Project ID 362

Competitive Research Grant

Sub-Project Completion Report

on

Prevalence and molecular characterization of shiga toxin producing *Escherichia coli* (STEC) in poultry and their products in Bangladesh

Project Duration

May2017 toSeptember 2018

Department of Microbiology & Hygiene
Bangladesh Agricultural University
Mymensingh

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
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Acronyms

%	:	Percentage
µl	:	Microliter
µg	:	Microgram
bp	:	Base pair
°C	:	Degree celcius
API	:	Analytical profile index
Co-PI	:	Co-Principal investigator
CLSI	:	Clinical and Laboratory Standard Institute
DNA	:	Deoxyribo nucleic acid
E.coli	:	*Escherichia coli*
EMB	:	Eosine Methylene Blue Agar
F	:	Forward
Fig.	:	Figure
MR	:	Methyl Red
ml	:	Milliliter
PCR	:	Polymerase chain reaction
PI	:	Principal investigator
R	:	Reverse
rpm	:	rotation per minute
STEC	:	Shiga toxin producing *E.coli*
Stx	:	Shiga toxin
TAE	:	Tris Acetate EDTA
USA	:	United States of America
VP	:	Voges-Proskauer

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Executive Summary

Shiga toxin producing *Escherichia coli* (STEC) is an enterotoxigenic diarrheal pathogen causing watery to bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome in humans. This study was designed to determine the shiga toxin producing genes and different serogroups *of E. coli* and to investigate antibiotic sensitivity or resistant patterns of the *E. coli* isolated from the healthy broiler, healthy layer, indigenous birds, egg washings and poultry meat samples collected from the selected live bird markets of Gazipur, Dhaka, Mymensingh and Bogura. A total of 300 samples comprising of 180 cloacal swabs, 80 egg washings and 80 poultry meat samples were collected and screened for the detection of *E. coli* on the basis of cultural, staining and biochemical properties followed by molecular characterization using genus specific 16S rRNA primers by Polymerase Chain Reaction (PCR). Out of 300 samples 159 were positive for *E. coli*. In Gazipur district, the prevalence of *E. coli* in healthy broiler, layer, indigenous birds, egg washings and poultry meat samples were 0%, 80%, 70%, 40% and 40% respectively. The prevalence of *E. coli* in healthy broiler, layer, indigenous birds, egg washings and poultry meat samples in Dhaka district were 60%, 60%, 60%, 40% and 40% respectively. In Mymensingh district, the prevalence of *E. coli* in healthy broiler, layer, indigenous birds, egg washings and poultry meat samples were 60%, 70%, 60%, 65% and 35% respectively. The overall prevalence of *E. coli* in Gazipur, Dhaka Mymensingh and Bogura districts were 46%, 52%, 58% and 52% respectively. Again, those 159 positive *E. coli* samples were analyzed for the presence of shiga toxin genes *Stx*1*, Stx*2*, rfb,* O157:H7, O111, O26 and O45 by PCR. From the results, only 34 (21.38%) isolates were confirmed to be positive for the presence of *Stx*1 gene and 9 (5.66%) were found positive for the *Stx*2 gene. Area wise prevalence of *Stx*1 gene was 13.04%, 19.24%, 20.69% and 26.93% in Gazipur, Dhaka, Mymensingh and Bogura districts respectively. The prevalence of *Stx*2 genes were 8.63% and 7.7% in Mymensingh and Bogura districts respectively. *Stx*2 gene was not detected in Gazipur and Dhaka districts. The overall prevalence of *E. coli* 0157:H7, O111, O26, O45 and O111 were 5.66%, 8.81%, 6.92% and 3.14% respectively. Area wise prevalence of *E. coli* O157:H7 were 17.39%, 7.69%, 3.45% and 1.93% in Gazipur, Dhaka, Mymensingh and Bogura districts respectively. On the contrary prevalence of O26 serogroup was 0%, 3.85%, 13.8% and 9.62% in Gazipur, Dhaka, Mymensingh and Bogura districts respectively. Serogroups O45 and O111 were not detected in Gazipur and Dhaka districts. But the prevalence of O45 was 8.63%, 11.5% and O111 was 3.45% and 5.77% in Mymensingh and Bogura districts respectively. Antimicrobial sensitivity and resistant pattern of the Shiga toxin (STX) producing *E. coli* was determined against 8 antimicrobial agents by disc diffusion method. Shiga toxins producing *E. coli* were found sensitive to Norfloxacin (74%), Azithromycin (62%) and Amikacin (56%) while the organism was found 100% resistant to Cephalexin, Ciprofloxacin and Colistin followed by Erythromycin (88%) and Neomycin sulphate (85%). Poultry meat and eggs were found to be contaminated with multidrug resistant STX producing *E. coli* which may cause public health hazard. These results underscore the need of implementation of good biosecurity practices in poultry production chain.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project:**
   Prevalence and molecular characterization of shiga toxin producing *Escherichia coli* (STEC) in poultry and their products in Bangladesh

2. **Implementing organization:**
   Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**
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4. **Sub-project budget (Tk):**
   a. Total: Tk. 21, 81, 753 BDT
   b. Revised (if any):

5. **Duration of the sub-project:**
   a. Start date (based on LoA signed) : May 2017
   b. End date : 30 September 2018

6. **Justification of undertaking the sub-project**:

Shiga toxin–producing *Escherichia coli* (STEC) have emerged as important enteric food borne zoonotic pathogens of considerable public health significance (Brooks *et al.*, 2005). STEC comprises of a diverse group that elaborate one or both Shiga toxins (Stx1 and Stx2) and can cause diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in human beings (Gyles, 2007). Poultry can harbor STEC and intimin producing (eae) *E. coli*. The STEC strains are classified into more than two hundred O serotypes. However majority of outbreak of hemorrhagic colitis and hemolytic uremic syndrome in humans have been caused by O26, O45, O111, and O157. Meat obtained from poultry contaminated with STEC has the potential to cause infections in human beings and while most of these *E. coli* infections caused by *E. coli* O157:H7, 20–70% of STEC infections throughout the world are attributed to non-O157 STEC (Brooks *et al.,* 2005). Occurrence of STEC has been investigated in livestock such as cattle, buffaloes and goats (Islam *et al.,* 2008), Human patients (Islam *et al.,* 2007), raw meat, milk and Juice samples (Islam *et al.*, 2010) in Bangladesh. However there is no detailed study regarding the prevalence of STEC in poultry and their products in Bangladesh. Hence we are particularly interested in estimating the prevalence of STEC in broiler, layer and indigenous chicken and their products to assess public health risk.

7. **Sub-project goal:**

i. Assessment of the role of chickens as reservoirs of shiga toxin (Stx) and intimin (eae) producing *E. coli*.
ii. Determination of the potential public health risks of STEC strains originated from poultry and their products in Bangladesh.

8. **Sub-project objective (s):**

i) Identification and serogrouping of *E. coli* isolated from live chicken, poultry meat and egg washings.
ii) Molecular characterization of shiga toxin producing genes (Stx1 and Stx2) of *E. coli* by polymerase chain reaction and sequence analysis of the amplified genes.
iii) Determination of antibiotic sensitivity profile of the STEC isolates.

9. **Implementing location (s):**

Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

10. Methodology in brief:

10.1 Isolation, Identification and Serogrouping of the agent:

Collection of samples

A total of 300 samples comprising of Cloacal swab (n=180), egg washings (n=60) and meat (n=60) samples of layers, broilers and indigenous chickens were collected from major live bird markets located at Gazipur (Mawna Chowrasta Bazar), Dhaka (Mohakhali Kacha Bazar), Mymensingh (Mechhowa Bazar and Chorpara Kacha Bazar) and Bogura (Fateh Ali Bazar) districts. The samples were carried in an ice box (4°C) and taken to the laboratory for processing. All works were performed in the Dept. of Microbiology and Hygiene, BAU, Mymensingh.

Isolation of bacteria in pure culture

Samples were enriched in nutrient broth at 37°C for 24 hours. The overnight bacterial broths were streaked onto EMB and incubated at 37°C for 24 hours. Single colony was further sub-cultured until a pure culture obtained.

Identification of bacteria

Colony characteristics of bacteria such as: shape, size, surface texture, edge, elevation and colour observed in pure culture, Gram's staining and biochemical tests (Sugar fermentation, Methyl red, Voges-Proskauer and Indole production tests) were used for identification of bacteria (Cheesbrough, 1985). Biochemically identified *E. coli* were confirmed by commercial API Kit.

10.2 Molecular characterization of *E. coli*:

Preparation of *E. coli* DNA for PCR assay

Bacterial DNA was extracted using Wizard® Genomic DNA purification kit, (Promega, USA). The extraction procedure was performed according to the protocol provided with the kit.

Detection of Stx1 and Stx2 genes by PCR

PCR were carried out using 2 sets of oligonucleotide primers for Stx1 and Stx2 (Table 1). The PCR mixture of 25.0µl contained 1×PCR buffer, 1.5 mM of $MgCl_2$ and each of the primers from the 2 primer sets at a concentration of 40 nM, 200 nM each of dNTPs, 1.0 U of *Taq* DNA polymerase and 2.0 µl of template DNA. The PCR reaction was performed in a thermal cycler (Thermo Electron, Germany) using the following standard cycling procedure: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 sec, primer annealing at 59°C for 45 sec and extension at 72°C for 1 min and a final extension at 72°C for 6 min. Amplified products were separated by agarose gel (2% agarose in 1XTris-borate-EDTA buffer) electrophoresis at 5v/cm for 2 h and stained with ethidium bromide (0.5 µg/ml). Standard molecular size marker (100 bp DNA ladder) was included in each gel. DNA fragments were observed by ultraviolet transilluminator and photographed in a gel documentation system (Alpha Imager, Germany). Oligonucleotide primers for amplification of O157: H7, O111, O26 and O45 genes of *E. coli* are shown in (Table 2).

Table 1. Oligonucleotide primers for amplification of Stx genes of *E. coli*

Target genes	Nucleotide sequence (5′-3′)	Product size (bp)	Reference
Stx1	CACAATCAGGCGTCGCCAGCGCACTTGCT (forward)	606	Talukdar *et al.,* 2013
	TGTTGCAGGGATCAGTCGTACGGGGATGC (reverse)		
Stx2	CCACATCGGTGTCTGTTATTAACCACACC (forward)	372	
	GCAGAACTGCTCTGGATGCATCTCTTGGTC (reverse)		

Identification of different serogroups of *E. coli* by PCR

PCR products were purified using rapid DNA purification kit (Bioneer DNA purification kit, Korea) using manufacturer's protocol. Amplified PCR products were sent to the commercial company for sequence analysis.

Table 2. Oligonucleotide primers for amplification of O157: H7, O111, O26 and O45 genes of *E. coli*

Target genes	Nucleotide sequence (5′-3′)	Product size (bp)	Reference
rfb	AACGGTTGCTCTTCATTTAG (forward)	678	Possé *et al.,* 2007
	GAGACCATCCAATAAGTGTG (reverse)		
O111	TAGAGAAATTATCAAGTTAGTTCC (forward)	406	Possé *et al.,* 2007
	ATAGTTATGAACATCTTGTTTAGC (reverse)		
O26	AGGGTGCGAATGCCATATT(forward)	417	Bai *et al.,* 2012
	GACATAATGACATACCACGAGCA (reverse)		
O45	GGGCTGTCCAGACAGTTCAT(forward)	890	Bai *et al.,* 2012
	TGTACTGCACCAATGCACCT (reverse)		

10.3 Antibiotic sensitivity test:

All shiga toxin producing *E. coli* isolates were tested for antimicrobial drug susceptibility against eight commonly used antibiotics such as: amikacin (30µg/disc), colistin (10µg/disc**)**, azithromycin (30µg/disc), Norfloxacin (10µg/disc**)**, Erythromycin (15µg/disc), cefalexin (30µg/disc), Neomycin sulphate (30µg/disc) and ciprofloxacin (5µg/disc) by disk diffusion or Kirby-Bauer method **[20].** Results of antibiotic sensitivity

tests were recorded as sensitive and resistant according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2016).

11. Results and discussion:

11.1 Isolation, identification and serogrouping of *E. coli*

Culture on Eosin Methylene Blue (EMB) agar

For presumptive identification of *E. coli*, the selective colonies on MacConkey agar for each sample were sub cultured onto Eosin Methylene Blue (EMB) agar. All suspected *E. coli* isolates produced greenish-black colonies with metallic sheen on EMB agar (Figure 1). For isolation of *E. coli,* selective culture media was used simultaneously in this study. The media used in this study was selected considering the experience of the past research works in various fields relevant to the present study by Islam *et al.* 2015; Pramanik, 2017. Colony growth character on different media exhibited characteristics reaction. In this study, colony characteristics of *E. coli* observed on EMB agar media were similar to the findings of other authors (Islam *et al.* 2015; Zakaria *et al.,* 2012; Mamun *et al*., 2016).

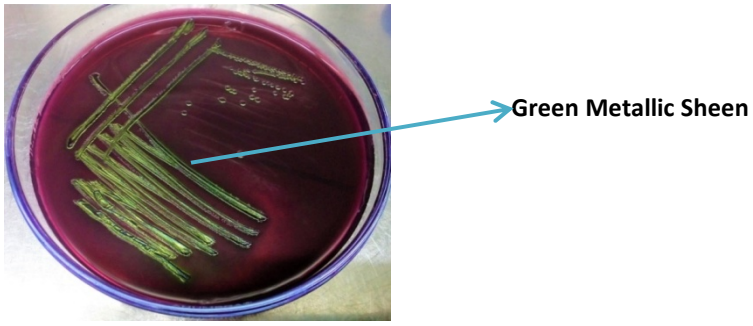

Figure 1: Growth of suspected *E. Coli* on EMB agar showing green metallic sheen colonies

Gram's staining

The pure cultures of suspected *E. coli* isolates subjected to Gram's staining for further identification. In Gram's staining, the organisms were found Gram negative, small rod shaped arranged in single or paired which is the characteristic of *E. coli* (Figure 2). In Gram's staining the morphology of the isolated bacteria exhibited pink, small rod shaped gram negative bacilli. This finding was agreed with Tanzin *et al.* (2016).

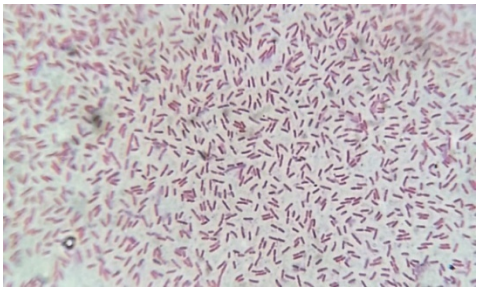

Figure 2: *E. coli* isolates showing gram negative, pink colored, small rod-shaped organisms (X100)

Biochemical tests

To confirm the isolates of *E. coli,* a series of biochemical tests were carried out. Among these, five basic sugar fermentation test, MR, VP, indole and API 20E tests were performed.

Sugar fermentation test

The colonies of suspected *E. coli* isolates were used for sugar fermentation test. All isolates produced both acid and gas by fermentation of sugars indicated by color change and deposition of gas in Durham's tube (Figure 3). Cloacal swab isolates revealed a complete fermentation of basic sugars as stated by Sandhu *et al.* (1996) and Mckec *et al. (*1995).

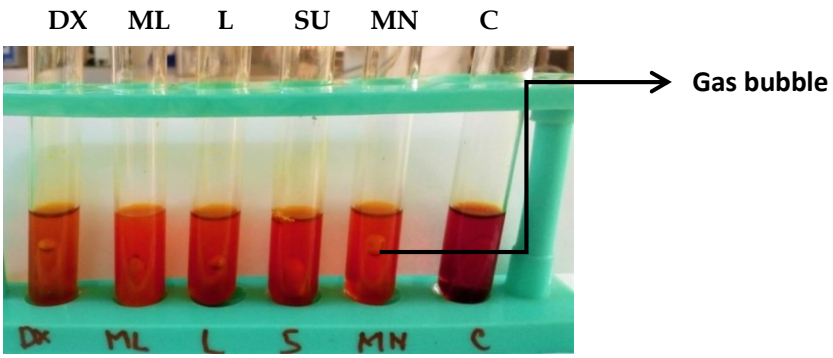

Figure 3: Sugar fermentation tests with five basic sugars showing the formation of acid and gas
DX: Dextrose, ML: Maltose, L: Lactose, S: Sugar, MN: Mannitol and C: Control

Other biochemical tests

All the isolates showed methyl-red (MR) positive, VP negative, indole positive (Figure 4) and were identified as *E. Coli* which was supported by several authors (Zinnah *et al.,* 2007; Momtaz *et al.,* 2012).

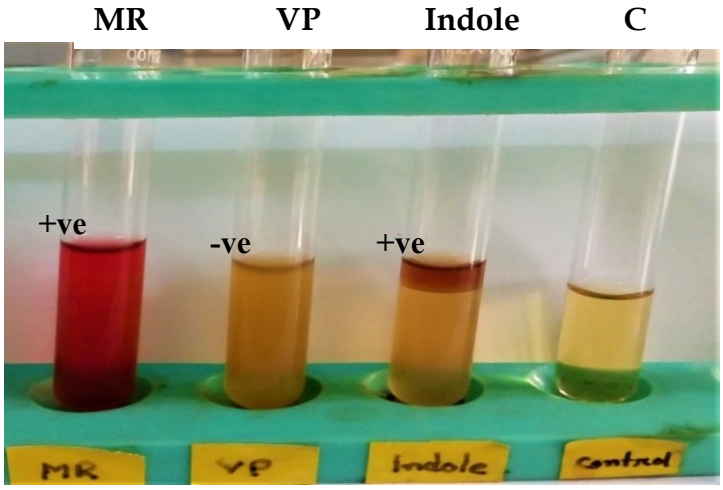

Figure 4: Biochemical tests for Methyl-red, Voges-Proskauer and Indole test
MR: Methyl-red, VP: Voges-Proskauer, C: Control

Catalase test

All isolates of *E. coli* showed production of oxygen bubbles indicative of positive result. The positive control also produced bubble whereas the negative control did not produce bubble. The catalase test was done by slide method (Figure 5).

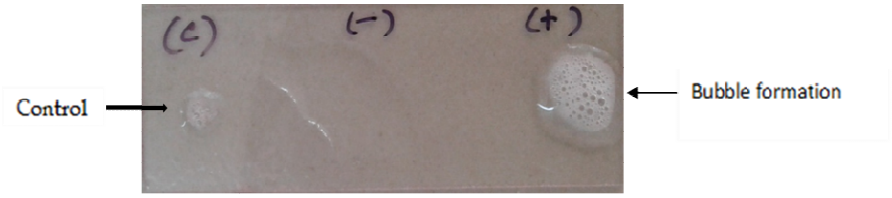

Figure 5: Catalase test for *E. coli*

Positive results indicated by formation of bubble due to release of hydrogen peroxide

Table 3. The summary of biochemical test results

MR	VP	Indole	Catalase	Sugar test					Interpretation
				Dextrose	Sucrose	Lactose	Maltose	Mannitol	
+	-	+	+	+	+	+	+	+	The organisms are *E. coli*

Results of API 20E test

After incubating the tray at 37°C for 18 to 24 hours, some of the compartments showed change in color straightway after 24 hours but for some we had to put reagents before reading. After addition of reagents the TDA, Indole took pink color indicating positive result and VP remained colorless which resembles negative results. Then marking was applied for all individual results which later matched with API (Analytical Profile Index) logbook or apiweb for identification (Figure 6).

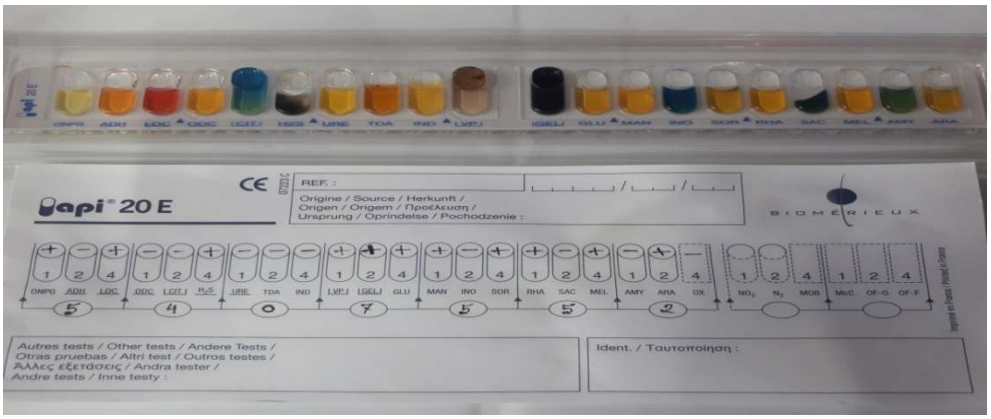

Figure 6: Result and Interpretation of API 20E test

Basic sugars took yellow color and MR, VP & Indole took light to dark pink color indicating positive result for E. coli

11.2 Molecular characterization of *E. coli*:

Prevalence of *E. coli* with their virulent genes

The virulence of STEC is known to mediate through *Stx* and *eae* or intimin (Barret *et al.,* 1992). Poultry can harbor STECs and intimin producing *E. coli* (Beutin *et al.,* 2004). Out of 300 samples, 159 isolates were found to be positive for *16S rRNA* genes. So, overall prevalence of *E. coli* was 53%. Prevalence of *E.*

coli on the basis of culture positive and *16S rRNA* genes positive are presented in a tabular form below (Table 4-10). This study revealed the 55% prevalence of *E. coli* which is higher to that of the findings of Majumder *et al.* (2017) who reported 43.33% prevalence of *E. coli in* cloacal swab samples. Homaira *et al.* (2015) found 54% prevalence of *E. coli* in cloacal swab, feed and water samples. In Bangladesh, slaughter animals like buffalo, cows and goats are known to carry STEC including strain *O157* (Islam *et al*. 2008). Mamun *et al.* (2016) determined prevalence of Shigatoxin producing *E. coli* in the cloacal swab of broiler chicken only. They recorded 10.20% isolates of *E. coli* positive for *stx*1 gene and 53.06% positive for *stx2* gene. Occurrence of Shiga toxin producing *E. coli* was also investigated in raw meat, raw milk and street vended juices in Bangladesh by Islam *et al*. (2010). Majumder *et al.* (2017) conducted a study where out of 60 samples, 26 (43.33%) were confirmed to be *E. coli* positive and among the *E. coli* positive samples, 12 (46.15%) samples were found positive for *Stx*-1 and 11 for *Stx*-2.

Table 4. Prevalence of *E. coli* in Gazipur district of Bangladesh

Types of samples	Total samples examined	Samples positive for *E. coli* by culture and PCR	Prevalence (%) of *E. coli*
Healthy Broiler	10	0	0
Healthy Layer	10	8	80
Indigenous birds	10	7	70
Egg washing	10	4	40
Poultry meat	10	4	40
Total	50	23	46

Table 5. Prevalence of *E. coli* in Dhaka district of Bangladesh

Types of samples	Total samples examined	Samples positive for *E. coli* by culture and PCR	Prevalence (%) of *E. coli*
Healthy Broiler	10	6	60
Healthy Layer	10	6	60
Indigenous birds	10	6	60
Egg washing	10	4	40
Poultry meat	10	4	40
Total	50	26	52

Table 6. Prevalence of *E. coli* in Mymensingh district of Bangladesh

Types of samples	Total samples examined	Samples positive for *E. coli* by culture and PCR	Prevalence (%) of *E. coli*
Healthy Broiler	20	12	60
Healthy Layer	20	14	70
Indigenous birds	20	12	60
Egg washing	20	13	65
Poultry meat	20	7	35
Total	100	58	58

Table 7. Prevalence of *E. coli* in Bogura district of Bangladesh

Types of samples	Total samples examined	Samples positive for *E. coli* by culture and PCR	Prevalence (%) of *E. coli*
Healthy Broiler	20	10	50
Healthy Layer	20	11	55
Indigenous birds	20	13	65
Egg washing	20	10	50
Poultry meat	20	8	40
Total	100	52	52

Table 8. Prevalence of *E. coli* according to types of samples

Types of samples	Total samples examined	Samples positive for E. coli by culture and PCR	Prevalence (%) of E. coli
Healthy Broiler	60	28	47
Healthy Layer	60	39	65
Indigenous birds	60	38	63
Egg washing	60	31	52
Poultry meat	60	23	38

Table 9. Prevalence of 16S rRNA, Stx-1 gene and Stx-2 gene in the areas studied

Areas of sample collection	No. of total samples	No. of *E. coli* culture positive and *16S rRNA* positive samples and prevalence (%)	No. of positive samples & prevalence (%) among isolates	
			Stx1	*Stx2*
Mawna Chowrasta Bazar, Mawna, Gazipur	50	23 (46)	3 (13.04)	0
Mohakhali Kacha Bazar, Dhaka	50	26 (52)	5 (19.23)	0
Mechhowa Bazar and Charpara Kacha Bazar, Mymensingh	100	58 (58)	12 (20.69)	5 (8.63)
Fateh Ali Bazar, Bogura	100	52 (52)	14 (26.93)	4 (7.7)
Total	300	159 (53)	34 (21.38)	9 (5.66)

Table 10. Prevalence of *E. coli* O157:H7, O26, O45 and O111 genes in the study areas

Areas of sample collection	No. of total samples	No. of *E. coli* culture positive and *16S rRNA* positive samples and prevalence (%)	No. of positive samples & prevalence (%) among isolates			
			Rfb O157:H7	***O26***	***O45***	***O111***
Mawna Chowrasta Bazar, Mawna, Gazipur	50	23 (46)	4 (17.39)	0	0	0
Mohakhali Kacha Bazar, Dhaka	50	26 (52)	2 (7.69)	1 (3.85)	0	0
Mechhowa Bazar and Charpara Kacha Bazar, Mymensingh	100	58 (58)	2 (3.45)	8 (13.8%)	5 (8.63)	2 (3.45)
Fateh Ali Bazar, Bogura	100	52 (52)	1 (1.93)	5 (9.62)	6 (11.5)	3 (5.77)
Total	300	159 (53)	9 (5.66)	14(8.81)	11(6.92)	5 (3.14)

Polymerase Chain Reaction (PCR) for confirmation of *E. coli*

All suspected *E. coli* isolates were confirmed by Polymerase chain reaction using genus specific *16S rRNA* primers. A total of 159 isolates out of 300 were confirmed as *E. coli* by amplifying genus specific *16S rRNA* primers (Figure 7).

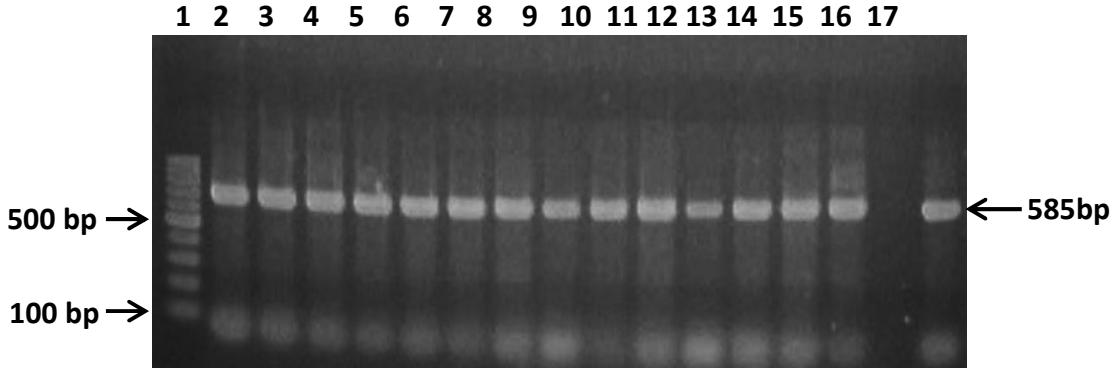

Figure 7: PCR amplification of *E. coli 16s rRNA* gene (585 bp)

Lane 1: 100 bp DNA marker; Lane 2 to 15: DNA of 16s rRNA positive E. coli isolates; Lane 16: Negative control; Lane 17: Positive control.

Detection of toxigenic genes in *E. coli* by PCR

To identify shigatoxin producing *E. coli* at genomic level a uniplex PCR was also performed using *Stx*-1 and *Stx*-2 gene specific primers. Out of 159 isolates of *E. coli* only 34 samples isolated from healthy broiler and healthy layer, indigenous birds, egg washing and poultry meat were found positive for *stx-1* gene and 9 samples were found stx-2 positive. This study reported 35 (31.82%) of STEC. Shigatoxigenic *E. coli* serotype *O157* has the highest prevalence in the world in recent years. For example, it has been reported that 6,658 of 13,524 STEC sourced infections in Europe were caused by the *O157* serotype of *E. coli* between 2007 and 2010 (Messens *et al.,* 2015). The result of *stx-1* and *stx-2* PCR are presented in (Figure 8 and 9).

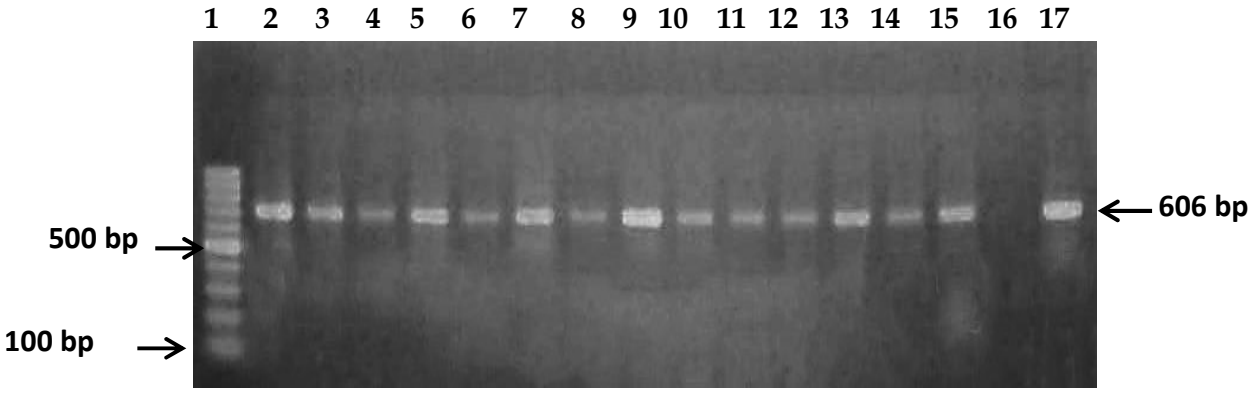

Figure 8: PCR amplification of *E. coli Stx-1* gene (606 bp)
Lane 1: 100 bp DNA marker; Lane 2 to 15: DNA of Stx-1 positive sample; Lane 16: Negative control; Lane 17: Positive control

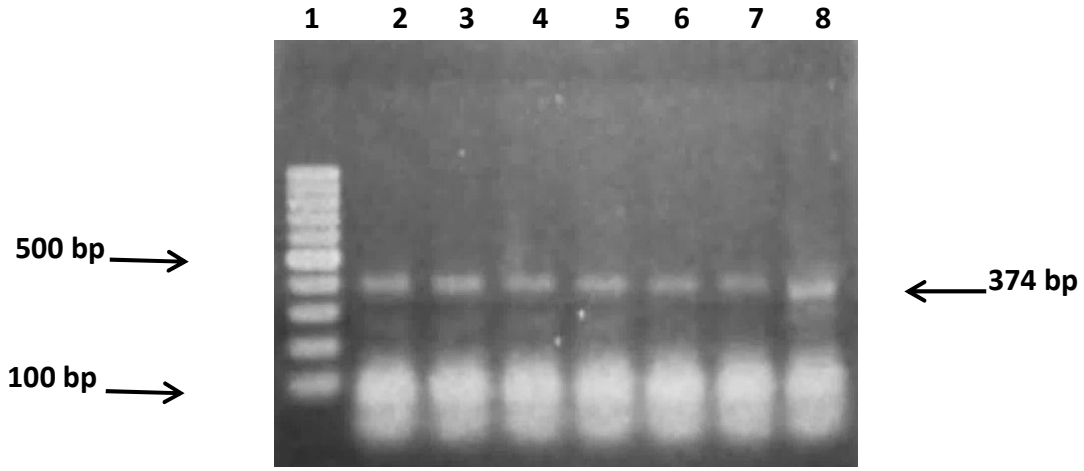

Figure 9: PCR amplification of *E. coli Stx-2* gene (374 bp)
Lane 1: 100 bp DNA marker; Lane 2 to 8: DNA of Stx-2 positive samples

Detection of serotype of *E. coli* by PCR

All the isolates of *E. coli* were screened for detection of *O157:H7, O26, O45* and *O111* serotype of *E. coli.* Among the isolates 9 were found positive for *O157:H7*, 14 isolates were *O26* positive, 11 isolates were found positive for O45 and 5 isolates were found positive for O111. The results of *O157:H7, O26, O45* and *O111* serotype PCR are presented in (Figure 10, 11, 12 and 13) respectively. In this study 3 (2.73%) of *rfb O157:H7*, 13 (11.82%) of *O26,* 11 (10%) *O45* and 5 (4.55%) *O111* were also found positive. Although slight variations in prevalence rate of *E. coli* in this study were found with some other studies but these may be due to their pattern of study.

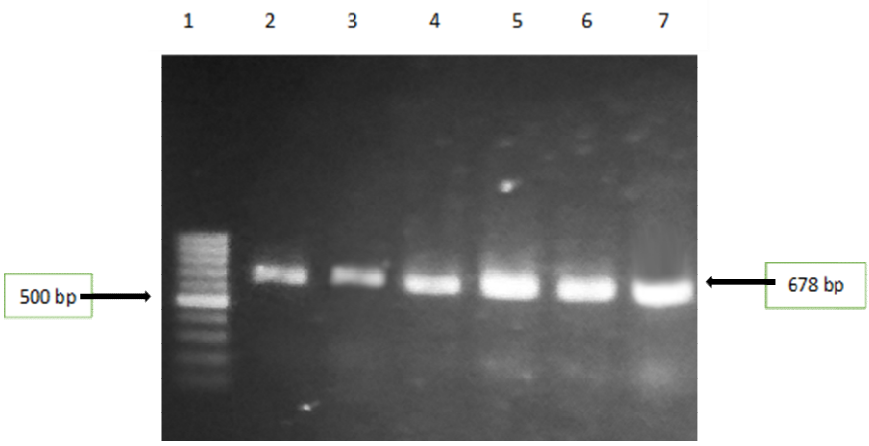

Figure 10: PCR for amplification of *rfb* O157: H7 gene (678 bp) and of *E. coli* of chicken
Lane 1: 100bp DNA ladder, Lane 2-7: DNA of rfb positive samples

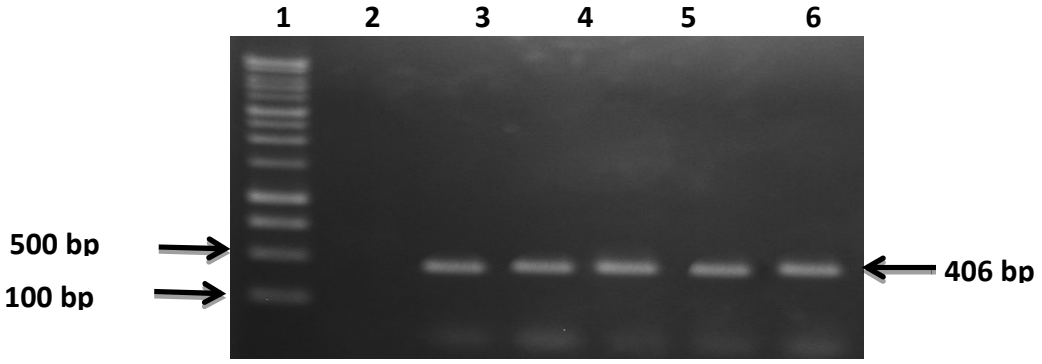

Figure 11: PCR amplification for *E. coli* O111 gene (406 bp)
Lane 1: 100 bp DNA marker; Lane 2: Negative control; Lane 3 to 7: DNA of O111 positive sample

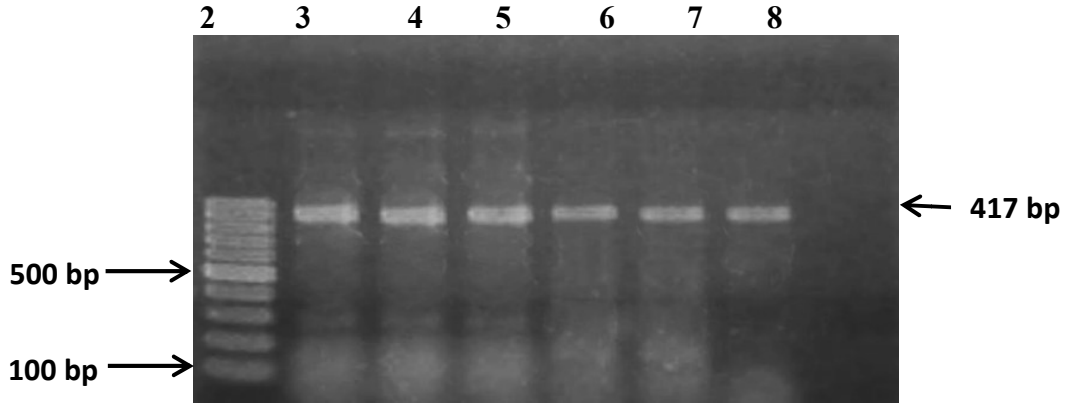

Figure 12: PCR amplification of *E. coli* O26 gene (417 bp)
Lane 1: 100 bp DNA marker; Lane 2 to 7: DNA of O26 positive sample; Lane 8: Negative control

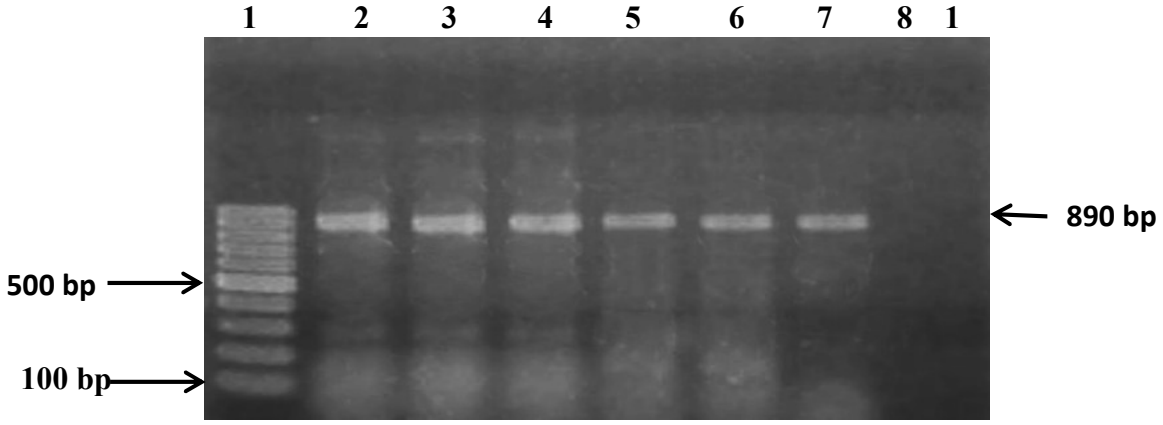

Figure 13: PCR amplification of *E. coli* O45 gene (890 bp)
Lane 1: 100 bp DNA marker; Lane 2 to 7: DNA of O45 positive sample; Lane 8: Negative control

Gene Sequencing

Among the positive isolates of STEC one sample was sequenced from main source "Invent Technologies Ltd" by using DNA *stx-1* gene containing forward and reverse primer with an amplicon size of 606 bp. After sequencing, the following sequenced data were found. The partially amplified DNA *stx-1* gene product was 606 bp. The nucleotide sequence obtained from the PCR products was aligned with the known sequence of STEC already available in the GenBank by NCBI BLAST. By using BLAST software homologous sequence of nucleotide from NCBI GenBank were downloaded and finally phylogenic tree was constructed using Mega 7 software. The sequence of the DNA *stx-1* gene Obtained in the study is given below:

***Stx-1* –F:**

5'TGACTGGTGTCTAACGGCAGGACATCACCGGCAAGCGCGGCTGATACTGCTCATTCAGCGCCACCGCTACATCT
TCCGCTTCGCCATCGTGACGCGCATGGTGAATCCCCGTACCACTGATCCCTGCATCAACGAATTTGCCACCAGTAC
GATTTCCACCGCACCGGTGACAAAGGTCAACAGACATGTTACCTGCGGCGTATAGCGCACTGCACACCAGATTAAT
GCAGGCAGCGGGAACGCCAGACTCCCCGCTCCGCCAATGACTACCGAGGCAATAACCGACACAATAAGCGCGAT
GGCGGGCATCATCTGTTCTGCTTTAAAGCGCGGCAGCACTCCAGGAATAGCCAACGTCAGCATACAAGGCACGAT
CAACACGCCCGTTGAGAATTGCTCACTGAACCAGTCCGCAAGCAAAGGCCAGAAATCCAGACTATCAATACTGACC
GAACCAATCGCCCCGACAATAGCGCATAATAATGCGGCAATCAGACAGTAATTAAATAGCCGTAAGGCACTTACT
GGCTCATACTTATTTTTCCCCAAACGCTTATCCCGCACGACCAGTAAGGCGACGGTAACAATAAACATCATATTGGA
GAAATTGATAGCCAGTGAAACCAGTCCCCATTCGGTGGTTATGGCATCATAAACCAGCATCCCCGTACCACTGATC
CCTGCAACAAAACAGNTNAA3'

***Stx-1* –R:**

5'CCATGTATGCGATCGATGCGCTAGCGACAGATGTTGCGGTGCGCTGAATGAGCAGTATCAGCCGCGCTTTGCCG
GTGATGACCTGCCGTCGAACCCGGTAGCAAGTGCGCTGGCGACGCCTGATTGTGACCAGTAAGTGCCTTACGGCT
ATTTAATTACTGTCTGATTGCCGCATTATTATGCGCTATTGTCGGGGCGATTGGTTCGGTCAGTATTGATAGTCTGG
ATTTCTGGCCTTTGCTTGCGGACTGGTTCAGTGAGCAATTCTCAACGGGCGTGTTGATCGTGCCTTGTATGCTGAC
GTTGGCTATTCCTGGAGTGCTGCCGCGCTTTAAAGCAGAACAGATGATGCCCGCCATCGCGCTTATTGTGTCGGTT
ATTGCCTCGGTAGTCATTGGCGGAGCGGGGAGTCTGGCGTTCCCGCTGCCTGCATTAATCTGGTGTGCAGTGCGC
TATACGCCGCAGGTAACATGTCTGTTGACCTTTGTCACCGGTGCGGTGGAAATCGTACTGGTGGCAAATTCGGTGA
TTGATATCTCCGGTCGGTTCCGCCGTTCTCCATTCCCGCAAATGTTCTCCCGCACGTCTCGGGTATTGCCCACGATG
GGCGATATGCCCAAATTATTGGTTTTCTTTTTAGCGGTGGCCAGCCGATCCAAATTCCCCTAAATGGAAGCCAAGG
TGCCGCTGGGCCGACCCCCTGGATTTGTTGAAGTGTTTTTT3'.

Sequencing also done for *E. coli* O157:H7 using DNA *rfb* gene containing forward and reverse primers with an amplicon size 678 bp. After sequencing, the following sequenced data were found. The partially amplified DNA *stx-1* gene product was 606 bp. The nucleotide sequence obtained from the PCR products was aligned with the known sequence of STEC already available in the GenBank by NCBI BLAST. By using BLAST software homologous sequence of nucleotide from NCBI GenBank were downloaded and finally phylogenic tree was constructed using Mega 7 software. The sequence of the DNA *rfb* gene Obtained in the study is given below:

***Rfb*-F:**

5'GCAGTGCAATCGGAGGAGATGAGTTATTGTTCCAACACTGACATATATAGCATCAGTTAATGCTATAAAATACAC
AGGAGCCACCCCCATTTTCGTTGATTCAGATAATGAAACTTGGCAAATGTCTGTTAGTGACATAGAACAAAAAATC
ACTAATAAAACTAAAGCTATTATGTGTGTCCATTTATACGGACATCCATGTGATATGGAACAAATTGTAGAACTGG
CCAAAAGTAGAAATTTGTTTGTAATTGAAGATTGCGCTGAAGCCTTTGGTTCTAAATATAAAGGTAAATATGTGGG
AACATTTGGAGATATTTCTACTTTTAGCTTTTTTGGAAATAAAACTATTACTACAGGTGAAGGTGGAATGGTTGTCA
CGAATGACAAAACACTTTATGACCGTTGTTTACATTTTAAAGGCCAAGGATTAGCTGTACATAGGCAATATTGGCA
TGACGTTATAGGCTACAATTATAGGATGACAAATATCTGCGCTGCTATAGGATTAGCCCAGTTAGAACAAGCTGAT
GATTTTATATCACGAAAACGTGAAATTGCTGATATTTATAAAAAAAATATCAACAGTCTTGTACAAGTCCACAAGG
AAAGTAAAGATGTTTTTCACACTTATTGAATTGGTCTCAAAA3'

***Rfb*-R:**

5'CAACGTTCGTGTGGTCTTGTACAGACTGTTGATATTTTTTTTATAAATATCAGCAATTTCACGTTTTCGTGATATAA
AATCATCAGCTTGTTCTAACTGGGCTAATCCTATAGCAGCGCAGATATTTGTCATCCTATAATTGTAGCCTATAACG
TCATGCCAATATTGCCTATGTACAGCTAATCCTTGGCCTTTAAAATGTAAACAACGGTCATAAAGTGTTTTGTCATT
CGTGACAACCATTCCACCTTCACCTGTAGTAATAGTTTTATTTCCAAAAAAGCTAAAAGTAGAAATATCTCCAAATG
TTCCCACATATTTACCTTTATATTTAGAACCAAAGGCTTCAGCGCAATCTTCAATTACAAACAAATTTCTACTTTTGG
CCAGTTCTACAATTTGTTCCATATCACATGGATGTCCGTATAAATGGACACACATAATAGCTTTAGTTTTATTAGTG
ATTTTTTGTTCTATGTCACTAACAGACATTTGCCAAGTTTCATTATCTGAATCAACGAAAATGGGGGTGGCTCCTGT
GTATTTTATAGCATTAACTGATGCTATATATGTCAGTGTTGGAACAATAACTTCATCTCCTTCCGATATACCTAACGC
TAACAAAGCTAAATGAAAGCCAACCGTTAAG3'

Direct nucleotide sequencing and phylogenic tree construction

The obtained nucleotide sequence of *stx-1* gene of 1 selected *E. coli* isolate (BAU-Bangladesh- *E. coli-stx*-1) and 6 isolates obtained from Genbank were put into phylogenic analysis. The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch length in the same units as those of the evolutionary distances used to infer the phylogenic tree. The evolutionary distances were computed using the Maximum Composite Likelihood Method (Tamura *et al.,* 2004). Evolutionary analyses were conducted in MEGA 7 software (Tamura *et al.,* 2011).

The phylogenic tree of *stx-1* derived from the sequence data is shown in Figure 14.

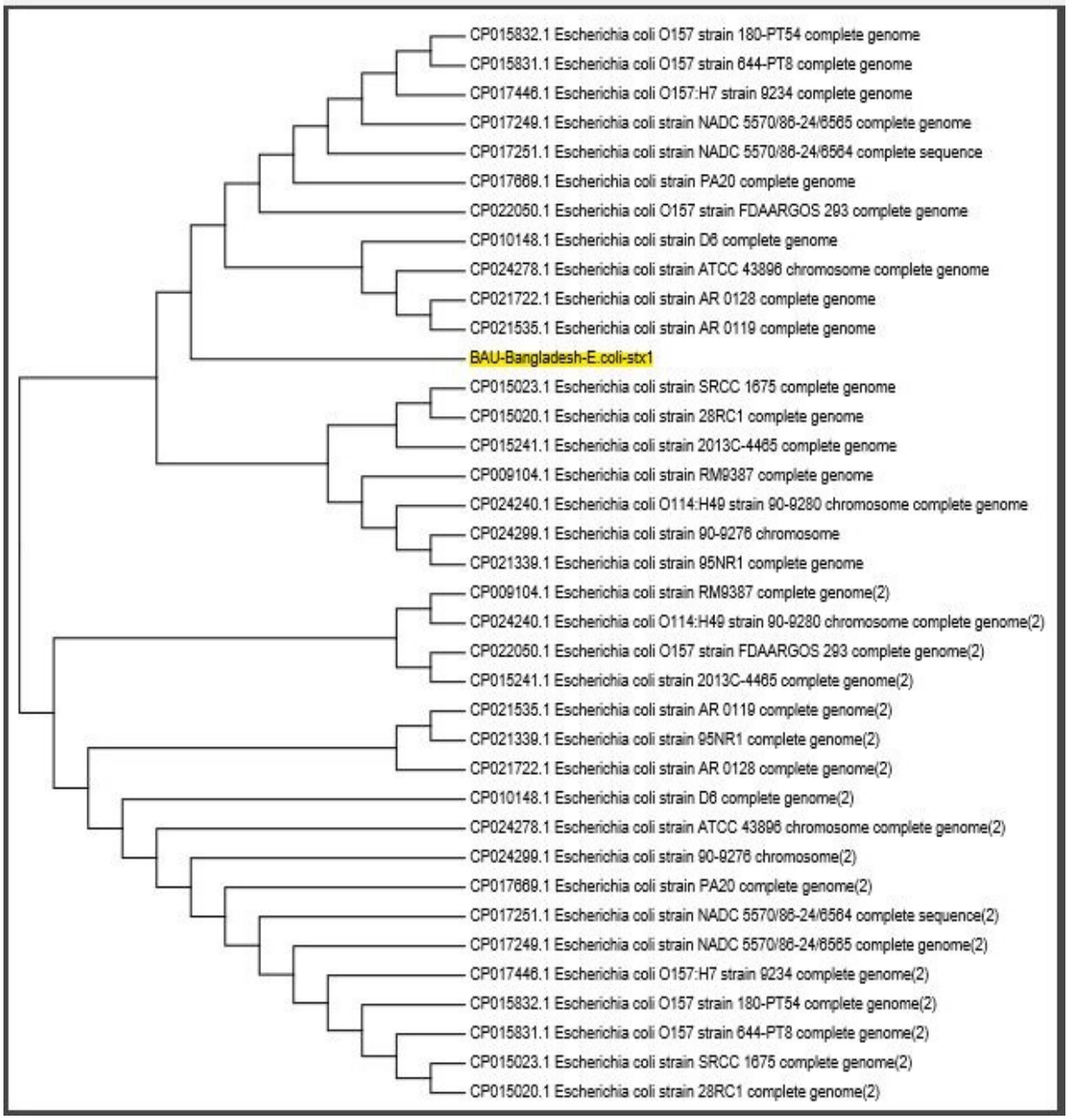

Figure 14. Sequence similarity tree showing relationship of STEC isolates

The tree was constructed using the neighbor joining method in Mega 7 software. Numbers on the nodes indicate bootstrap values calculated using 1000 replicates. Yellow color indicates the study isolates

The phylogenic tree of *rfb* gene of *E. coli* O157: H7 derived from the sequenced data is shown in Figure 15.

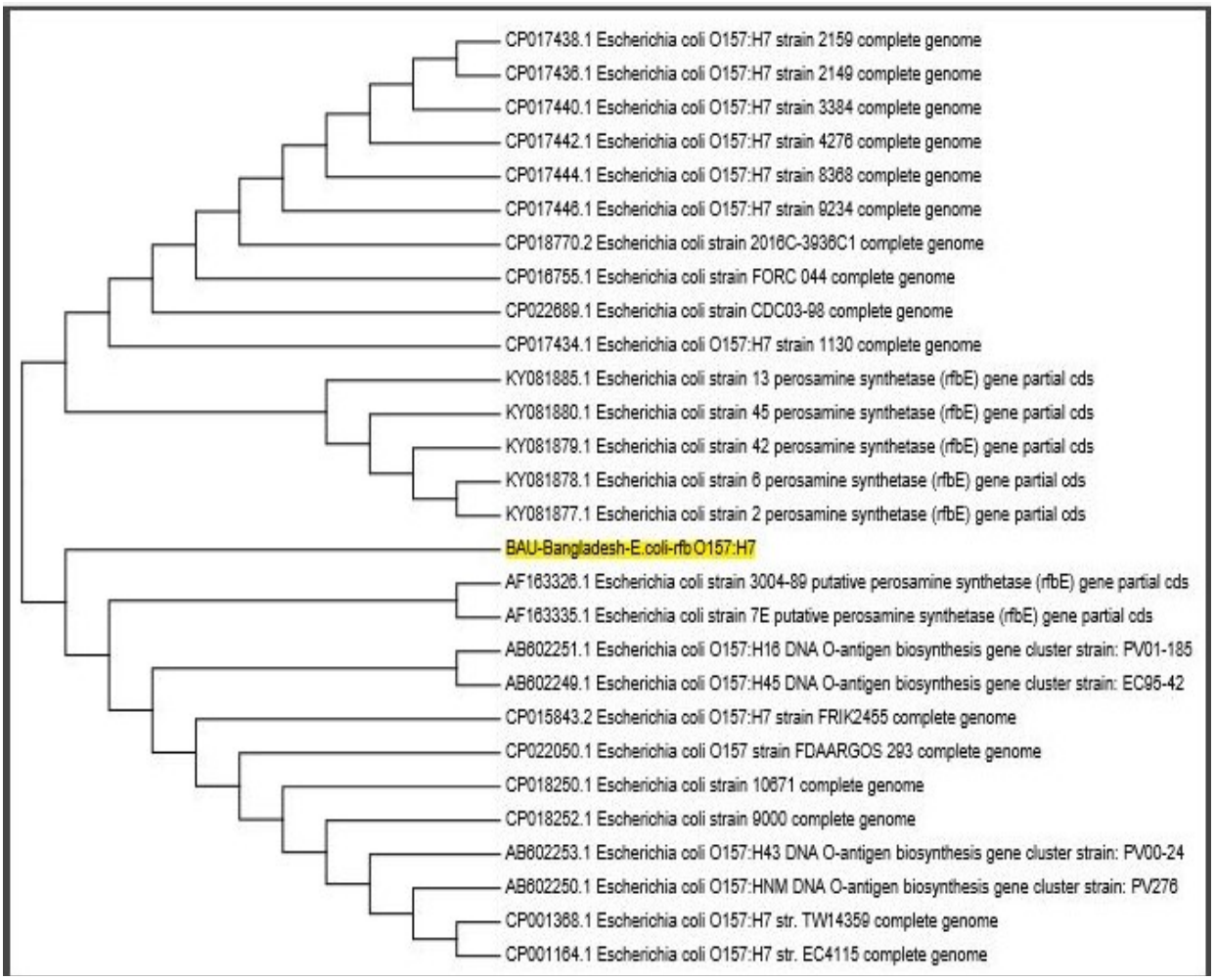

Figure 15. Sequence similarity tree showing relationship of *rfb* O157:H7 isolates

The tree was constructed using the neighbor joining method in Mega 7 software. Numbers on the nodes indicate bootstrap values calculated using 1000 replicates. Yellow color indicates the study isolates

11.3. Antibiotic sensitivity test

Results of antibiotic sensitivity test for STEC are shown in Table 11, Figure 16 and Figure 17. All 34 STEC isolates were tested against 8 different antibiotics. Highest sensitivity pattern was found in Norfloxacin (74%), Azithromycin (62%) and Amikacin (56%) and highest resistant pattern was found in Colistin (100%), Cephalexin (100%), Ciprofloxacin (100%) and Neomycin sulphate (85%) respectively. So, Norfloxacin, Azithromycin and Amikacin can be recommended as the effective drugs to control the STEC infections in poultry**.** Twenty six *Stx-1* positive *E. coli* isolates were tested against 8 different antibiotics by disc diffusion method. Among them Norfloxacin (73.38%), Azithromycin (61.54%) and Amikacin (57.7%) showed highest sensitivity pattern. Highest resistant pattern was showed by Colistin (100%), Cephalexin (100%) and Ciprofloxacin (100%). Several *E. coli* isolates were found resistant to multiple antibiotics. This study conforms with the study of Akond *et al.* (2009) who tested 50 *E. coli* isolates to 13 antimicrobial agents to check their susceptibility. 88%, 82%, 80%, 76%, 70%, 68%, 64%, 58%, 52%, and 20% of the tested *Escherichia coli* strains from poultry sources were found resistant to Penicillin, Ciprofloxacin, Rifampicin, Kanamycin, Streptomycin, Cefixime, Erythromycin, Ampicillin, Tetracycline, Chloramphenicol and Neomycin. None of the strains showed resistance to Norfloxacin and Gentamicin. Data of this study indicated that poultry harbor multidrug resistant *Stx-1* producing *E. coli* which may

cause public health hazard if transmitted to human through food chain. The study showed that most of the *E. coli* isolates are multi-drug resistant. Such high incidence of multidrug resistance may apparently have occurred due to indiscriminate use of antimicrobial agents which may ultimately replace the susceptible microorganisms. New strategies and proper food safety management are needed to prevent the contamination of food materials and to reduce the drug resistance. Developing a new and natural antibiotic with a novel mode of action is necessary for the treatment of such multi-drug resistant bacteria.

Table 11. Antibiogram profile of *Stx*-1 producing *E. coli* isolates

Antimicrobial agents	**Group**	**No. of isolates (%)**		
		Resistant	Intermediate	Sensitive
Amikacin	Aminoglycoside	5 (15)	9 (26)	19 (56)
Colistin	Polymyxin B	34 (100)	0(0.0)	0(0.0)
Azithromycin	Macrolide	13(38)	0 (0.0)	21 (62)
Norfloxacin	Fluroquinolones	0(0.0)	9 (26)	25(74)
Erythromycin	Macrolide	30 (88)	4 (12)	0(0.0)
Cephalexin	Cephalosporin	34 (100)	0(0.0)	0(0.0)
Neomycin sulphate	Aminoglycoside	29 (85)	5(15)	0(0.0)
Ciprofloxacin	Fluoroquinolones	34 (100)	0(0.0)	0(0.0)

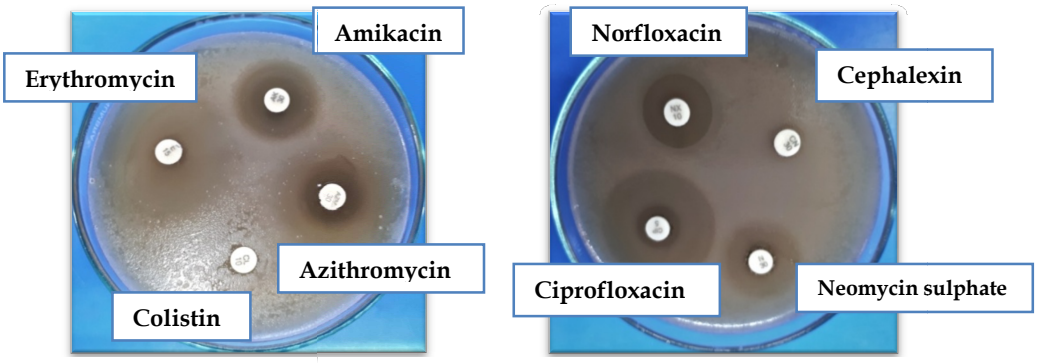

Figure 16: Antibiotic sensitivity test for Shiga toxin producing *E.coli* using disc diffusion method

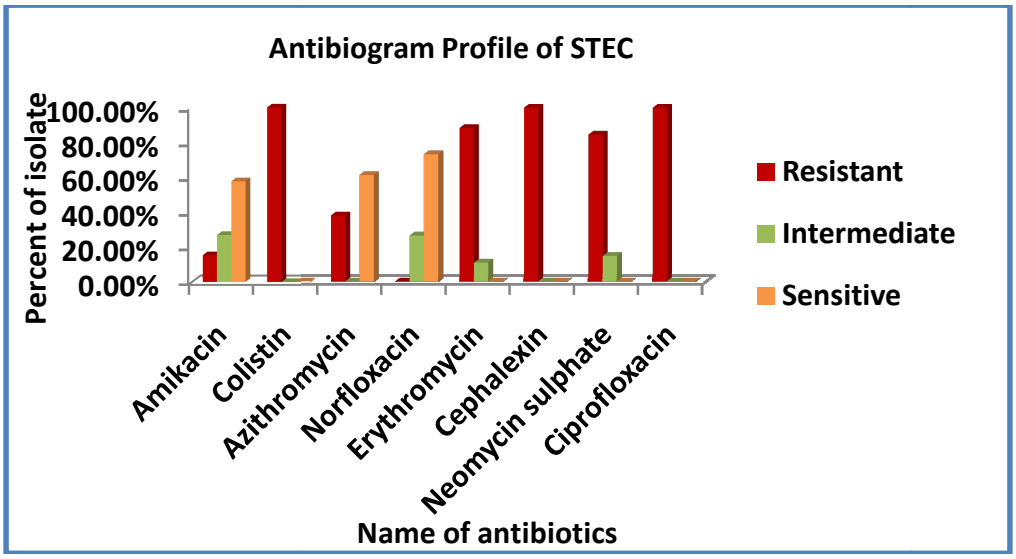

Figure 17: Antibiogram profile of Shiga toxin producing *E. coli* isolates

12. Research highlights (findings)

- Out of 300 samples, 159 (53%) samples were found positive for *E. coli*.
- The prevalence of *stx*1 gene was found higher (21.38%) than *stx*2 gene (5.66%).
- Presence of shiga toxin producing *E. coli, E. coli 157:H7* and O26 were confirmed by sequencing of bacterial DNA.
- Different serogroups of *E. coli* such as O157:H7, O111, O26 and O45 has been successfully isolated and identified from the poultry and their products.
- Sensitivity pattern was found be for Norfloxacin 74%, Azithromycin 62% and Amikacin 58% while the resistant pattern was found in Colistin 100%, Cephalexin 100%, Ciprofloxacin 100% and Neomycin sulphate 85%.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment	GD_3 & GD_5	83000.00	GD_3 & GD_5	82850.00	Tk. 150 could not spend
(b) Lab &field equipment	GD_1, GD_2, GD_4 & GD_6	1196798.00	GD_1, GD_2, GD_4 & GD_6	1195890.00	Tk. 908 could not spend
(c) Other capital items					

2. Establishment/renovation facilities: Not applicable

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized:

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					
(C) Seminar	35	10	45	One day (19/02/2019)	

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	464955	454717	443181	11536	95.3%	
B. Field research/lab expenses and supplies	1137548	1137548	1136972	576	99.9%	
C. Operating expenses	110000	110000	110000	0	100%	
D. Vehicle hire and fuel, oil & maintenance	50000	50000	50000	0	100%	
E. Training/workshop/ seminar etc.	40000	40000	40000	0	100%	
F. Publications and printing	90000	30000	30000	60000	33.3%	
G. Miscellaneous	30000	30000	30000	0	100%	
H. Capital expenses	259250	259250	258900	350	99.9%	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
i) Isolation, identification and serogrouping of *E. coli* field isolates from broiler, layers and indigenous chickens.	• Collection and Processing of samples • Isolation and Identification of *E. coli* in the different cultural media • Identification using different biochemical tests • Serological tests for serogrouping	• Out of 300 samples, 159 (53%) isolates were confirmed as *E. coli*.	The data generated from this study related to occurrence of shiga toxin producing *E. coli* along with their serogroups and antibiogram will be helpful to formulate a guideline to protect the public health
ii) Molecular characterizatio n of shiga toxin producing genes (*stx1 and stx2* genes) using PCR assay	• Identification of Stx producing *E. coli* by PCR • Sequence analysis of PCR products	• The prevalence of *stx*1 gene was found higher (21.38%) than *stx*2 gene (5.66%). • Presence of shiga toxin producing *E. coli, E. coli 157: H7* and O26 were confirmed by sequencing of bacterial DNA.	
iii) Determination of antibiotic sensitivity profile of the STEC isolates.	• Antibiotic sensitivity test using disc diffusion method	• Sensitivity to *E. coli* for Norfloxacin, Azithromycin and Amikacin was 73.38%, 61.54% and 57.7% respectively while; the resistant pattern for Colistin, Cephalexin and Ciprofloxacin was 100%.	

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer, etc.			
Journal publication			
Information development			
Other publications, if any: MS thesis		2	1. Prevalence and molecular characterization of shiga toxin producing *Escherichia coli* in poultry and their products at Gazipur and Dhaka districts of Bangladesh. 2. Prevalence and molecular characterization of shiga toxin producing *Escherichia coli* and its serogroups in poultry and their products at Mymensingh and Bogura districts of Bangladesh.

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

Methods of detection of different serogroups of *E. coli* by PCR assay

ii. **Generation of new knowledge that help in developing more technology in future**

Detection of *E. coli* O157: H7 in poultry

iii. Technology transferred that help increased agricultural productivity and farmers' income

Not applicable

iv. Policy Support

This data will be helpful to formulate a guideline to protect the public health

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring (description &output of consultation meeting, monitoring workshops/seminars etc.):

ii) Field Monitoring (time & No. of visit, Team visit and output):

Date	No. of visit	Team visit	Output
04/03/2018	1	Technical Division/ Unit, BARC	Satisfactory
07/03/2018	1	PIU-BARC, NATP-2	Satisfactory

H. Lesson Learned: None

I. Challenges (if any): None

Signature of the Principal Investigator
Date : December 31, 2018

Seal

Counter signature of the Head of the organization/authorized representative
Date ……..
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