Original Article

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.

Introduction

Land application and or disposal of both human and cattle wastes of fecal origin is becoming a routine procedure in many areas of the world [1,2]. The use of such wastes in agricultural farming might spread antibiotic resistant bacteria to humans if the wastes are contaminated, as several research have proven [3,4]. Human urine has been used as a source of nutrients for crop fertilization in countries such as Mexico, Germany, USA, Sweden, Denmark, Zimbabwe, China, Korea, and Japan [5,6]. Manures contribute to the soil fertility by adding organic matters and nutrients, such as nitrogen, that are trapped by bacteria in the soil [7]. It naturally activates the microorganisms found in the soil, restoring the soil's natural fertility and stimulates plant growth [7]. In countries like China, India,

Vietnam, sub-Saharan Africa and Latin America, grains and vegetables are harvested by nearly 200 million farmers in fields that use untreated human waste as fertilizer [8]. Faecal sludge may contain disease causing microorganisms even after keeping it undisturbed in the pit for months. Farmers of low income countries tend to use human feces as fertilizer due to availability and lower cost [9]. Therefore, they ultimately face the greatest risk of suffering from diseases if such fecal sludge is not treated well before applying to the field. In Bangladesh, farmers who deal with these are generally poor and illiterate and are highly susceptible to infection by pathogenic bacteria present in fecal sludge as they rarely maintain good hygiene practices. On the other hand, due to rain, flood and poor sanitation, ponds and rivers become contaminated from

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), cefixime (5 μg), ceftazidime(30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), sulfamethoxazole/ trimethoprim (25 μg), gentamicin (10 μg), chloramphenicol (30 μg), tetracycline (30 μg) and polymixin 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)
*bla*TEM	TEM-F: TEM-R:	TCG GGG AAA TGT GCG CG TGC TTA ATC AGT GAG GCA CC	971
*bla*SHV	SHV-F: SHV-R:	CAC TCA AGG ATG TAT TGT G TTA GCG TTG CCA GTG CTC G	885
*bla*OXA	OXA-F: OXA-R:	ACCAGATTCAACTTTCAA TCTTGGCTTTTATGCTTG	590
*bla*CTX-M	CTX MU1: CTX-MU2:	ATGTGCAGYACCAGTAARGT TGGGTRAARTARGTSACCAGA	593
elt	LT-F: LT-R:	CACACGGAGCTCCTCAGTC CCCCCAGCCTAGCTTAGTTT	508
est	ST-F: ST-R:	GCTAAACCAGTAGAGGTCTTCAAAA CCCGGTACAGAGCAGGATTACAACA	147
bfp	bfp-F: bfp-R:	GGAAGTCAAATTCATGGGGG GGAATCAGACGCAGACTGGT	300
eae	eae- F: eae-R:	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	881
aat	Pcvd-F: Pcvd-R:	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	650
aai	aaiC-F: aaiC-R:	ATTGTCCTCACGCATTTCAC ACGACACCCCTGATAAACAA	215

Detection of antibiotic resistance genes in ESBL-positive isolates by PCR Assay

All the ESBL positive isolates were tested for the presence of *bla*$_{ESBL}$ genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, and *bla*$_{CTX-M}$). 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 *bla*$_{TEM}$ and *bla*$_{SHV}$ used the basic set-up: 96ºC for 5 min followed by 28 cycles of 20 sec at 96ºC, 20 sec at $T_{Annealing}$ 50ºC and $Ti_{Elongate}$(min) at 72ºC for 2 min [31]. On the other hand, PCR for *bla*$_{CTX-M}$ was 94ºC for 5 min followed by 32 cycles of 1 min at 94ºC, 1 min at $T_{Annealing}$ 57ºC and $Ti_{Elongate}$(min) at 72ºC for 5 min [32]. The PCR program of *bla*$_{OXA}$ was 96ºC for 5 min followed by 32 cycles of 20 sec at 96ºC, 30 sec at $T_{Annealing}$ 55ºC and $Ti_{Elongate}$(min) at 72ºC for 2 min [33]. In this PCR assay, four *E. coli* strains specific for *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$ 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 *(st)*, heat labile *(lt)*, bundle forming pilus *(bfp)*, attaching and effacing gene *(eae)*, antiaggregation protein transporter gene *(aat)*, and gene for AggR-activated island *(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 *lt*, *st*), *E. coli* O157:H7 12079 (for *bfp*, *eae*) and a well-characterized EAEC (for *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: *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 E. coli

To test whether fecal sludge samples are positive for the presence of *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 *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 *E. coli.*
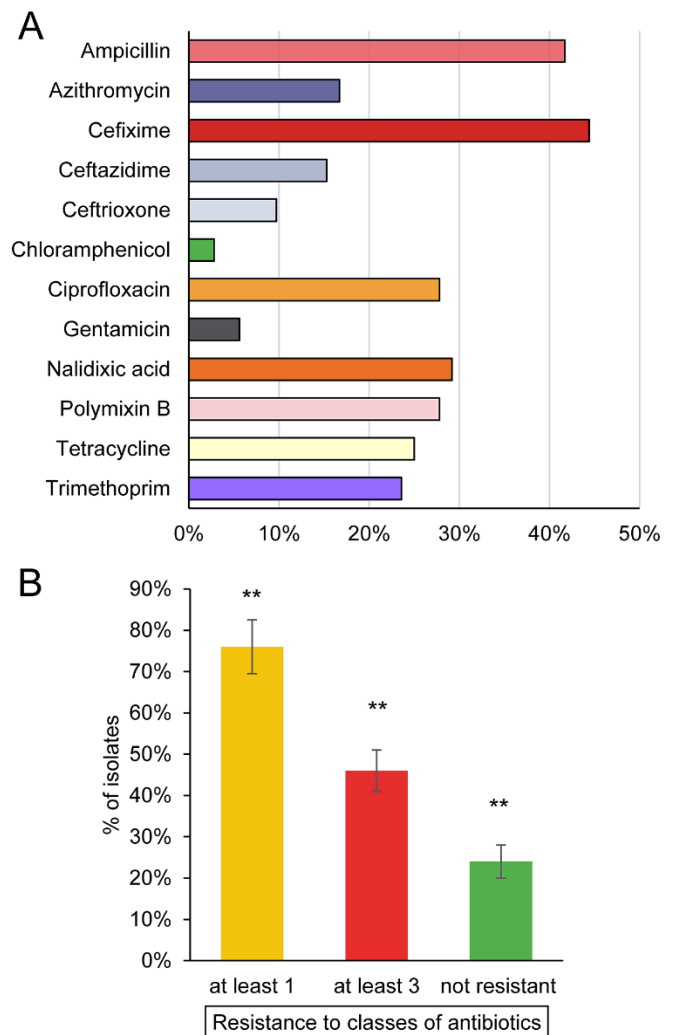
E. coli isolation and antibiotic resistance test were performed as described in the methods and materials section. (A) Percent of *E. coli* isolates (of 72 isolates) resistant to different antibiotics. (B) Percent of *E. coli* isolates resistant to one or more classes of antibiotics. Results are expressed as mean ± SD of three individual experiments. "**" denotes P < 0.01.

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

ESBL specific genes				Size of gene product (bp)	No. of isolates
*bla*TEM	*bla*CTX-M	*bla*OXA	*bla*SHV		
+				971	6
	+			593	4
		+		590	1
			-	-	0
+	+			971, 593	2
				Total no. of ESBL isolates = 13	

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 representative *E. coli* 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, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, polymixin B, tetracycline, and trimethoprim. Of the 72 isolates tested, 44.4% (32 isolates) were resistant to cefixime, followed by 41.7% to ampicillin, 29.2% to nalidixic acid, 27.8% to

polymixin B and ciprofloxacin, 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 *bla*TEM, 18.2% positive for *bla*CTX-M, and 4.4% positive for *bla*OXA. Of note, 9.0% (n = 2) isolates carried both *bla*TEM and *bla*CTX-M genes. On the other hand, none of them was positive for *bla*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 3. Detection of pathogenic genes in the *E. coli* isolates.

Pathogenic gene profile						No. of isolates	Pathotype	Antibiotic resistance genes	Resistance to antibiotics
lt	*st*	*bfp*	*eae*	*aat*	*aai*				
+	+					1		*bla*TEM	AMP, CFM, CRO, CAZ, CIP, C, SXT, NA
	+					2	ETEC	**Isolate 1-** *bla*CTX-M, *bla*TEM **Isolate 2-** none	**Isolate 1-** AMP, CFM, CRO, C, AZ, SXT, PB **Isolate 2-** TE, NA
		-				0	No EPEC		
			-			0			
				-		0	No EAEC		
					-	0			

AMP- Ampicillin, AZ- Azithromycin, C- Chloramphenicol, CAZ- Ceftazidime, CFM- Cefixime, CIP- Ciprofloxacin, CRO- Ceftriaxone, NA- Nalidixic Acid, SXT- Salphamethoxazole/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 *bla*$_{TEM}$ 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 *bla*$_{CTX-M}$ and *bla*$_{TEM}$ 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).
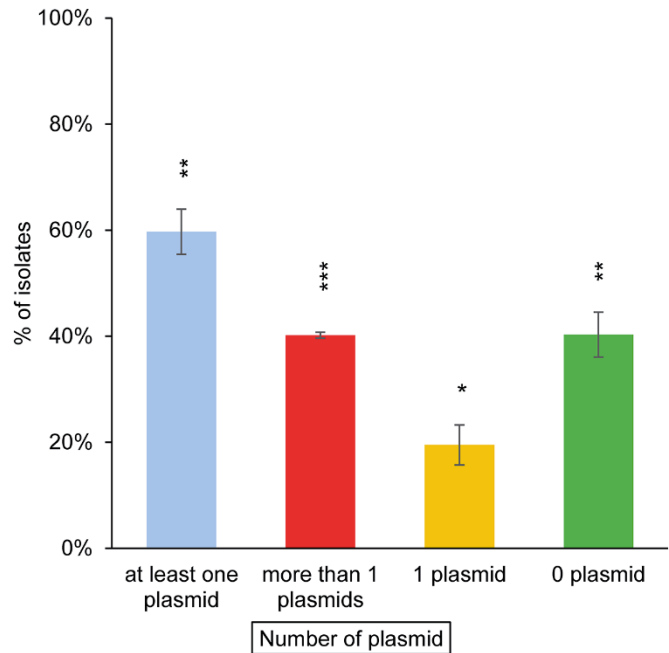
Plasmid profile of isolates

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].

Figure 2. Plasmid profiles of isolated *E. coli.*

Profiling of plasmid was performed as described in the methods and materials section. Results are expressed as mean ± SD of percent of isolates that contained different number of plasmid(s). * states P < 0.05, ** states P < 0.01 and *** states P < 0.001.

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 (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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