical countries have been found to harbor pathogenic *E. coli* strains and their transmission can occur while bathing and/or using water for cooking[4]. These forms of transmission are common in areas where it is endemic both in the local populations and among the international travelers across these areas. Contaminated food and water sources both contribute to the seasonal outbreaks which affect tourists[4].

In the line of these evidences and facts, present study attempted to conduct the isolation and identification of *E. coli* strains from ponds, lakes, drains and stool samples. Antibiogram patterns of the isolates were also examined. In addition, presence of any drug resistance confering plasmid was tested.

Materials and Methods

Samples and Sampling Sites

A total of 12 samples, 6 environmental and 6 clinical, were included in this study. Environmental samples were collected

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from ponds, lakes, and drain water (areas of Dhaka University, Gulisthan, Dhanmondi and Siddeswari). For clinical samples, stool specimens were collected from suspected patients admitted to Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) hospital. Sample collection was carried out in between February 2009 and March 2009.

Sample collection

The environmental samples were collected aseptically in sterile polyethylene terephthalate (PET) bottles. For clinical samples, stool specimens from suspected diarrheal patients were collected aseptically in sterile containers. Samples were transported to the laboratory as quick as possible. Environmental samples were kept at room temperature and the clinical samples were preserved at 4 °C.

Isolation of escherichia coli

Fifty microliter of each sample was inoculated onto MacConkey agar medium by spread plate method. Two or three loopful of the samples were also inoculated onto MacConkey agar by streak plate method. Culture plates were then incubated at 37 °C for 24 h. Presumptive *E. coli* colonies were subcultured onto MacConkey agar to obtain pure cultures. The pure colonies of isolates were again grown on Eosin Methylene Blue (EMB) agar and the characteristic greenish metallic sheen was produced. Xylose Lysine Deoxycholate (XLD) agar were also used to observe the characteristic yellow colonies.[8]

Identification of escherichia coli isolates

According to Cappuccino and Shernan (1996), confirmative biochemical tests including the sugar fermentation, citrate utilization, motility indole urease and oxidase tests were performed to identify *E. coli* isolates.[9] Microscopic observation of the isolates was also performed.[9]

Determination of antimicrobial susceptibility by disc-diffusion method

Susceptibility of environmental isolates against different antimicrobial agents was determined *in vitro* by employing the standardized agar disc-diffusion method, more commonly known as the 'Kirby-Bauer Method'.[10] A suspension of the test organism was prepared by adjusting the turbidity of the broth in phosphate buffer saline by comparing with that of 0.5 McFarland solution. By means of a sterile cotton swab, a uniform lawn of bacterial growth (10^8 cfu/ml) was prepared on Muller-Hinton agar plates (pH 7.0). Before streaking, the swab was passed against the wall of the tube containing the suspension to let the excess fluid drain out. Commercially available antimicrobial discs (Oxoid, UK) were used for the test. Ampicillin (AMP), gentamicin (CN), trimethoprim-sulfamethoxazole (SXT), nalidixic acid (NA), ciprofloxacin (CIP) and ceptriaxone (CRO) were tested at a concentration of 10 µg, 10 µg, 25 µg, 30 µg, 5 µg, and 30 µg, consecutively against the isolates. Antibiotic discs were applied aseptically on the surface of the inoculated plates at appropriate spatial arrangement by means of sterile forceps. Plates were then inverted and incubated at 37°C for 24 h. Susceptibility against the specific drug was interpreted by the presence of clear zone around the disc. The zone diameters for individual antimicrobial agent were translated into susceptible, intermediate and resistant according to the interpretations described by Barry and Thornberry (1985).[10]

Observation of plasmid profile

Plasmid extraction was carried out according to the modified method of Birnboim and Doly (1979)[11]. Fresh *E. coli* suspension (1.5 ml) from the log phase culture was taken in eppendorf tubes and was spun for 10 min at 13,000 rpm in a micro-centrifuge (NF 800R, Niive). The supernatant was aspirated and the cells were resuspended in 500 µl solution I (2 mg/ml lysozyme, 50 mM glucose, 10 mM cyclohexane diamine tetracetate and 25 mM Tris-HC1, pH 8.0) by recentrifugation. The supernatants were removed and the pellets were completely resuspended in 100 µl of solution I. The tubes were then kept at 30 °C for 30 minutes. 200 µl solution II (0.2 N NaOH and 1% sodium dodecyl sulfate) was added to the tubes and was mixed gently by sample inversion following incubation on ice for 5 min. 150 µl of 3 M sodium acetate (pH 4.8) was added immediately and was mixed gently by inversion until the DNA precipitated. The mixtures were kept on ice for 60 min. Afterwards, the mixtures were centrifuged for 10 min at 13,000 rpm to precipitate the chromosomal DNA. The clear supernatants (400 µl) were transferred to sterile eppendorf tubes. Ice-cold ("20 °C) 95% ethanol (1 ml) was mixed with the samples, then kept on ice for 30 min following centrifugation for 10 min at 13,000 rpm. The supernatants were removed and the pellets were washed with 70% alcohol and dried for 1-2 min. DNA samples were then resuspended in 25 µl of Tris-Borate (TB) buffer, and the DNA suspension was kept at 20°C until use. Presence of plasmid DNA was checked by 1% agarose gel electrophoresis.

Agarose gel was prepared by dissolving 1% agarose in 40 ml Tris-Borate-EDTA (TBE) buffer. Electrophoresis was carried out at 60 volts for 1 h, gel was placed in a container containing 10 ìl of ethidium bromide in 100 ml of distilled water for 30 min. Then the gel was observed under a UV-transilluminator for the presence of plasmid bands at 260 nm.

Results

Phenotypic and biochemical characterization of the escherichia coli isolates

Since the isolation and identification of *E. coli* is very crucial for the culture-based detection purpose, our study used MacConkey agar for primary isolation. Bright pink or red lactose fermenting colonies were selected as presumptive *E. coli*. Presumptive colonies were grown on EMB agar and production of greenish metallic sheen was observed. Typical

circular yellow colonies of *E. coli* was observed on XLD media. Thus, all the 12 isolates were selected as *E. coli* strains for further biochemical analysis.

Detailed biochemical study revealed that all the strains had the biochemical properties typical for *E. coli*. Both environmental and clinical isolates showed the similar biochemical properties (Tables 1 and 2). All the isolates fermented glucose with acidic reactions and produced gas in the tubes of triple sugar iron (TSI) agar. However, it was observed that more gas was produced in cases of the clinical isolates compared to that of the environmental isolates. All the strains exhibited motility and were found to be negative for citrate and urease test.

Table 1. *Biochemical characteristics of environmental E. coli isolates.*

Strain ID	TSI			MIU			Citrate	Oxidase
	Slant	Butt	H_2S	Mot	Ind	Urease		
C-1	A	AG	–	+	–	+	–	–
C-2	A	AG	–	+	–	+	–	–
C-3	A	AG	–	+	–	+	_	–
C-4	A	AG	–	+	–	+	–	–
C-5	A	AG	–	+	–	+	–	–
C-6	A	AG	–	+	–	+	–	–

TSI Triple Sugar Iron test,
MIU Motility Iodole Urease test
Mot Motility
Ind Iodole
K Alkaline reaction
A Acidic reaction
AG Acidic reaction and gas
\+ Positive
– Negative
C Clinical isolates

Table 2. *Biochemical characteristics of clinical E. coli isolates.*

Strain ID	TSI			MIU			Citrate	Oxidase
	Slant	Butt	H_2S	Mot	Ind	Urease		
E-1	A	AG	–	+	–	+	–	–
E-2	A	AG	–	+	–	+	–	–
E-3	A	AG	–	+	–	+	–	–
E-4	A	AG	–	+	–	+	–	–
E-5	A	AG	–	+	–	+	–	–
E-6	A	AG	–	+	–	+	–	–

TSI Triple Sugar Iron test,
MIU Motility Iodole Urease test
Mot Motility
Ind Iodole
K Alkaline reaction
A Acidic reaction
AG acidic reaction and gas
\+ Positive
– Negative
E Environmental isolates

Antibiotic susceptibility pattern of the isolates

All *E. coli* strains were tested for antibiotic susceptibility against 6 commonly used antibiotics. The isolates showed variations in the susceptibility pattern against the antibiotics used in the study (Tables 3 and 4). In case of environmental isolates, 5 (80%) were susceptible against ceptriaxone (CRO), 4 were sensitive (65%) against ciprofloxacin (CIP) and gentamicin (CN), 3 were resistant (50%) against ampicillin (AMP), trimethoprim-sulfamethoxazole (STX) and nalidixic acid (NA) (Table 3 and Figure 1A). On the other hand, clinical isolates showed 100% sensitivity against CN, 4 isolates were resistant (65%) against AMP and NA, while 4 were found to be sensitive (65%) against CRO and 3 were resistant (50%) against CIP and SXT (Table 4 and Figure 1B). A total of 10 isolates (83%) were found to be resistant against more than one drug (Table 3 and 4). The antibiogram pattern of the isolates of clinical and environmental origions varied significantly (Figure 1).

Table 3. *Resistance/ Susceptibility pattern of environmental E. coli isolates against different antibiotics.*

Strain ID	Antibiotic Susceptibility					
	AMP (10 µg)	CRO (30 µg)	CIP (5 µg)	CN (10 µg)	SXT (25 µg)	NA (30 µg)
E-1	R	S	S	S	R	S
E-2	R	S	R	S	R	R
E-3	S	S	S	S	S	R
E-4	S	R	R	R	S	R
E-5	S	S	S	S	S	S
E-6	R	S	S	R	R	S

AMP Ampicillin
CN Gentamicin
SXT Trimethoprim-Sulfamethoxazole
NA Nalidixic acid
CIP Ciprofloxacin
CRO Ceptriaxone
S Sensitive
R Resistant
E Environmental isolates

Table 4. *Resistance/ Susceptibility pattern of clinical E. coli isolates against* different antibiotics.

Strain ID	Antibiotic Susceptibility					
	AMP (10 µg)	CRO (10 µg)	CIP (25 µg)	CN (30 µg)	SXT (5 µg)	NA (30 µg)
C-1	R	S	S	R	S	S
C-2	S	S	S	S	S	S
C-3	R	S	R	R	R	S
C-4	R	S	R	R	R	S
C-5	S	S	R	S	S	R
C-6	R	S	S	R	R	R

AMP Ampicillin
CN Gentamicin
SXT Trimethoprim-Sulfamethoxazole
NA Nalidixic acid
CIP Ciprofloxacin
CRO Ceptriaxone
S Sensitive
R Resistant
C Clinical isolate

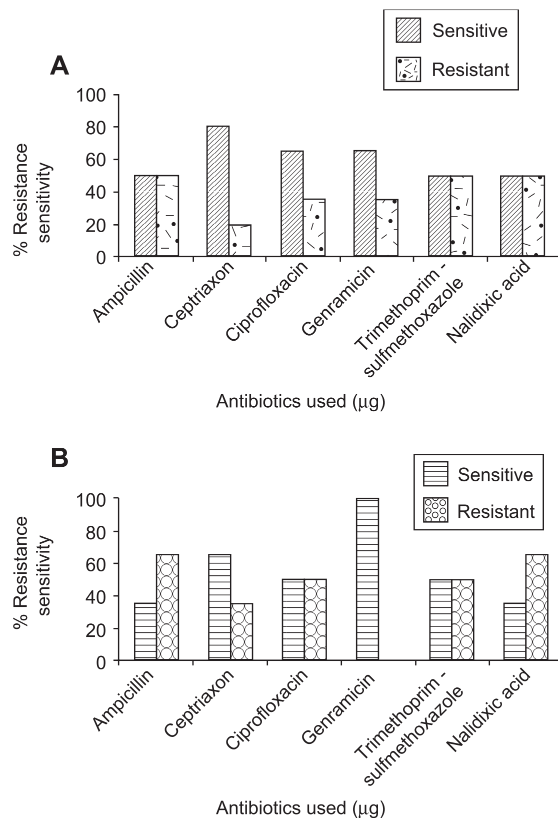

Fig 1. *Relative antibiotic resistance/ susceptibility pattern of A. environmental and B. clinical E. coli isolates through the disc-diffusion assay. Ampicillin (10 µg), ceptriaxon (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (25 µg) and nalidixic acid (30 µg) were used.*

Absence of plasmid bearing the drug-resistance gene

The modified method of Birnboim and Doly (1979) was applied to screen antibiotic resistant plasmids. Seven randomly selected isolates were analyzed for this purpose. However, after performing the 1% agarose gel electrophoresis, no plasmid band was visualized (data not shown).

Discussion

The presence of virulent strains of *E. coli* in the environment may be a potential source of contamination of food and the water supply, hence posing the public health risk. These strains may comprise a potential reservoir of virulence genes acquired from different sources (e.g. bacteriophages and plasmids). *E. coli* strains in aquatic environment may be subjected to a sort of genetic exchange through the lateral gene transfer.[12,13] Being a competent organism, it has the capacity of horizontal gene transfer to increase the genetic diversity, and under certain circumstances, this can lead to the emergence of new pathogenic strains.[12] According to the previous suggestive data,[14] the present study dealt with drug resistance/sensitivity patterns of the environmental and clinical *E. coli* strains as well with the probable existence of their drug resistance plasmids.

From our study, *E. coli*, both from environmental and clinical samples, exhibited nearly similar morphological and biological traits. However, their antibiogram profile showed some variations. *E. coli* recovered in this study expressed a high level of resistance against antimicrobials that are commonly used in clinical medicine. This could contribute to the spread and persistence of the antimicrobial-resistant bacteria. Surprisingly, in the most developing countries, without identifying the pathogen first, diarrheal diseases are treated by an inadequate quantity of antimicrobials[12]. Thus, the present study is of significance regarding the treatment of enteric diseases in Bangladesh. The emergence of resistance (mostly due to irrational use of antibiotics and inappropriate self-medication) and the decreasing levels of susceptibility of *E. coli* to a wide spectrum of antimicrobials are noteworthy since they may limit the availability of antimicrobials for clinical management of waterborne outbreaks in the future.

Some earlier studies reported that the multi-drug resistant *E. coli* isolates from surface waters were found to be positive for virulence determinants of enterohaemorrhagic *E. coli* (EHEC).[13] Some other studies have shown that the clinical and surface water isolates of *E. coli* resistant to ciprofloxacin were multi-drug resistant. The resistance against nalidixic acid was significantly associated to cephalothin and tetracycline.[13] Webster *et al*. (2004) reported that *E. coli* isolates from urban areas/point sources had resistance against more antimicrobials than those from rural/non–point sources, possibly because of frequent exposure to antimicrobials.[12,15] In accordance with these reports, we also extended the investigation towards the screening of drug-resistant plasmid.

As plasmids play a major role in transferring this resistance among different strains, first we aimed to detect the presence of such plasmids. However, no plasmid could be detected in our study and hence, no further investigation on the presence of the drug-resistance gene was carried out. The possible reasons for the absence of antibiotic resistance conferring plasmid might be that the isolates might have possessed small sized plasmid or due to the plasmid instability or incompatibility. Extensive study using molecular detection methods on such plasmid might unveil the drug resistance in *E. coli*.

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