International Journal of Natural and Social Sciences
Print: 2617-6637 < ISSN > Online: 2313-4461

Phenotypic and genotypic diversity of *Escherichia coli* isolated from cattle in the dairy farms in Chittagong

Aungshuman Das Gupta[1], Shamima Najmin[1]*, Muhammad Gias Uddin[2], Md. Omar Qaium[3], Mohammad Yasrib Haider[4], Paritosh K. Biswas[1]

[1]Department of Microbiology, Chittagong Veterinary and Animal Sciences University (CVASU), Chittagong, Bangladesh
[2]Paediatric, Lohagora Health Complex, Chittagong, Directorate General of Health Services, Bangladesh
[3]Institute of Epidemiology, Disease Control and Research (IEDCR), Directorate General of Health Services, Bangladesh
[4]Jahurul Islam Medical College, Bhagalpur, Bajitpur, Kishoreganj, Bangladesh

ARTICLE INFO

Article history

Accepted 7 July 2019
Online release 20 July 2019

Keyword

Dairy farm, Escherichia coli, EHEC, plasmid profiling, PF antimicrobial resistance, CT-SMAC

***Corresponding Author**

Shamima Najmin
✉ momydvm@gmail.com

ABSTRACT

A cross -sectional survey was conducted to assess the diversity among fecal *Escherichia coli* from cattle in the dairy farms of Chittagong region at phenotypic and genotypic level. A total of 21 farms were selected and swabs were collected from recto anal junctions(RAJ) of randomly selected 419 cattle on them. The diversity of *E. coli*, particularly those belonging to probable entero hemorrhagic *E. coli* (EHEC)0157 serogroup was discriminated from others based on observation of sorbitol non-fermenting colonies of a selective medium named potassium tellurite added sorbital MacConkey (CT-SMAC)agar. Any member of the EHEC 0157 serogroup is sorbital non-fermenter and thus produces colorless colonies. *E. coli* fidelity on four selected isolates was tested for the presence of seven house-keeping genes,and their genetic diversities were verified by plasmid profiling and pulse field gel electrophoresis(PFGE). Finally,their antimicrobial susceptibility profiles were also assessed by disk-diffusion method, against 11 antimicrobials. The results showed that all the 21 cattle farms were positive for probable EHEC 0157, but the magnitude of colonized animals with it varied from farm to farm. The overall prevalence of probable EHEC was 7.6%(32/419) (95% CI 4.3–12.7%) and its prevalence in the adult cows,heifers and calves were 6.4% (95% CI 0.07-11.9%),9%(95% CI 0.01-14.6%) and 10%(95%CI 6.4-24.6%)respectively.Its prevalence in the cows suffering from mastitis (2.4%) was 0.29(95% CI 0.12-0.86) times lower compared with others (p=0.015).The population of probable EHEC 0157 in the dairy cattle in Chittagong seemed genetically diverse based on plasmid and PFGE profiles of four selected isolates. The antimicrobial resistance profiles of the probable EHEC isolates varied substantially. Most EHEC 0157 isolates showed diversity in their resistance patterns against 11 antimicrobials tested. O-serotype identities of the of the probable EHEC 0157 isolates need to be confirmed by the *rfb* gene or serogroup-specific agglutination test and the possession of the virulent genes, such as *stx1,stx2*,*eae* and *HlyA* in them also remain unexplored from the study.

INTRODUCTION

Enterohaemorrhagic *E. coli* (EHEC) are important gastrointestinal pathogens of humans. *E.coli* serotype O157:H7 is the EHEC most commonly associated with human illness(Gyles, 2007). *E. coli* O157:H7 is carried asymptomatically by cattle which form an important reservoir for the bacterium. *E. coli* O157:H7 has been found to colonise at the terminal rectum of cattle in preference to other sites in the bovine gastrointestinal tract (Borczyk *et al.*, 1987). This strain produces Shiga-like toxins, causing severe illness by eating cheese and contaminated meat. VTEC O157:H7 is the prototype bacterium for EHEC and is the serotype most frequently isolated from outbreaks and severe human disease worldwide (Karmali *et al*., 2003). Cattle and other ruminants are the most important reservoir of zoonotic STEC, which are transmitted to humans through the ingestion of foods or water contaminated with animal faeces, or through direct contact with the infected animals or their environment.. Life treatening disease may be resulted because of consuming milk contaminated

How to cite this article: Gupta AD, Najmin S, Uddin MG, Qaium MO, Haider MY and Biswas PK (2019). Phenotypic and genotypic diversity of Escherichia coli isolated from cattle in the dairy farms in Chittagong. International Journal of Natural and Social Sciences, 6(2): 01-13.

with shiga toxin producing 0157 :H7 E. coli (Grant *et al,* 2011).

The main sources of STEC infection of cattle on-farm are the drinking water, the feed, and the immediate environment of the animal (Fairbrother & Nadeau, 2006). *E. coli* strains belonging to over 200 serotypes can express Stx, but within most serotypes both Stx-positive and Stx-negative strains can be found (Nataro & Kaper, 1998). In 1983, STEC strains of serotype O157:H7 were definitively proved to be linked, for the first time, to several major outbreaks of HC and HUS in the United States of America (USA) and Canada. Zoonotic STEC-related disease has been observed worldwide, and in most industrialized countries O157:H7 remains the predominant serotype. Cattle are the main reservoir for zoonotic STEC throughout the world. The advent of selective media and kits for the rapid identification of O157:H7strains have permitted a more accurate assessment of the role of this serotype in human disease outbreaks and the transmission of the infection from animal reservoirs. However, a lack of similar tests for the rapid and easy identification of zoonotic non-O157 STEC and of other STEC, which are found in the intestinal tract of animals but have not yet been implicated in human infections, has impeded assessment of the geographical distribution of these strains, the mode of transmission to humans, and the prevalence of these strains in human outbreaks and in animal reservoirs (Fairbrother & Nadeau, 2006).

Considerable effort has been taken to determine the prevalence of *E. coli* O157 in cattle and identify possible risk factors for carriage. Moreover, there are many different strains of *E. coli*; each of these strains differs in its genotype from wild-type *E. coli*. The genotype can then affect the phenotype that is expressed, and further influences the physiology and life cycle of each strain. Therefore, different strains of *E. coli* can live in different kinds of animals. The natural biological process of mutation in genomes is the major cause to produce so many different strains of *E. coli*. In addition, similar to most bacteria, *E. coli* can transfer its DNA materials through bacterial conjugation with other related bacteria to produce more mutation and add more strains into the existing population.

However, a very little is known on the prevalence of EHEC O157 in cattle reared in Bangladesh and probably little or nothing is published hither to in the literature on its magnitude in cattle raised in dairy farms in any part of the world including Bangladesh.

Polymerase chain reactions (PCR) to identify the presence of the major virulent genes in EHEC O157 of different animal and food sources have been optimized and published (Ji-Yeon *et al*., 2005; Deising and Thompson, 2004; Osek and Gallien, 2002). Because any EHEC O157 strain produces colorless colony on SMAC, relatedness of the strains isolated from large and diverse populations cannot be established just by seeing the colonial morphology. Molecular techniques targeting some of the virulent genes might be useful as alternative tools towards exploration of a clonal relationship of a particular genotype(s) circulated in a population of a defied geo-location. Plasmid profiling might also add some values. However, more sophisticated and a gold-standard molecular diagnostic test, such as Pulsed Field Gel Electrophoresis (PFGE), is needed to discriminate and to establish the clonal relationship of the isolates belonging to the common circulating serotype(s).

Antibiotics are used in cattle even in the rural areas in Bangladesh to control infectious disease. Use of sub-optimal doses is a triggering factor towards the emergence of antimicrobial-resistant *E. coli* strains which can then be passed on to humans via food or direct contact with infected animals. Recent reports indicate that antimicrobial resistance of *E. coli* O157 is on the rise (Galland et al., 2001). Because antimicrobials can lyse bacterial cell walls liberating shiga toxins to aggravate any clinical case (Wong et al., 2000), antibiotics are not recommended to treat any hemolytic uremic syndrome (HUS) case in humans, although some recent studies suggested that some antimicrobials, if administered early in the course of infection, might prevent disease progression to HUS (Ikeda et al., 1999). However, examination of antimicrobial resistance for bacterial isolates could also be a phenotypical assay in unveiling clonal diversity of EHEC O157 isolates from a defined population (Zhang et al., 2000). E. coli was found in 18% raw milk in

Chittagong.The antimicrobial succeptibility of E. coli isolates showed a high prevalenvce of resistance to most of the antibiotics use (Alam *et al.,*2017).

Bangladesh has a huge cattle population reared in smallholdings and in the commercial dairy farms. The interactions between humans and these dairy farm cattle are very intensive. Therefore, there could be probable transmission of any zoonotic pathogen, such as EHEC O157 from these farm cattle to humans through raw milk and milk products where raw milk is used (e.g. cheese). Baseline information on the rate at which the commercial dairy cattle are harboring this pathogen is necessary before advocating any approach targeting the public from being infected with EHEC O157.

Cefixime-tellurite added SMAC (CT-SMAC), a primary selective growth medium for EHEC O157 can be used for assessing the harboring rate of EHEC O157 in commercial dairy cattle in Chittagong, Bangladesh on the basis of colorless colonies developed on it. Fecal materials collected from recto anal junction (RAJ) can be the best sample of choice because EHEC O157 specifically colonizes the most distal few centimeters of the intestine (Naylor et al., 2003). Isolates displaying characteristic colorless colonial morphology need to be investigated further to produce evidence as to whether they are truly *E. coli* or not and what percentage of them are carrying at least one virulent gene of EHEC O157, and whether there is any clonal relationship among the isolates, particularly in those that lack any of the four virulent genes. This study was carried out to determine the phenotypic and genotypic diversity of probable EHEC O157 isolates and to estimate their prevalence in the cattle in dairy farms in Chittagong and also discriminate phenotypically the antimicrobial-resistant diversity of the probable EHEC O157 strains isolated on CT-SMAC from dairy cattle in the farms of Chittagong.

METHODOLOGY

Study population and collection of fecal samples

A cross sectional survey was undertaken to isolate probable EHEC O157 and its prevalence of harboring in cattle in dairy farms of Chittagong, between June-2011 to June-2012, in the randomly selected dairy farms in Chittagong district. In total, 21 farms and 419 cattle were sampled and Fecal material was collected from RAJ of each animal by sterile swab that was placed in a tube (5ml) containing buffered peptone water (Oxoid, Basingstoke, Hampshire, UK), and sent to the Microbiology Laboratory, Chittagong Veterinary and Animal Sciences University (ML-CVASU). Additional demographic and epidemiological information of each farm sampled was recorded using a data collection sheet. Global Positioning System (GPS) coordinates were also collected to mark the location of the farms using a personal navigator (eTrex venture, GARMIN, USA)

Bacteriological investigation for EHEC O157:

For bacteriological investigation, each collected sample was streaked onto the selected medium for EHEC O157, cefixime and potassium tellurite added sorbitol MacConkey (CT-SMAC) agar plate, incubated at 37 °C for 18-24 h, and then presence of sorbitol fermenting (colorness) colonies that was slightly transparent, colorless with a weak pale brownish appearance, and with a diameter of 1mm indicates the probability of presence of EHEC O157. (Krishnan et al., 1987; March and Ratnam, 1986; Wells et al., 1983). Five such cross-sectional colonies were picked up and transferred to a 10 ml test tube containing 5 ml of tryptic soy broth (TSB), incubated at 37 °C for 6 h with continuous shaking**.** Any growth in TSB was inoculated onto CT-SMAC and when only homogenous colorless colonies were seen then 5 cross-sectional colonies of them were mixed together again and this mixture was inoculated onto an Eosin Methylene Blue (EMB) (Oxoid, Basingstoke, Hampshire, UK) agar plate, incubated at 37 °C for 24 h to verify whether the bacterial population was *E. coli*, or not. Dyes Eosin Y and Methylene Blue react with products released by *E. coli* from lactose or sucrose as carbon and energy source, forming metallic green sheen. The isolates from CT-MAC through TSB that produced metallic sheen on EMB were considered as probable EHEC O157. These were re-inoculated into TSB, incubated at 37 °C for 20

h and were preserved at -80 °C with 15% glycerin until investigation for more diversity at molecular level.

Testing diversity of the sorbitol non-fermenting *E. coli* isolates

Four selected strains were investigated for the presence of seven housekeeping genes as *adk, fumC, gyrB, icd, mdh, purA* and recA (http://mlst.ucc.ie/mlst/dbs-/Ecoli/documents/primersColi html) to verify whether, by key functional genes, these were truly *E. coli*. The functions of the genes, the sequences of the primers used to their PCR amplifications and the product sizes are shown in Table 1. The selected isolates were shipped to the Department of Veterinary Disease Biology, Copenhagen University (DVDB-CU) to have the PCR assays for the seven genes done. Having found them positive for all these genes, the diversity of the isolates were further characterized by plasmid profiling and pulsed field gel electrophoresis (PFGE), also at DVDB-CU. Plasmid profiling was conducted by alkaline-lysis according to the protocol described by Kado and Liu (1981) with minor modifications. One ml overnight shaking culture grown in LB broth at 37°C was used for plasmid isolation.

The extracted plasmid DNA was subjected to electrophoresis using 0.8% agarose (Sea Kem LE® agarose; Lonza, Rockland, ME USA) gel in Tris-acetate-EDTA (TAE) buffer at 120V for 3 hours at room temperature and subsequently stained with ethidium bromide (10 µg/ml; E1510; Sigma-Aldrich, USA). The gel picture was taken under UV-transillumination using GelDoc EQ system with Quantity One® (Version 4.2.1) software (Bio-Rad Laboratories, Hercules, California, USA).

Table 1
Seven housekeeping gene of *E. coli* along with their PCR primer.

Gene	Phenotypic expression	Primer Sequence(5'– 3')	Annealing temp.	Size of product (bp)
Adk	Adenylate kinase	F'-ATTCTGCTTGGCGCTCCGGG R –CCGTCAACTTTCGCGTATTT	54° C	583
fumC	Fumarate hydratase	F -TCACAGGTCGCCAGCGCTTC R –GTACGCAGCGAAAAAGATTC	54° C	806
gyrB	DNA gyrase	F –TCGGCGACACGGATGACGGC R-ATCAGGCCTTCACGCGCATC	60° C	911
Icd	Isocitrate/isopropy lmalate dehydrogenase	F –ATGGAAAGTAAAGTAGTTGTTCC GGCACA R –GGACGCAGCAGGATCTGTT	54° C	878
Mdh	Malate dehydrogenase	F –ATGAAAGTCGCAGTCCTCGGCGC TGCTGGCGG R-TTAACGAACTCCTGCCCCAGAGCG ATATCTTTCTT	60° C	932
purA	Adenylosuccinate dehydrogenase	F -CGCGCTGATGAAAGAGATGA R –CATACGGTAAGCCACGCAGA	54° C	816
recA	ATP/GTP binding motif	F -CGCATTCGCTTTACCCTGACC R –TCGTCGAAATCTACGGACCGGA	58° C	780

Plasmids in *E. coli* 39R861 (Threlfall et al., 1986) and *E. coli* V517 (Macrina et al., 1978) were used as references for standard plasmid sizes which were estimated by calculating the migration of plasmid mobility relative to that of the reference plasmids (Rochelle et al. 1985). PFGE was performed following the standardized CDC PulseNet protocol (CDC, 2009). Briefly, overnight culture of bacteria grown on brain heart infusion (BHI) broth (CM1135; Oxoid Ltd., England) was used. Genomic DNA was prepared using 1% agarose (SeaKem® gold agarose, Lonza, Rockland, MEUSA). The agarose blocks were placed to lysis buffer (1% sarcosyl, 50 mM Tris, 50 mM EDTA, pH 8.0) with proteinase K (Sigma-Aldrich, USA) solution in a shaking water bath for 3 hours at 55°C. The chromosomal DNA was digested using 60U of the restriction enzyme XbaI (R0145L; New England BioLabs Inc.) for 15 hours at 37°C. The DNA fragments were isolated using 1% SeaKem® gold agarose gel in 0.5x TBE buffer. CHEFF DR III (Bio-Rad Laboratories, Hercules, California, US) system was used to perform electrophoresis at 14°C. Running condition was set as follows – initial switch time 2.2s, final switch time 63.8 sec, current 6V/cm, included angle 120 and run time 19 h. The gel was stained with 1% ethidium bromide (E1510; Sigma-Aldrich, USA) solution for 30 minutes and destined in deionzed water for 3 times with 20 minutes interval. Using UV transillumination, gel image was captured by GelDoc EQ system with Quantity One® (Version 4.2.1) software (Bio-Rad Laboratories, Hercules, California, U.S.) and obtained images were saved in TIFF format in computer. The analysis of the fingerprints was performed using GelCompar®II (version 4.6) software (Applied Maths, Belgium). Dice coefficient with a band position tolerance of 1% and 0.5% optimization level were used to determine similarity between fingerprints. The unweighted pair group method with arithmetic averages (UPGMA) was applied to produce the dendrogram. The DNA restriction patterns of the isolates were interpreted according to the criteria described by Tenovar et al. (1995)

Diversity among the uprobable EHEC O157 isolates based on their antimicrobial susceptibility profiles

All the *E. coli* isolates producing homogenous colorless colonies for the second time on CT-SMAC were investigated for their diversity in antimicrobial susceptibility profiles. Bauer-Kirby disk-diffusion procedure (Bauer et al., 1966) was used on Mueller-Hinton (MH) agar, prepared according to the manufacturer's instructions (Oxoid). A bacterial turbidity equivalent of 0.5 McFarland standards was used for each isolate. A 0.5 McFarland standard was prepared by adding 0.5 ml of 1% (11.75g/L) $BaCl_2.2H_2O$ to 99.5ml of 1% (0.36N) H_2SO_4 (Carter and Cole, 1990).The panel of antibiotics used for the assays along with the sizes of zone of inhibition of them to be considered as "resistant (R)", "intermediately resistant (I)" and "sensitive (S)" against the tested isolates are shown in Table 5. These characterizations were based on the recommendations from Clinical and Laboratory Standards Institute (CLSI, 2007). A sterile swab was dipped into the prepared inoculums, rotated against the side of the tube with firm pressure to remove excess fluid and the dried surface of a MH agar plate was inoculated by streaking the swab three times over the entire agar surface. The plate was rotated approximately 60 degrees each time to ensure an even distribution of the inoculums.

Table **2**
Panel of antibiotics used, their concentrations and Zone diameter interpretative standards for *E. coli* (CLSI, 2007)**.**

Group of Antimicrobial agents	Antimicrobial agents	Disk contents	Zone diameter, nearest whole mm			Manufacturer
			R	I	S	Oxoid Ltd. Basingstoke, Hampshire, England
Penicillin	Ampicillin	10 µg	≤ 13	14-16	≥ 17	
B-lactamase inhibitor combination	Amoxicillin-clavulanic acid	20/10 µg	≤ 13	14-17	≥ 18	
Cephems	Ceftriaxone	30 µg	≤ 13	14-20	≥ 21	

Amino glycosides	Gentamicin	10 µg	≤ 12	13-14	≥ 15
Tetracycline	Tetracycline	30 µg	≤ 11	12-14	≥ 15
	Doxycycline	30 µg	≤ 10	11-13	≥ 14
Fluoroquinolones	Ciprofloxacin	5 µg	≤ 15	16-20	≥ 21
Quinolones	Nalidixic acid	30 µg	≤ 13	14-18	≥ 19
Folate pathway inhibitor	Trimethoprim-sulfomethoxazole	1.25/23.7 µg	≤10	11-15	≥ 16
Phenicoles	Chloramphenicol	30 µg	≤ 12	13-17	≥ 18

Table **3**
On-farm population statistics of cattle and farm status on the days of sampling of animals for the study.

	Median	Mean	Standard deviation	Minimum	Maximum	S. E. of Mean
Milking cow	21	30.85714	31.5187	12	145	6.877945
Dry cow	6	9.47619	9.579243	4	45	2.090362
Heifers	7	11.66667	13.33917	4	56	2.910845
Calves	15	21.14286	16.76092	9	76	3.657533
Bull	0	2.238095	4.83637	0	17	1.055382
Total	50	75.38095	75.23462	30	339	16.41754

The antimicrobial micro-disks were placed on the surface of the inoculated agar using forceps to dispense each antimicrobial disk at a time**.** An antimicrobial disk was carefully placed on the surface with a gentle pressure to make a complete contact and when all the disks were dispensed the agar plate was incubated at 35°C for 16 to 18 hours. After the said period of incubation the size of zone of inhibition around a micro-disk was measured in millimeter with a digital slide calipers and the result was deduced according to CLSI, 2007**.**

Statistical analysis

All data were entered into a spreadsheet programme (Excel 2003, Microsoft Corporation) and transferred to Stata 11.0 (Intercooled Stata 11.0, Stata Corp., College Station, Texas, USA) for analyses. The difference in the occurrence rate of *Stx1/Stx2/hly* gene in the probable EHEC O157 isolates between variables was shown using a Pearsons $\chi 2$ test.

RESULTS AND DISCUSSION

Population statistics of cattle and status of the dairy farms sampled for the investigation

From each of the 21 farms, randomly selected adult cows, heifers, bulls and calves were sampled. Bulls and bull calves were reared for selling or breeding purposes. Table 6 shows their population statistics on the days of sampling. All the farms reported that they sold their non-pasteurized raw milk for home delivery as well as to the sweet shops. All the farms had open drains to dispose of liquid or semi-liquid wastes to the outlets. Overall, the hygienic conditions were poor to satisfactory levels. Slurries were destined for the use in the cultivation of crops. The farm staffs including milkers were unaware of hygienic procedures needed to be applied during milking or milk delivery.

Prevalence of probable EHEC O157 based on the growth on CT-SMAC

From 21 farms, 419 bovine animals sampled, 77 yielded colorless colonies along with other pink-colored colonies on CT-SMAC. When 5 cross-sectional homogenized colorless colonies from each initially positive case were re-verified only 47 produced pure colorless colonies across the CT-SMAC plates and from those 32 of them were found to be *E. coli* based on their characteristic colonial morphology on EMB agar plates.

So, the prevalence of probable EHEC O157 in the study population could be 7.6% (95% CI 4.3–12.7%) (Table 4).

Table **4**.
Prevalence of probable EHEC O157 in dairy farm cattle of Chittagong

No. animals tested	No. (%) probable EHEC O157	(%) probable EHEC O157	95% CI
419	32	7.6%	4.3 – 12.7%

Table **5**
Prevalence of probable EHEC O157 in cattle in dairy farms of Chittagong

Variable	**(N)**	**Prevalence % (No. positive)**	**95% CI**	**P**	**OR (95% CI)**
Age	Calf (110)	10.0 (11)	6.4 – 24.6	0.1854	1.56 (0.73-1.41)
	Heifer(44)	9.0 (4)	0.01 – 14.6	0.8088	1.4 (0.28-3.58)
	Adult (265)	6.4 (17)	0.07 - 11.9		
Sex	Male (27)	7.4 (2)	0.1 – 16.8	0.7719	0.96 (0.15-2.8)
	Female (392)	7.7 (30)	2.4 – 10.6		
Health status	Diseased (15)	0.0 (0)	0.03 – 6.0	0.2595	0.00 (0.0-3.06)
	Healthy (404)	7.9 (32)	1.3 – 22.4		
Suffered Mastitis	Yes (83)	2.4 (2)	0.1 – 9.7	0.0146	0.29 (0.12-0.86)
	No(182)	8.2 (15)	1.1 – 17.8		
Antibiotic used	Yes (74)	5.4 (4)	0.04 – 8.4	0.4059	0.67 (0.2-1.78)
	No (345)	8.1 (28)	2.2 – 18.2		
Total	419	7.6 (39)	5.3 – 9.9	-	-

N = Number of animals sampled; CI = Confidence interval; OR = Odds ratio

Diversity among probable EHEC O157 and their prevalence

Diversity among the probable EHEC isolates and their prevalence among the different categories of sampled populations are shown in table 5.

Diversity among the isolates based on plasmid and PFGE profiling

The four probable EHEC O157 isolates possessed all the seven housekeeping genes - *adk, fumC, gyrB, icd, mdh, purA* and recA, reconfirming their fidelity as *E. coli* at the molecular level. PCR products of four housekeeping genes *gyrb, icd, recA* and *adk* of some of them are shown in Figure 1.

All the four isolates harbored different sized plasmids. The results obtained in PFGE showed that all the four probable EHEC O157 isolates investigated for PFGE were genetically diverse owing to differences in > 9 bands (>3 genes) (Figure 2). Therefore, four different pulsotypes were identified.

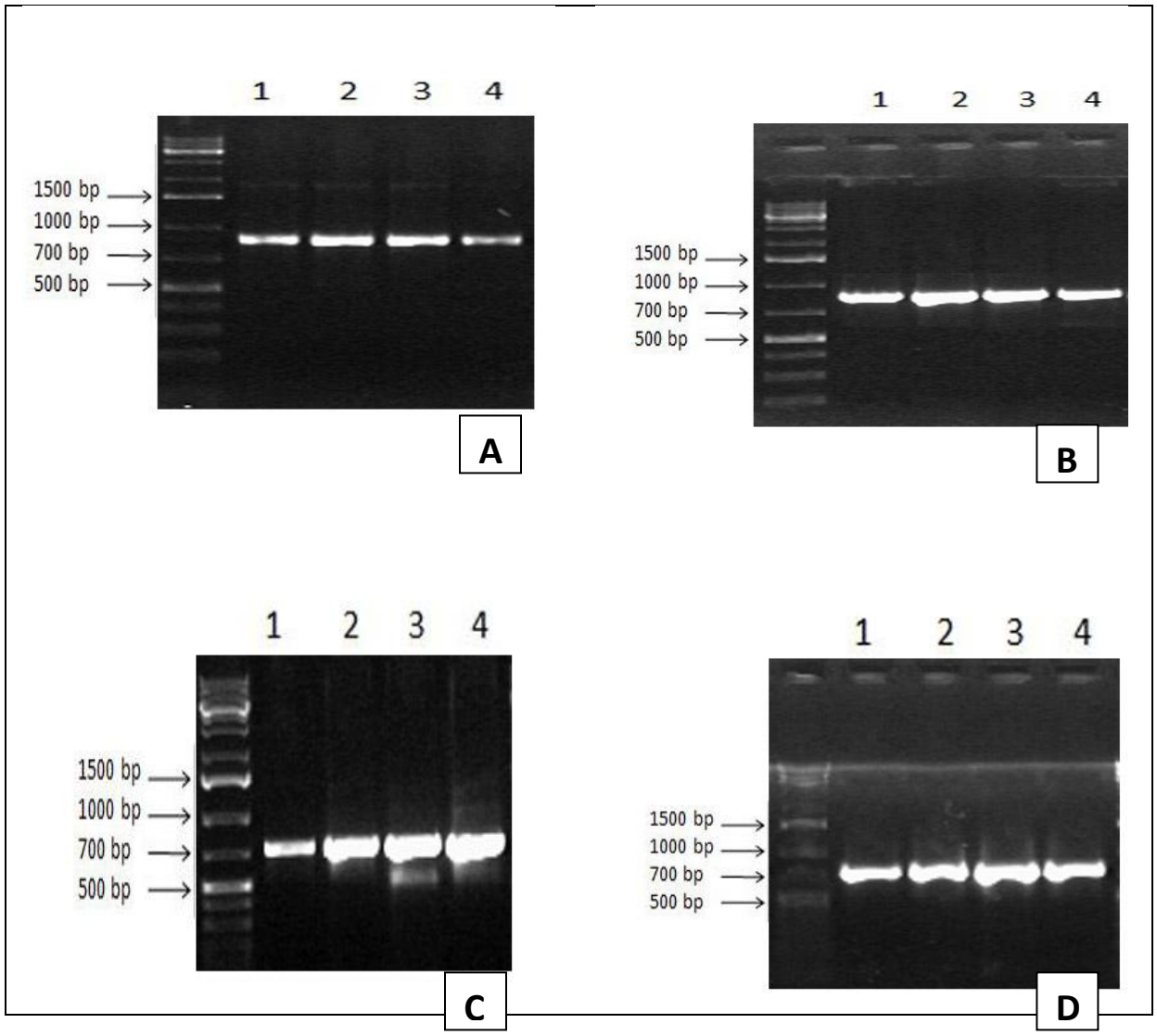

Figure 1
Results of PCR assay for four housekeeping genes of *E. coli***.** (A) Lane 1, 1kb plus DNA ladder, Lanes: 2-7 - 911 bp size PCR products of *gyrb* gene *of* 4 *E.coli* isolates;(B) Lane 1, 1kb plus DNA ladder, Lanes: 2-5 - 878 bp size PCR products of *icd* gene of 4 isolates; (C) Lan 1, 1kb plus DNA ladder, Lanes: 2-5 - 780 bp size PCR products of *recA* gene of 4 isolates; (D) Lane 1, 1kb plus DNA ladder, Lanes: 2-7 - 583 bp size PCR products of *adk* gene of 4 isolates.

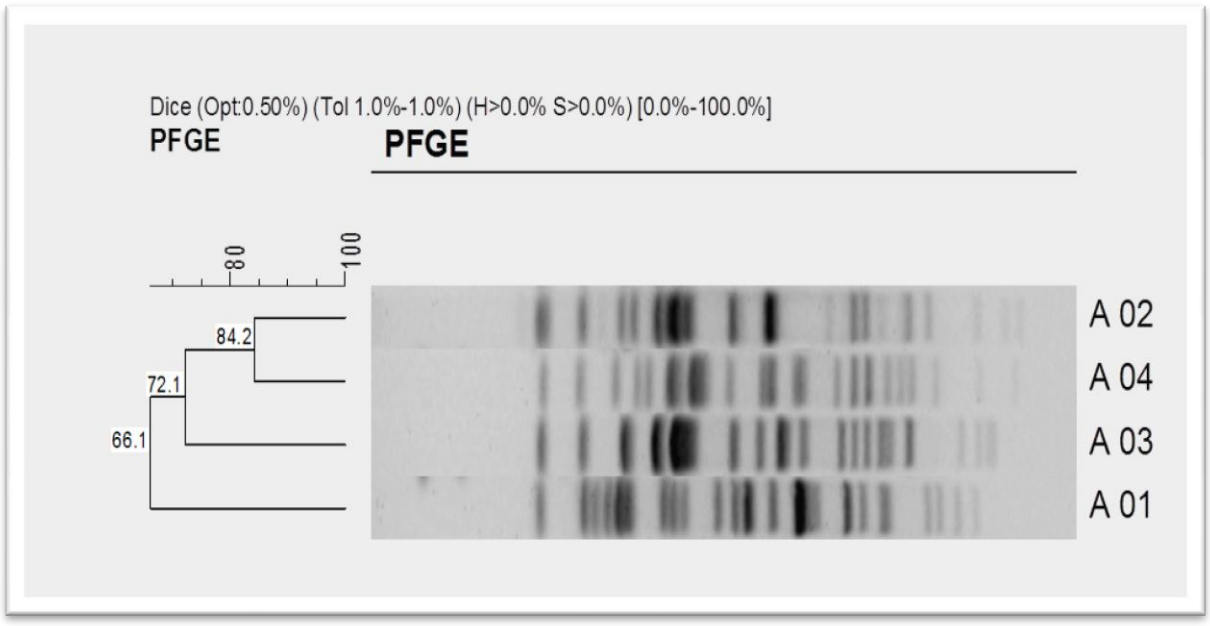

Figure **2**
Dendogram showing the cluster analysis on the basis of XbaI-PFGE of the 4 probable EHEC O157 isolates. Dice coefficient was used to perform similarity analysis, and clustering was performed by using unweighted pair-group method with arithmetic means (UPGMA) with 1% band position tolerance and 0.5% optimization parameter.

Diversity based on antimicrobial susceptibility patterns of the probable EHEC isolates

All the 32 isolates that yielded colorless colonies on CT-SMAC for the second time as well as showed typical colonial morphology in EMB agar were tested for susceptibility to 11 different antimicrobial agents**.** The frequencies of isolates showing sensitive, intermediately resistant and resistant to the antimicrobials tested are shown.

Of the tested isolates 87.2% (95% CI 76.2-98.2%), 79.5% (95% CI 66.2-92.7%) and 69.2% (95% CI 54.1-84.3%) were sensitive to ciprofloxacin, ceftriaxone and chloramphenicle, respectively; 100% isolates were resistant to penicillin, and 38.5% (95% CI 32.3-65.1%), 35.9% (95% CI 20.1-51.7%), 35.9% (95% CI 20.1-51.7%), to trimethoprim-sulfomethoxazole, tetracycline, ampicillin, respectively which were frequently used antibiotics in the dairy farms.

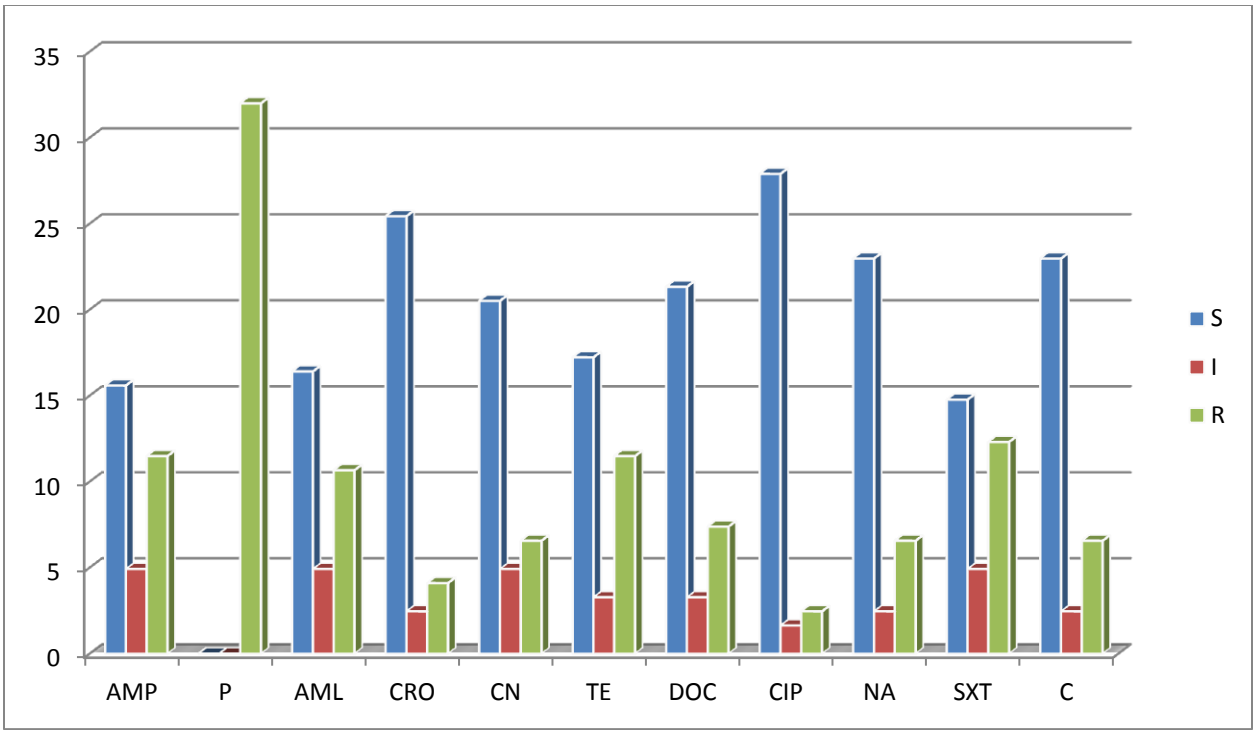

Figure **3**
Results of antimicrobial susceptibility testing; S, I, R = proportional representations of sensitive, intermediately-resistant and resistant isolates, respectively, against the antimicrobials tested; AMP,Ampicillin;P, Penicillin;AML, Amoxycillin;CRO, Ceftriaxone;CN, Gentamycin;TE, Tetracyclin;DOC, Doxicyclin;CIP, Ciprofloxain;NA, Nalidixic Acid;SXT, Sulfomethoxazole-Trimethoprim;C, Chloramphenicol.

There are no common biochemical characteristics associated with the great majority of EHEC serotypes. Only the recognition of the serogroup O157 was facilitated by its inability to ferment sorbitol after overnight incubation but there is always the need of serotype confirmation through other tests. Moreover, over the past decade it has emerged that STEC strains of serotype O157:H-**,** which do ferment sorbitol rapidly, is an important cause of human disease. Thus, as a result of phenotypic variability, there could be an increased likelihood of misdiagnosing O157 infections using only classical methods. Therefore, inspection services would enhance awareness to the possibility of mislaying O157 serogroup if sorbitol fermentation is regarded as a discriminating test for the pathogen identification. Indeed, the emergence of antibiotic-resistant strains is a major therapeutic problem. This phenomenon has been brought about, and extended, by the transmission of resistant isolates, through the mobility of both local and worldwide populations, and by the consumption of foodstuffs derived from animals treated with antimicrobial agents. The present study has revealed some strains to have multiple resistance to different antibiotics.

All the 47 sorbitol non-fermenting isolates, as evidenced with the production of colorless colonies on CT-SMAC (a selective medium recommended for EHEC O157) may probably be not EHEC O157. It can only be confirmed based on the possession of ≥ 1 of the three virulent genes – *Stx1*, *Stx2* and *hly*. The identities of those 32 (68%) bacterial isolates that yielded colorless colonies on CT-SMAC and showing typical *E. coli* colonial morphology on EMB need to be investigated further; however based on just seeing some colorless colonies on a CT-SMAC agar plate the growth of a probable EHEC O157 strain could be warranted, but should not be confirmed unless other confirmatory tests are applied, such as

identification of the presence of at least one virulent gene by a PCR assay.

PFGE and plasmid assays of the 4 *E. coli* isolates showed that they were also diverse, and as many as 4 pulsotypes existed in them. EHEC O157 starins harbours virulent genes named "*stx"*and "*eae*". Because, the presences of these genes were not investigated in this study, it cannot entirely be confirmed that the isolates belonged to EHEC O157 serogroup.

Another important highlight of the present study was that it provided an illustration on the probable prevalence of EHEC O157 in dairy farm cattle in Chittagong, Bangladesh. In this study the term "probable EHEC O157" was used to estimate the prevalence of this group in the study population because the study missed to include the investigation of the fourth virulence genes–*stx* and *eae* because of time and resource limitations.

There is a little information on the isolation and characterization of EHEC O157 strains in dairy farm cattle in Bangladesh (Arifeen et al., 2005), but there is published report on the pathogens from children with diarrhea (Islam et al., 2007). Reports of prevalence and persistency of EHEC O157 in farmed dairy cattle have probably not been published before of this study.

The prevalence that we have found in the dairy cattle was 7.6% (95% CI 4.3 – 12.7%). The detection rates of EHEC O157 were variable among countries. In general, prevalence rates ranged from 0.3 to 19.7% in feedlot cattle, 0.7 to 27.3% in cattle on irrigated pasture, and 0.9 to 6.9% in cattle grazing rangeland forages (Hussein and Bollinger, 2005).

This strain exists in freshly deficated cowdung, compost and soil near cow shed where it is higher in composts and soils compared to fresh cowdung (Hossain et al., 2015).

The prevalence of EHEC O157 was 3.3% in bovine feces in Thailand and 8.7% in Spain (Orden et al., 2002). The differences in the detection among these studies are probably due to the fact that the patterns of shedding rates might be affected by diet, age, environmental condition, and seasonal variation (Kudva et al., 1997). This study reveals that the proportion of EHEC O157 varies in milking cows, heifers and calves. The rate is slightly high in the heifers and calves comparing to dairy cows which is in agreement with Cobbold and Desmarchelier, 2000, Orden et al., 2002 and Shinagawa et al., 2000. This might be because the dairy cattle are frequently treated with antibiotics for mastitis and other diseases so the bacterial loads are low in those. Also we can find that the isolation rates are low in those cattle suffering from diseases or which have suffered mastitis earlier which illustrates the point of antibiotic use as the dairy farms are more prompt to use antibiotics at the occurrence of any diseases. As we have also found that the prevalence is higher in the non-antibiotic used group compared to the antibiotic used group, this also gives us a little insight into the frequent use of antibiotics in diseases which might not be indicated by the professionals.

In recent years, DNA macro-restriction analysis by PFGE has been used increasingly for the molecular subtyping of a wide range of bacterial and fungal pathogens. Different authors (Heuvelink et al. 1998; Radu et al. 2001) demonstrated that PFGE is a technique of very high discriminating power for STEC isolates, and we found that our stains are diverse genetically on the basis of PFGE band patterns. However, the antimicrobial resistance profile that was observed in the study could not be correlated with specific PFGE subtype because of analyzing only four isolates for PFGE.

Isolates showing resistance to antimicrobials tested were also of diverse nature based on the variable numbers of antimicrobials against which they showed resistance. However, in the present study, it was observed that a high prevalence of resistant isolates to sulfamethoxazole, tetracycline and ampicillin, an agreement with some previous reports (Galland et al., 2001; Meng et al., 1998; Zhao et al., 2001). Because antimicrobial-resistant bacteria from food animals may colonize in humans and most of the farm reported that they sell un-pasteurized raw milk to household delivery which means the contacts with their produce are close and more frequent with consumers could be the mode of transmission. In such cases

antimicrobial resistant strains might have more zoonotic consequences. Most of the farm staff responsible for the operation of milking and milk delivery is not well trained to be aware of the risk of the contamination of the milk or the transmission of the EHEC through milk to consumers. Also the dung is frequently used in the cultivation field which might also be a source for the contamination and transmission of EHCE if they are used in the fields of vegetables which can be consumed without cooking.

CONCLUSION

All the 21 cattle farms investigated for probable EHEC O157 were positive for the organism and the magnitude of colonized animals with it varied from farm to farm. Overall, of the 419 animals sampled, 32 were positive for probable EHEC O157, indicating that its prevalence might be7.6% (95% CI4.3-12.7%). Phenotypic differentiation of probable EHEC O157 from other members of *E. coli* resulted from the RAJ samples was made based on sorbitol non-fermenting property. The strains finally identified as probable EHEC O157 were not verified by the presence of any virulent genes, such as *stx1, stx2, eae* and *HlyA*, but PGFE profiles of the four isolates indicated that each was diverse from one another, although they all had the seven house-keeping genes. Not all the probable EHEC O157 was susceptible to the 11 antimicrobials tested, but most showed diversity in their resistance patterns. Further studies are needed to explore the presence of virulent genes in the probable EHEC strains obtained from this survey, and their potential to cause human infections.

REFERENCES

Alam MK, Akther S, Sarwar, N, Morshed S and Debnath GK (2017). Prevalene and Antimicrobial Succeptibility of Escherichia coli 0157 isolated from raw milk marketed in Chittagong, Bangladesh. Turkish Journal of Agriculture-Food science and Technology, 5(3):214-220.

Bauer AW, Kirby WMM, Sherris JC and Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology, 36: 493-496.

Gyles CL (2007). Shiga toxin-producing *Escherichia coli:* an overview. Journal of Animal Science, 85: E45- E62.

Borczyk AA, Karmali MA, Lior H and Duncan LM (1987). Bovine reservoir for verotoxin-producing *Escherichia coli* O157:H7. *Lancet* 1: 98.

Carter GR and Cole JRJ (1990). Diagnostic Procedure in Veterinary Bacteriology and Mycology, Fifth eition, Academic Press, Inc., Harcourt Brace Jovanovich, Publishers, New York, 484 p.

Centers for Disease Control and Prevention (CDC) (2009). One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, Salmonella serotypes, Shigella sonnei, and Shigella flexneri by Pulsed Field Gel Electrophoresis (PFGE), 5: 1-16.

Clinical Laboratory Standards Institute (CLSI) (2007). Performance standards for antimicrobial susceptibility testing; Seventeenth information Supplement. CLSI document M100-S17. 27: 3

Cobbold R and Desmarchelier P (2000). A longitudinal study of Shiga-toxigenic *Escherichia coli* (STEC) prevalence in three Australian dairy herds. Veterinary Microbiology, 71: 125-137.

Deising AK and Thompson M (2004). Strategies for the detection of *Escherichia coli* O157:H7 in foods, Journal of Applied Microbiology, 96: 419-429

Fairbrother J.M. & Nadeau É., (2006). Escherichia coli : on-farm contamination of animals. Rev. sci. tech. Off. int. Epiz., 2006, 25 (2), 555-569

Galland JC, Hyatt DR, Crupper SS and Acheson DW (2001). Prevalence, antibiotic susceptibility, and diversity of *Escherichia coli* O157:H7 isolates from a longitudinal study of beef cattle feedlots. Applied Environmental Microbiology, 67: 1619–1627.

Heuvelink AE, Van Den Biggelaar FLAM, De Boer E, Herbes RG, Melchers WJG, Huis Intveld JHJ and Monners LAH (1998). Isolation and characterization of verocytotoxins producing Escherichia coli O157 strain from Dutch cattle and sheep. Journal of Clinical Microbiology, 36: 878-882.

Hossain MA, Sultana N and Akter S (2015). Screening of Escherichia coli 0157 isolates of bovine origin. Microbs and Health.,4(1):25-28.

Hussein, H. S. and Bollinger, L. M. 2005. Prevalence of Shiga toxin-producing *Escherichia coli* in beef cattle. Journal of Food Protection, 68: 2224-2241.

Ikeda K, Ida O, Kimoto K, Takatorige T, Nakanishi N and Tatara K (1999). Effect of early fosfomycin treatment on prevention of hemolytic uremicsyndrome accompanying *Escherichia coli* O157:H7 infection. Clinical Nephrology, 52: 357–362.

Ji-Yeon, K, So-Hyun K, Nam-Hoon K, Won-Ki B, Ji-Youn L. et al. (2005). Isolation and identification of *Escherichia coli* O157:H7 using different detection methods and molecular determination

by multiplex PCR and RAPD. Journal of Veterinary Science, 6(1): 7-9

Kado CI and Liu ST (1981). Rapid procedure for detection and isolation of large and small plasmids. Journal of Bacteriology, 145: 1365-1373.

Karmali MA, Mascarenhas M, Shen S, Ziebell K, Johnson S, Reid-Smith R, Isaac- Renton J, Clark C, Rahn K and Kaper JB (2003). Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing Escherichia coli seropathotypes that are linked to epidemic and/or serious disease. Journal of Clinical Microbiology, 41(11): 4930-40.

Kudva IT, Hatfield PG and Hovde CJ (1997). Characterization of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* serotypes isolated from sheep. Journal of Clinical Microbiology, 35: 892-899.

Macrina FL, Kopecko DJ, Jones KR, Ayers DJ and McCowen SM (1978). A multiple plasmid-containing Escherichia coli strain: convenient source of size reference plasmids molecules. Plasmid, 1: 417-420.

Meng J, Zhao S, Doyle MP and Joseph SW (1998). Antibiotic resistance of *Escherichia coli* O157:H7 and O157: NM from animals, food, and humans. Journal of Food Protection, 61: 1511–1514.

Nataro JP and Kaper JB (1998). Diarrheagenic *Escherichia coli*. Clinical Microbiology Review, 11: 142–201.

Naylor SW, Low JC, Besser TE, Mahajan A, Gunn GJ, Pearce MC, McKendrick IJ, Smith, DG and Gally DL (2003). Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. Infection and Immunology, 71(3): 1505-12.

Orden JA, Cid D, Ruiz-Santa-Quiteria JA, Garcia S, Martinez S and de la Fuente R (2002). Verotoxin-producing *Escherichia coli* (VTEC), enteropathogenic *E. coli* (EPEC) and necrotoxigenic *E. coli* (NTEC) isolated from healthy cattle in Spain. Journal of Applied Microbiology, 93: 29-35.

Osek J and Gallien P (2002). Molecular analysis of *Escherichia coli* O157 strains isolated from cattle and pigs by the use of PCR and pulsed-field gel electrophoresis methods, Veterinary Medicine of Czech, *47: 149-158*

Radu S, Wai Ling O, Karim MIA and Nishibuchi M (2001). Detection of Escherichia coli O157 by Multiplex PCR and Their Characterization by Plasmid Profiling, Antimicrobial Resistance, RAPD and PFGE Analyses. Journal of Microbiology Methods, 46, 131–139.

Rochelle PA, Fry JC, Day MJ and Bale MJ (1985). An accurate method for estimating sizes of small and large plasmids and DNA fragments by gel electrophoresis. Journal of Genetic Microbiology, 132: 53-59.

Shinagawa K, Kanehira M, Omoe K, Matsuda I, Hu D, Widiasih DA and Sugii S (2000). Frequency of Shiga toxin-producing *Escherichia coli* in cattle at a breeding farm and at a slaughterhouse in Japan. Veterinary Microbiology, 76: 305-309.

Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH and Swaminathan B (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. Journal of Clinical Microbiology, 33(9): 2233–2239.

Threlfall EJ, Rowe B, Ferguson JL and Ward LR (1986). Characterization of plasmids conferring resistance to gentamicin and apramycin in strains of Salmonella typhimurium phage type 204c isolated in Britain. Journal of Hygiene, (Lond), 97: 419-426.

Wong CS, Jelacic S, Habeeb RL, Watkins SL and Tarr PI (2000). The risk of hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. North England Journal of Medicine, 342: 1930–1936.

Zhang X, McDaniel AD, Wolf LE, Keusch GT, Waldor MK and Acheson DW (2000). Quinolone antibiotics induce Shiga toxin-encodingbacteriophages, toxin production, and death in mice. Journal of Infectious Disease, 181: 664–670.

Zhao S, White DG, Ge, D, Ayers S, Friedman S, English L, Wagner D, Gaines S and Meng J (2001). Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. Applied Environment and Microbiology, 67: 1558–1564.