Presence of virulence factors and antibiotic resistance among *Escherichia coli* strains isolated from human pit sludge

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Abstract

Introduction: In Bangladesh, human sludge from dry pit latrines is commonly applied directly to agricultural lands as manure. This study was conducted to investigate the presence of antibiotic resistance, virulence factors and plasmid contents of *E. coli* strains isolated from sludge samples.

Methodology: *E. coli* were isolated from human feces from closed pit latrines and identified by culture method. Antibiotic susceptibility patterns of the isolates were determined by Standard Kirby-Bauer disk diffusion method. Pathogenic genes and antibiotic resistance genes of ESBL producing isolates were determined by PCR assay.

Results: Of the 34 samples tested, 76.5% contained *E. coli*. Of 72 *E. coli* isolates, 76.4% were resistant to at least one of the 12 antibiotics tested and 47.2% isolates were resistant to three or four classes of antibiotics. Around 18% isolates were extended spectrum β-lactamase producing and of them 6 were positive for *bla*TEM specific gene, 4 for *bla*CTX-M gene, 1 for *bla*OXA gene and 2 for both *bla*TEM and *bla*CTX-M genes. Moreover, among 72 isolates, 4.2% carried virulence genes of enterotoxigenic *E. coli*; two isolates were positive for *st* and one was positive for both *st* and *lt* genes. In addition, 59.7% of the isolates contained plasmids (range 1.4 to 140 MDa) of which 19.5% isolates contained a single plasmid and 40.2% contained multiple plasmids.

Conclusions: The presence of pathogenic, drug resistant *E. coli* in human sludge necessitates a regular surveillance before using as a biofertilizer.

Key words: Human sludge; ESBL; *E. coli*; Biofertilizer; Antibiotic resistance.
untreated manures used in irrigation fields. People living near ponds, come in contact with contaminated water when they drink it and bathe in it and this causes diarrheal diseases in Bangladesh [10].

Food and waterborne diseases remain as the prevailing cause of mortality and morbidity in many developing countries including Bangladesh [11]. *Escherichia coli*, widely used as an indicator organism for the microbiological quality of water and food, are also an important causative agent of diarrhea and other enteric diseases [12]. Persistence of *E. coli*, particularly of pathogenic strains, in open environment and factors influencing their survival rate are very critical issues in regard of disease occurrence [13].

While most *E. coli* are usually harmless, certain strains of *E. coli* have virulence properties that may account for life-threatening infections. Currently, six *E. coli* pathotypes are recognized that can cause diarrhea in humans [14]: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC) and diffusely adhering *E. coli* (DAEC) [14]. In Bangladesh pathogenic *E. coli* are the second leading causes of diarrhea next to Rotavirus [15]. ETEC is the most common cause of traveler's diarrhea. Every year, ETEC causes more than 200 million cases of diarrhea and 380,000 deaths, mostly in children in developing countries [16]. There are several virulence genes that are associated with specific pathotypes of diarrheagenic *E. coli* which are st, lt (ETEC); bfp, eae (EPEC); aat, aai (EAEC) [17]. Diarrhea caused by ETEC strains happens by the action of the enterotoxins ST and/or LT. Similarly, EPEC strains cause diarrhea through the help of eae and bfp gene products along with other virulence factors [17]. The diagnostic genes of EAEC namely aggregative adherence genes (aaI and aaT) helps EAEC strains to adhere to host cell and subsequently cause diarrhea [17].

Antibiotic resistance in enteric pathogens is of particular interest in developing countries as it is considered one of the most serious threats to the treatment of infectious diseases and is one of the leading public health concerns of the 21st century [18]. There is also a growing concern about resistance in enteric pathogens including *Salmonella* species, *Shigella* species, *Campylobacter* species, and *E. coli*. Extended-spectrum β-lactamase (ESBL) producing members of the family *Enterobacteriaceae* (enterobacteria) have become a worldwide problem [19] and prevalence of ESBL producing *E. coli* in environmental components such as surface water [20,21] and poultry manure [20] has been observed. The emergence of ESBL producing bacteria, particularly *E. coli* and *Klebsiella pneumoniae*, is now a critical concern for the development of therapies against bacterial infection [21]. Almost all beta-lactam antibiotics and also other classes of antibiotics are ineffective to such β lactamase producing bacteria and this enforces the use of carbapenems, example of so-called ‘last-resort antibiotics’ [22]. This novel enzyme along with other antibiotic resistance factors is carried by mobile genetic elements such as plasmids or transposons [23].

Numerous reports confirmed isolation of pathogenic and antibiotic resistant *E. coli* from environmental samples [24–26]. Some reports also showed evidence for transfer of resistance genes containing plasmids between different species of bacteria [27,28]. These evidences are particularly alarming because, some reports suggested horizontal transfer of resistance gene and others suggested outbreaks sourced from environmental samples or contaminated food and water sources [1].

The goal of this study was to look for the presence of *E. coli* in human fecal sludge (before applying as manure) and characterize them for their antibiotic resistance, pathogenic types, presence of ESBL genes and plasmids.

**Methodology**

*Sample types, sources and study sites*

Fecal sludge samples were collected from closed pit latrines, which had not been used for last six months from the date of sampling. Since, usually farmers do not apply in-use latrines sludge in agricultural fields, we selected closed pit latrines for this study. Sampling sites were Bogra and Dhaka, Bangladesh and the experiments were performed at the Environmental Microbiology Laboratory of International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

*Collection and transportation of samples*

A total 34 fecal sludge samples were collected. For each area, sampling sites were selected randomly and samples were collected aseptically. Samples were collected in 2014 in three visits with one month of gap. Sterile plastic bottles (Nalgene, Rochester, USA) of 500 mL capacity were used for sample collection. All samples were placed in an insulated box filled with ice packs maintaining temperature 4°C to 10°C and transported to the Environmental Microbiology Laboratory of icddr,b for bacteriological analysis immediately after collection.
Processing of samples and isolation of E. coli

Samples were at first mixed with sterile normal saline and four fold dilutions were made. To get isolated E. coli presumptive colonies, 100µl of each diluted samples were cultured on modified mTEC agar medium (Difco, Sparks, MD, USA) using spread plate technique. Plates were incubated at 37°C for the initial 2 hours, and then at 44.5°C for 18–24 hours. Purple colored colonies were assumed as E. coli and selected for further confirmation. The selected colonies were subcultured on MacConkey agar (Difco, Sparks, MD, USA) plate and incubated at 37°C for 18-24 hours. From each plate, suspected dry pink colonies with typical E. coli colony morphology were taken and again subcultured on MacConkey plates to obtain pure culture. Isolates identified on the culture plates were also further tested by using a battery of biochemical tests according to standard methods described in Manual for Laboratory Investigation of Acute Enteric Infections [29]. The following biochemical tests were performed: Kliger’s Iron Agar (KIA) test, Motility Indole Urease (MIU), Citrate utilization test, Catalase and Oxidase test. In case of E. coli, butt and slant were turned into yellow color with formation of gas in KIA test. Isolates those were positive for indole, catalase tests and negative for urease, citrate, oxidase were considered to be E. coli and were stored at -70°C in tryptic soy broth supplemented with 30% (vol/vol) glycerol. E. coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as positive and negative controls respectively for each biochemical tests.

Antibiotic susceptibility tests

Standard Kirby-Bauer disk diffusion method was followed to determine the antibiotic susceptibility patterns of the E. coli isolates according to the Clinical and Laboratory Standards Institute guideline [30]. We interpreted the susceptibility results for 12 antibiotic agents including ampicillin (10 μg), azithromycin (15 μg), ceftaxime (5 μg), ceftriaxone (30 μg), cefepime (5 μg), nalidixic acid (30 μg), sulfamethoxazole/ trimethoprim (25 μg), gentamicin (10 μg), chloramphenicol (30 μg), tetracycline (30 μg) and polymyxin B (300μg) according to the guidelines recommended by the Clinical and Laboratory Standards Institute-CLSI [30]. Antibiotic discs were placed on Mueller Hinton agar medium (Difco, MD, USA) seeded with young culture of E. coli suspension and incubated for 18h (± 2 hours) at 37°C. The plates were examined, and the diameters (in millimeters) of the clear zones of growth inhibition around the antibiotic discs, including the 6-mm disc diameter, were measured. The zone diameter for individual antibiotic agents was then translated into susceptible, intermediate or resistant categories according to the interpretation guideline provided by CLSI [30]. Isolates that showed resistance to third generation cephalosporins were further tested for the presence of ESBL by performing double disc synergy test (DDST) [30].

Table 1. PCR primers used in the study.

| Target gene | Primer | Nucleotide sequences (5’-3’*) | Product size (bp) |
|-------------|--------|------------------------------|------------------|
| blatem      | TEM-F: | TCG GGG AAA TGT GCG CG       | 971              |
|             | TEM-R: | TGC TTA ATC AGT GAG GCA CC   |                  |
| blashv      | SHV-F: | CAC TCA AGG ATG TAT TGT G    | 885              |
|             | SHV-R: | TTA GCG TTG CCA GTG CTC G    |                  |
| blaxoxa     | OXA-F: | ACCAGATTCAACTTTCAA           | 590              |
|             | OXA-R: | TCTTGGCTTTATGCTTG            |                  |
| blactx-m    | CTX MU1: | ATGTGCGAYACCAGTAARGT | 593 |
|             | CTX-MU2: | TGGGTRAARTARGTSACCAGA       |                  |
| eae         | eae-F: | CACACGGAGCTTCTCAGTC          | 508              |
|             | eae-R: | CCCCCAGCCTAGCTTTAGTT         |                  |
| elt         | LT-F: | GCTAAACCAGTAGGTGCTCAAAAA     | 147              |
|             | LT-R: | CCCGCTAGAGCAGATGATTACAACA   |                  |
| est         | ST-F: | GGAAGTCAAATTCTGAGGGG        | 300              |
|             | ST-R: | GGAATCAGACGGACGACTGTT       |                  |
| bfp         | bfp-F: | CCCGAGGCCG4C4T7C4GAGT4AC7    | 881              |
|             | bfp-R: | CAAGGACAACGAGATGATCAT       |                  |
| eae         | eae-F: | CTGGCGAAAGACGTGATCAT        | 650              |
|             | eae-R: | CAATGATAGAAATCCGCTT       |                  |
| aat         | Pevd-F: | ATTTGACCCGACAGATCAG        | 215              |
|             | Pevd-R: | CTGGCGAAGACGTGATCAT        |                  |
| aai         | aai-F: | ACGACACCCCTGATAAACA         |                  |
|             | aai-R: | ACGACACCCCTGATAAACA         |                  |
Detection of antibiotic resistance genes in ESBL-positive isolates by PCR Assay

All the ESBL positive isolates were tested for the presence of \textit{bla} \textit{ESBL} genes (\textit{bla}\textit{TEM}, \textit{bla}\textit{SHV}, \textit{bla}\textit{OXA}, and \textit{bla}\textit{CTX-M}). PCR assays were performed in a DNA thermal cycler (PTC-0200 DNA Engine® Cycler, BIO-RAD) using Taq DNA polymerase (Promega, WI, USA). PCR for \textit{bla}\textit{TEM} and \textit{bla}\textit{SHV} used the basic set-up: 96°C for 5 min followed by 28 cycles of 20 sec at 96°C, 20 sec at \textit{T} \text{Annealing} 50°C and \textit{T} \text{Elongate(min)} at 72°C for 2 min [31]. On the other hand, PCR for \textit{bla}\textit{CTX-M} was 94°C for 5 min followed by 32 cycles of 1 min at 94°C, 1 min at \textit{T} \text{Annealing} 57°C and \textit{T} \text{Elongate(min)} at 72°C for 5 min [32]. The PCR program for \textit{bla}\textit{OXA} was 72°C for 5 min followed by 32 cycles of 20 sec at 96°C, 30 sec at \textit{T} \text{Annealing} 55°C and \textit{T} \text{Elongate(min)} at 72°C for 2 min [33]. In this PCR assay, four \textit{E. coli} strains specific for \textit{bla}\textit{TEM}, \textit{bla}\textit{SHV}, \textit{bla}\textit{OXA}, \textit{bla}\textit{CTX-M} were used as positive control. The primer sequences and their product size are listed in Table 1.

Multiplex PCR for pathogenic genes

All isolates were tested for the presence of heat stable (\textit{st}), heat labile (\textit{lt}), bundle forming pilus (\textit{bfp}), attaching and effacing gene (\textit{eae}), anti aggregation protein transporter gene (\textit{aat}), and gene for AggR-activated island (\textit{aai}) by multiplex PCR assay according to the procedures described earlier [34]. The primer size for these genes is listed in Table 1. A well-characterized ETEC strain (for \textit{lt, st}), \textit{E. coli} O157:H7 12079 (for \textit{bfp, eae}) and a well-characterized EAEC (for \textit{aat, aai}) strain were used as positive control.

Plasmid profile analysis

During plasmid DNA extraction, the modified alkaline lysis method of [35] was applied and plasmids were separated by horizontal electrophoresis in 0.7% agarose slab gels in 1X Tris-borate EDTA (TBE) buffer at room temperature at 100 volt (50 mA) for 3 hours. The molecular weight of the unknown plasmid DNA was determined on the basis of its mobility through agarose gel and was compared with the mobility of the known molecular weight plasmids [36,37]. Plasmids present in the following strains were used as molecular weight standards: \textit{E. coli} R1 (62 MDa), pDK9 (140, 105, 2.7, and 2.1 MDa), RP4 (36 MDa), Sa (23 MDa) and V517 (35.8, 4.8, 3.7, 3.4, 3.1, 2.0, 1.8 and 1.4 MDa).

Statistical analyses

Data are expressed as the mean ± standard deviation (SD). Significance level between different groups were defined by 2 tailed t-test using IBM SPSS 20 software. A p value of < 0.05 was considered statistically significant.

Results

Fecal sludge samples are contaminated with \textit{E. coli}

To test whether fecal sludge samples are positive for the presence of \textit{E. coli} or not, first we performed conventional plate culture method using mTEC agar plate. Typical colonies those showed similar characteristics with the positive control of \textit{E. coli} on modified mTEC agar plate were subcultured on MacConkey agar plate for further confirmation. On MacConkey agar plate round, dry, pink, lactose

![Figure 1. Antibiotic resistance patterns of isolated \textit{E. coli}.](image-url)
fermenting colonies were taken as suspected *E. coli* for further confirmation. Additional confirmation was done by a battery of biochemical tests (results not shown here) with positive control to match with the sample results. As we expected, of 34 fecal sludge samples, 26 (76.5%) were found to be positive for the presence of *E. coli*. No pattern of the presence of *E. coli* found between the two sampling sites (Result not shown). Multiple colonies were selected from the positive samples (at least one colony from each positive sample) based on proper match with the positive control and later sample redundancy was removed by antibiotic resistance pattern. A total of 72 isolates were used in this study.

**E. coli isolates were found resistant to some common antibiotics**

Numerous recent report suggested multiple antibiotic resistant isolates from various environmental samples [20,38]. We hypothesized that, these *E. coli* isolated from fecal sludge might be resistant to different antibiotics due to the abuse of antibiotics. To test our hypothesis, we tested all 72 isolates for the resistance to available and frequently used antibiotics including ampicillin, azithromycin, cefixime, ceftriaxone, chloramphenicol, ciprofloxacins, gentamicin, nalidixic acid, polymixin B and trimethoprim, 25% to tetracycline, 23.6% to trimethoprim, 16.7% to azithromycin, 15.3% to ceftazidime, 9.7% to ceftriaxone and 5.6% to gentamicin and 2.8 % to chloramphenicol (Figure 1A). Around 76% isolates were resistant to at least one antibiotic and 47% of the isolates were resistant to three or more classes of antibiotics thus defined as multi-drug resistant (MDR) (Figure 1B). Moreover, it was found that among all the isolates 30.5% showed positive result in double disc synergy test done for ESBL detection.

**Presence of antibiotic resistance genes in ESBL-positive organisms**

The alarming presence of ESBL positive organism from double disc synergy test intrigued us to test these isolates for the presence of ESBL specific genes. As a result we found that, out of 22 ESBL positive isolates, 59.1% were positive for the presence of these genes responsible for ESBL-production. As shown in Table 2, 27.3% (n = 6) were positive for *bl*\textsubscript{TEM}, 18.2% positive for *bl*\textsubscript{CTX-M}, and 4.4% positive for *bl*\textsubscript{OXA}. Of note, 9.0% (n = 2) isolates carried both *bl*\textsubscript{TEM} and *bl*\textsubscript{CTX-M} genes. On the other hand, none of them was positive for *bl*\textsubscript{SHV} gene (Table 2).

**Pathogenic *E. coli* were detected from the isolates**

Antibiotic resistant *E. coli* become more alarming if they are pathogenic. Next we tested for the presence of pathogenic *E. coli* from the all 72 isolates by using PCR pathogenic gene detection method. Out of 72 isolates, 3

### Table 2. Distribution of ESBL specific genes in the *E. coli* isolates.

| ESBL specific genes | Size of gene product (bp) | No. of isolates |
|---------------------|---------------------------|----------------|
| *bl*\textsubscript{TEM} | 971 | 6 |
| *bl*\textsubscript{CTX-M} | 593 | 4 |
| *bl*\textsubscript{OXA} | 590 | 1 |
| *bl*\textsubscript{SHV} | 971, 593 | 2 |

**Total no. of ESBL isolates = 13**

### Table 3. Detection of pathogenic genes in the *E. coli* isolates.

| Pathogenic gene profile | No. of isolates | Pathotype | Antibiotic resistance genes | Resistance to antibiotics |
|-------------------------|-----------------|-----------|----------------------------|--------------------------|
| +                       | 1               | ETEC     | *bl*\textsubscript{TEM}    | AMP, CFM, CRO, CAZ, CIP, C, SXT, NA |
| +                       | 2               | Isolate 1- *bl*\textsubscript{CTX-M}, *bl*\textsubscript{TEM} | AMP, CFM, CRO, C, AZ, SXT, PB |
| -                       | 0               | Isolate 2- none | AMP, CFM, CRO, C, AZ, SXT, PB |
| -                       | 0               | No EPEC   | NA |
| -                       | 0               | No EAEC   | NA |

AMP- Ampicillin, AZ- Azithromycin, C- Chloramphenicol, CAZ- Cefazidime, CFM- Cefixime, CIP- Ciprofloxacins, CRO- Ceftriaxone, NA- Nalidixic Acid, SXT- Sulphamethoxazole/trimethoprim, TE- Tetracycline, PB- Polymyxin B.
(4.2%) isolates possessed either st (n = 3) gene or lt gene (Table 3), where two isolates were positive for st genes and one was positive for both st and lt genes and thus belonged to ETEC [39]. All other isolates were negative for any of the pathogenic genes tested in the study (Table 3). Of note, all of the three pathogenic isolates were resistant to at least two antibiotics. Isolate that was positive for both st and lt genes, carried blaTEM and was found to be resistant to eight antibiotics of six groups (Table 3). Isolates that were positive for st gene showed difference in resistance profiles of which one carried both blaCTX-M and blaTEM genes and was resistant to seven antibiotics of five groups, other isolate carried no ESBL gene but was resistant to tetracycline and nalidixic acid (Table 3).

**Plasmid profile of isolates**

In medical microbiology the study of plasmids is important because plasmids can encode genes for virulence factors or antibiotic resistance [17]. Next we tested the presence of plasmid(s) in the all 72 isolates. Our result of plasmid profile analysis showed that 59.7% E. coli isolates contain plasmids of different sizes ranged from 1.40 to 140 MDa (Figure 2). Among these plasmid containing strains, around 49.23% isolates contained more than one plasmid and 19.5% isolates contained a single plasmid (Figure 2).

**Discussion**

The present study was aimed to investigate the rate of antibiotic resistance and the presence of virulence factors of E. coli strains isolated from different fecal sludge samples. In typical rural areas of Bangladesh, people use open type pit latrines by digging hole in deep to the soil and defecate into the pit until it is fully filled. So, the presence of both pathogenic and non-pathogenic E. coli strains is expected in such pit sludge since E. coli constitutes the major indigenous gut flora of human [40]. These organisms might also originate from the environment, which might enter the pit latrine through rain water from surrounding household waste, soil or groundwater. Farmers in underdeveloped or developing countries use dried human and cattle fecal sludge as manure in the agricultural field [41]. Use of untreated or undertreated pit sludge as manure or biofertilizer in agricultural land may cause the bared exposure of pathogenic E. coli to the environment posing an imminent health risk to humans. In Bangladesh, diarrheal diseases are major health problem and ETEC accounts for about 20% of all diarrheal cases in children less than 2 years of age [42].

In the present study, a total of 34 fecal sludge samples from two sites were investigated among which 76.5% of samples were found contaminated with E. coli. In the antibiotic susceptibility pattern test, 76% of the E. coli isolates were resistant to at least one of the 12 antibiotics tested and surprisingly the two highest resistant antibiotics are β-lactam ring containing ones (ampicillin and cefixime). Another interesting finding is that the third generation cephalosporin (cefixime) is the highest resistant type of antibiotic among other third generation of cephalosporins. Previous study also suggested a similar high rate of resistance for cefixime (57.9%) in children stool samples in Iran [43]. The presence of ESBL producing E. coli indicates the reason of high resistant to β-lactam ring containing ones (ampicillin and cefixime) antibiotics. A considerable percent of multi drug resistant (MDR) isolates indicate that some E. coli are resistant to β-lactam ring containing antibiotics as well as at least two other classes of antibiotics. Previously, it was reported that cephalosporins accounted for more than 55% of the total antibiotics used in Bangladesh while no mention of chloramphenicol use in the 150 cases studied [44]. Several reports have also claimed that the occurrence of resistance to the antibiotics is related to the frequency of its use [45,46]. It has been reported that in Europe,
the shift to the use of new broad-spectrum antibiotics from the old narrow-spectrum has increased the resistance of the broad-spectrum types [46]. This might be the cause of higher resistance to cefixime than to chloramphenicol.

In case of susceptibility to a particular antibiotic, the present study showed highest sensitivity against chloramphenicol (97.2%) and it has a resemblance with the study carried out in the year 2013 in Dhaka, Bangladesh, where E. coli was isolated from household water supply and tested for their susceptibility to commonly used antibiotic agents [34]. In that report, 73% E. coli were found resistant against at least one antibiotic and the highest sensitivity was against gentamicin (99%) and second highest sensitivity was against chloramphenicol (around 92%) [34]. This concurs with our findings of the lowest resistance (2.8%) to chloramphenicol with presumptive indication to a decreased use of it, although the data from Bangladesh is not sufficient enough to interpret and require further investigations.

A previous study reported that 11.8% of E. coli isolates obtained from medical hospitals in urban and rural areas of Bangladesh were ESBL producing [47]. Similar to other studies, a higher frequency of resistance against β-lactam antibiotic was observed among the isolates in this study. A significantly high proportion (18.1%) of E. coli isolates tested in the present study were ESBL-producing. This might be due to the residual effect of antibiotics, which have been used extensively in human population as well as in the food chain creating a selective antibiotic pressure in the environment [34].

With the beginning of the twenty-first century, E. coli strains producing bla_{CTX-M-15} have emerged and disseminated worldwide and are now one of the cause of both nosocomial and community-onset urinary tract and bloodstream infections in humans [48]. Our findings pointed out the importance of carrying out this kind of study to identify clinically significant bla_{TEM}\* gene and bla_{CTX-M} gene positive strains and also signifies the importance to find out a solution for it. The prevalence of CTX-M type β-lactamases in Enterobacteriaceae is increasing and in some geographic locations they are more prevalent than TEM and SHV types [49]. But in this study, presence of TEM type β-lactamases has been found more than that of CTX-M type. The exact reason for this presence were not studied in this study and needs further investigation.

Although human or animal manures are considered as important route of transmission of pathogenic E. coli, only a few reports are available that describe its transmission. In a previous study it was observed that, raw or improperly composted manures are more likely to contaminate fresh produce with E. coli O157:H7 [50]. In the present study, a small percentage (4.2%) of E. coli isolates was found pathogenic. These isolates belonged to only ETEC among six different pathotypes. In an ongoing birth cohort study in Bangladesh, ETEC was found to be the most common cause of diarrhea in children of less than 2 years of age, accounting for 18% of all diarrhea cases [42]. At present, there is no vaccine available for diarrhea caused by E. coli and besides, the different treatment modalities including antibiotic therapy are not very efficient due to the emergence of MDR organisms.

The results in this study indicated that a significant proportion of collected fecal sludge samples was contaminated with E. coli of which most of them were resistant to various antibiotics and some of them were pathogenic. If these contaminated manures are not composted well at required temperature, the sludge will remain contaminated with E. coli in the biofertilizers. Presence of E. coli in manures indicates the presence of microorganisms potentially hazardous for human health. Such manures might contaminate the crops growing in the fertilized field and can be hazardous to the handlers. These contaminated manures might flow with rain into the nearby water bodies and cause fecal contamination in surface water sources. Although not alarming, a few of the isolates were pathogenic and a significantly high proportion of isolates were MDR. The isolates were individually resistant to common antibiotics such as ampicillin, azithromycin, cefixime, ceftrizidime, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, polymixim B, trimethoprim, and tetracycline. The adverse effects of antibiotic resistant bacteria on humans might happen in several ways. When contaminated food is ingested, the bacteria might transfer resistance determinants to other bacteria in the human gut by horizontal gene transfer [51]. Thus, the occurrence of pathogenic E. coli with multiple antibiotic resistances in fecal sludge will create a potential threat to the public health.

**Conclusion**

To overcome this problem, use of unnecessary and uncontrolled antibiotic therapies should be restricted as well as proper treatment of manure should be performed before using it in the field. So, effective control measures are strongly recommended to prevent contamination of food and water bodies with pathogenic E. coli coming from contaminated manures.
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References
1. Fenlon DR, Ogden ID, Vinten A, Svoboda I (2000) The fate of Escherichia coli and E. coli O157 in cattle slurry after application to land. J Appl Microbiol: 88.
2. Menzies JD (1977) Pathogen considerations for land application of human and domestic animal wastes. Soils Manag Organ wastes waste waters: 573–585.
3. Ungemach FR, Müller-Bahrdt D, Abraham G (2006) Guidelines for prudent use of antimicrobials and their implications on antibiotic usage in veterinary medicine. Int J Med Microbiol 296: 33–38.
4. Linton AH, Howe K, Bennett PM, Richmond MH, Whiteside EJ (1977) The colonization of the human gut by antibiotic resistant Escherichia coli from chickens. J Appl Microbiol 43: 465–469.
5. Mkeni P, Cisneros JBP, Austin L (2006) Use of human excreta from urine diversion toilets in food gardens. Water Res Comm 3: 1–40.
6. Heinonen-Tanski H, van Wijk-Sijbesma C (2005) Human excreta for plant production. Bioreour Technol 96: 403–411.
7. Stevenson FJ, Cole MA (1999) Cycles of soils: carbon, nitrogen, phosphorus, sulfur, micronutrients. 2nd edition. New York: John Wiley & Sons 448p.
8. Eichenseher Tasha (2008) Human waste used by 200 million farmers, study says. Available: https://news.nationalgeographic.com/news/2008/08/080821-human-waste.html. Accessed 14 March 2018.
9. Lagerkvist CJ, Shikuku K, Okello J, Karanja N, Ackello-Ogutu C (2015) A conceptual approach for measuring farmers’ attitudes to integrated soil fertility management in Kenya. NJAS-Wageningen J Life Sci 74: 17–26.
10. Knappett PSK, Escamilla V, Layton A, McKay LD, Emch M, Williams DE, Huq R, Alam J, Farhana L, Mailloux BJ (2011) Impact of population and latrines on fecal contamination of ponds in rural Bangladesh. Sci Total Environ 409: 3174–3182.
11. Prüss A, Kay D, Fewtrell L, Bartram J (2002) Estimating the burden of disease from water, sanitation, and hygiene at a global level. Environ Health Perspect 110: 537.
12. Sack RB (1975) Human diarrheal disease caused by enterotoxogenic Escherichia coli. Annu Rev Microbiol 29: 333–354.
13. Van Elsas JD, semenov A V, Costa R, trevors JT (2011) Survival of Escherichia coli in the environment: fundamental and public health aspects. ISME J 5: 173.
14. Levine MM (1987) Escherichia coli that cause diarrhea: Enterotoxogenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J Infect Dis 155: 377–389. Available: http://www.jstor.org/stable/30105047 Accessed: 30 November 2014.
15. Talukder KA, Islam Z, Dutta DK, Islam MA, Khajanchi BK, Azmi JJ, Iqbal MS, Hossain MA, Faraque ASG, Nair GB (2006) Antibiotic resistance and genetic diversity of Shigella sonnei isolated from patients with diarrhoea between 1999 and 2003 in Bangladesh. J Med Microbiol 55: 1257–1263.
16. Wennérás C, Erling V (2004) Prevalence of enterotoxigenic Escherichia coli-associated diarrhoea and carrier state in the developing world. J Heal Popul Nutr: 370–382.
17. Nataro JP, Kaper JB (1998) Diarrheagenic Escherichia coli. Clin Microbiol Rev 11: 142–201.
18. World Health Organization (2014) Global report on surveillance: Antimicrobial resistance. 1-7 p. Available: http://www.who.int/drugresistance/documents/surveillancereport/en/. Accessed: 12 January 2015.
19. Okeke IN, Laxminarayan R, Bhutta ZA, Duse AG, Jenkins P, O’Brien TF, Pablos-Mendez A, Klugman KP (2005) Antimicrobial resistance in developing countries. Part I: recent trends and current status. Lancet Infect Dis 5: 481–493.
20. Biaa H, van Hoek AHAM, Hamidjaja RA, van der Plaats RN, Kerkhof-de Heer L, de Roda Husman AM, Schets FM (2015) Distribution, numbers, and diversity of ESBL-producing E. coli in the poultry farm environment. PLoS One 10: e0135402.
21. Franz E, Veeman C, Van Hoek AHAM, de Roda Husman A, Biaa H (2015) Pathogenic Escherichia coli producing extended-spectrum β-lactamases isolated from surface water and wastewater. Sci Rep 5: 14372.
22. Cantón R, Akóva M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Miriagou V, Naas T, Rossolini GM (2012) Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. Clin Microbiol Infect 18: 413–431.
23. Cantón R, Coque TM (2006) The CTX-M β-lactamase pandemic. Curr Opin Microbiol 9: 466–475.
24. Ongeng D, Geeraerd AH, Winokur PL, Vonstein DL, Hoffman LJ, Uhlenhopp EK, carattoli A (2009) Resistance plasmid families in Enterobacteriaceae. Annu Rev Microbiol 63: 223–252.
25. Truchado P, Hernández N, Gil MI, Ivanek R, Allende A (2018) Correlation between E. coli levels and the presence of foodborne pathogens in surface irrigation water: Establishment of a sampling program. Water Res 128: 226–233.
26. Bibbal D, Lô FT, Mbengue M, Sarr MM, Diouf M, Sambe Y, Kérourédan M, Alambédji R, Thiongane Y, Oswald E (2017) Prevalence of pathogenic and antibiotics resistant Escherichia coli from effluents of a slaughterhouse and a municipal wastewater treatment plant in Dakar. African J Microbiol Res 11: 1035–1042.
27. Carattoli A (2009) Resistance plasmid families in Enterobacteriaceae. Antimicrob Agents Chemother 53: 2227–2238.
28. Winokur PL, Vonstein DL, Hoffman LJ, Uhlenhopp EK, Doern G V (2001) Evidence for transfer of CMY-2 AmpC β-lactamase plasmids between Escherichia coli and Salmonella isolates from food animals and humans. Antimicrob Agents Chemother 45: 2716–2722.
29. Bopp C, Ries AA, Wells JG, Koplan JP, Hughes JM (2011) Laboratory methods for the diagnosis of epidemic dysentery and cholera. Geneva. Available: https://stacks.cdc.gov/view/cdc/6669/cdc_6669_DS1.pdf. Accessed 20 April 2016.
30. Clinical and Laboratory standard institute (CLSI) (2012) Performance standards for antimicrobial susceptibility testing, 22nd informational supplement. CLSI document M100-S22 (ISBN 1-56238-785-5).

31. Briñas L, Zarazaga M, Sáenz Y, Ruiz-Larraí F, Torres C (2002) β-Lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. Antimicrob Agents Chemother 46: 3156–3163.

32. Pagani L, Dell’Amico E, Migliavacca R, D’Andrea MM, Giacobone E, Amicosante G, Romero E, Rossolini GM (2003) Multiple CTX-M-type extended-spectrum β-lactamases in nosocomial isolates of Enterobacteriaceae from a hospital in northern Italy. J Clin Microbiol 41: 4264–4269.

33. Li R, Lai J, Wang Y, Liu S, Li Y, Liu K, Shen J, Wu C (2013) Prevalence and characterization of Salmonella species isolated from pigs, ducks and chickens in Sichuan Province, China. Int J Food Microbiol 163: 14–18.

34. Talukder PK, Rahman M, Rahman M, Nabi A, Islam Z, Hoque MM, Endtz HP, Islam MA (2013) Antimicrobial resistance, virulence factors and genetic diversity of *Escherichia coli* isolates from household water supply in Dhaka, Bangladesh. PLoS One 8: e61090.

35. Kado C, Liu S (1981) Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145: 1365–1373.

36. Haider K, Huq MI, Talukder KA, Ahmad QS (1989) Electropherotyping of plasmid DNA of different serotypes of *Shigella flexneri* isolated in Bangladesh. Epidemiol Infect 102: 421–428.

37. Talukder KA, Islam MA, Khajanchi BK, Dutta DK, Islam Z, Safa A, Alam K, Hossain A, Nair GB, Sack DA (2003) Temporal shifts in the dominance of serotypes of *Shigella dysenteriae* from 1999 to 2002 in Dhaka, Bangladesh. J Clin Microbiol 41: 5053–5058.

38. Natvig EE, Ingham SC, Ingham BH, Cooperband LR, Roper TR, Arment AR (2003) Salmonella enterica Serovar *Typhimurium* and *Escherichia coli* contamination of root and leaf vegetables grown in soils with incorporated bovine manure. Appl Environ Microbiol 69: 3686.

39. Gaasta W, Svennerholm A-M (1996) Colonization factors of human enterotoxicogenic *Escherichia coli* (ETEC). Trends Microbiol 4: 444–452.

40. Hussain SA, Khalil A, Shaukat A, Arif-un-Nisa N, Muhammad A, Sujjad H, Qamar A, Salar A, and Najam-ul-Hassan S (2014) Health risk assessment of pit compost latrine at Oshkhandas Valley, Gilgit-Baltistan, Pakistan. J Biodivers Environ Sci 5: 195–203.

41. Cofie OO, Kranjac-Berisavljevic G, Drechsel P (2005) The use of human waste for peri-urban agriculture in Northern Ghana. Renew Agric Food Syst 20: 73–80.

42. Qadri F, Svennerholm A-M, Faruque ASG, Sack RB (2005) Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev 18: 465–483.

43. Ayatollahi J, Shahcheraghi SH, Akhondi R, Soluti SS (2013) Antibiotic resistance patterns of *Escherichia coli* isolated from children in Shahid Sadoughi hospital of Yazd. Iran J Pediatr Hematol Oncol 3: 78–82.

44. Fahad BM, Matin A, Shill MC, Asish KD (2010) Antibiotic usage at a primary health care unit in Bangladesh. Australas Med J: 3.

45. Barbosa TM, Levy SB (2000) The impact of antibiotic use on resistance development and persistence. Drug Resist Updat 3: 303–311.

46. Goossens H, Ferech M, Vander Stichele R, Elseviers M (2005) Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. Lancet 365: 587–591.

47. Lina TT, Khajanchi BK, Azmi IJ, Islam MA, Mahmood B, Akter M, Banik A, Alim R, Navarro A, Perez G (2014) Phenotypic and molecular characterization of extended-spectrum beta-lactamase-producing *Escherichia coli* in Bangladesh. PLoS One 9: e108735.

48. Pitout JDD (2010) Infections with extended-spectrum β-lactamase-producing *Enterobacteriaceae*. Drugs 70: 313–333.

49. Falagas ME, Roussos N, Gkegkes ID, Rafaillidis Pl, Karageorgopoulos DE (2009) Fosfomycin for the treatment of infections caused by Gram-positive cocci with advanced antimicrobial drug resistance: a review of microbiological, animal and clinical studies. Expert Opin Investig Drugs 18: 921–944.

50. Beuchat LR, Ryu JH (1997) Produce handling and processing practices. Emerg Infect Dis 3: 459.

51. Oloomi M, Javadi M, Bouzari S (2015) Presence of pathogenicity island related and plasmid encoded virulence genes in cytolysin producing toxin producing *Escherichia coli* isolates from diarrheal cases. Int J Appl basic Med Res 5: 181–186.

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