Prevalence, Isolation and Detection of Virulent Gene in *Escherichia coli* From Duck

ABSTRACT

Aims: This study was conducted to determine virulent genes in *Escherichia coli* prevalent in duck population by multiplex Polymerase Chain Reaction.

Methodology: A total of 60 cloacal swab samples were collected from two duck farms of Bangladesh Agricultural University and Shamvuganj. Initially the samples were screened for the detection of *E. coli* on the basis of cultural, staining and biochemical properties, followed by molecular detection of *E. coli* using genus specific primers to amplify 16s RNA.

Results: According to the result, out of 60 samples, 26 (43.33%) were confirmed to be *E. coli* positive. Among the *E. coli* positive samples, 12 (46.15%) samples were found positive for *Stx*-1 and 11 for *Stx*-2. Among 26, 11 (42.31%) samples possess both *Stx*-1 and *Stx*-2 genes, whereas only one isolate had *Stx*-1 gene. The prevalence of both *Stx*-1 and *Stx*-2 in Bangladesh Agricultural University poultry farm is 41.66% and the prevalence of *Stx*-1 and *Stx*-2 in Shamvuganj is 50% and 42.86%, respectively.

Conclusion: This is the first report on the detection of virulence genes in *E. coli* of duck origin in the context of Bangladesh. This study indicates that duck may play role for the transmission of STEC to human or its environment through fecal contamination or eggs or meat.

Keywords: Prevalence, STEC, Isolation, PCR, Importance

1. INTRODUCTION

Shiga-like toxin producing *Escherichia coli* (STEC) is known as Verotoxin producing *E. coli*. Infections due to STEC can result in severe bloody diarrhea (hemorrhagic colitis, HC), which may evolve towards the life-threatening hemolytic-uremic syndrome (HUS).

A major virulence factor of STEC is the production of one or more shiga toxins (*Stx*). The production of and *Stx*-2 in *E. coli* is conferred by toxin-converting lysogenic bacteriophages [1]. The involvement of these phages could explain the production of shiga toxins in more than 150 different serotypes of *E. coli* [2]. Only few *Stx*-1 variants and more than 20 *Stx*-2 variants have so far been reported [3].

The natural hosts for STEC are ruminants like sheep, goats, and in particular, bovines [4]. Other animals such as pigs and dogs can also harbour STEC strains [5].

E. coli O157:H7 is an enterohemorrhagic strain of *E. coli* and a cause of illness through food [6].

The *E. coli* is widely used in laboratory research and extensive works have been performed throughout the world regarding its isolation, molecular characterization, prevalence and risk factors associated with the outbreaks of *E. coli* O157:H7 in cattle [7-9].

Several works have been done in Bangladesh regarding the isolation and molecular characterization of *E. coli* from the intestinal content and meat cattle, diarrheic human patient, and environmental [10-12].

The presence of *Stx* positive fecal cultures in asymptomatic individuals [13,14] suggested that other virulence factors besides *Stx* are required to cause serious disease in humans. Fratamico et al. [15] described a multiplex PCR capable of detecting *Stx*-1, *Stx*-2, *eae*A, and EHEC *hly*A genes. However, this PCR was not tested with fecal samples; primers for each target gene sequence showed differential sensitivities, and *Stx* primers were unable to distinguish *Stx*-1 from *Stx*-2 by agarose gel electrophoresis. Ideally, PCR-based detection methods should be rapid and sensitive without requiring extensive sample preparation. More recently Paton and Paton, [14] developed a multiplex PCR utilizing four PCR primer pairs for the detection of *Stx*-1, *Stx*-2, *eae*A, and EHEC *hly*A in human feces and foodstuffs. However, the relatively lengthy PCR template preparation protocol used was considered inappropriate for testing large numbers of samples.

Ruminants, particularly cattle [5] and sheep [16] are natural reservoirs of EHEC, although other domestic animals, including goats, pigs, poultry, cats and dogs, can also harbour these bacteria [16]. However, methodologies which provide comparatively rapid (24h) and sensitive detection of *Stx*-1, *Stx*-2, *eae*A, and *hly*A gene sequences in animal feces have not been reported.

As per literature review no work was yet performed for the isolation and molecular characterization of *E. coli* O157:H7 from the cloacal swab of diarrheic and apparently healthy duck in Bangladesh.

2. MATERIAL AND METHODS

2.1 Sample collection and transportation

Diarrheic and apparently healthy ducks were selected for the experimental study. A total number of 60 cloacal swab samples were collected by sterile cotton bud and put into eppendorf tube containing nutrient broth brought to the laboratory of the Department of Microbiology and hygiene, BAU by maintaining cool chain.

2.2 Processing and enrichment of samples

Samples were processed for bacteriological analysis immediately after arrival to the bacteriological lab. At first samples were vortexed separately and then it was enriched in nutrient broth and incubated at 37℃ overnight.

2.3 Isolation of bacteria

Primary growth was performed in nutrient broth followed by inoculation into selective media and incubated at 37℃ for overnight. After primary culture of the organism, a 10 fold dilution was made to reduce overgrowth of the organisms. After that 100 µl was inoculated onto Mac-Conkey agar. The colonies showed typical cultural characteristics of *E. coli* were selected for subculture on selective media such as EMB. The colonies showed typical characteristics of *E. coli* in Mac-Conkey agar were further inoculated into EMB agar to confirm the isolates as *E. coli.*

2.4 Identification of bacteria

Appearance of pink/red and greenish black with metallic sheen colony on Mac-Conkey and EMB agar plates respectively was considered positive for *E. coli* and stained with Gram's stain [17]. After that microscopic examination was performed with high power objectives (100x) using immersion oil. *E. Coli* was characterized by their ability to ferment dextrose, sucrose, lactose, maltose and mannitol to produce gas (CO_2), positive for MR and indole test, and negative for VP test [18].

2.5 Molecular detection of bacteria by PCR

The genomic DNA of each *E. coli* isolates was extracted by mixing of one colony into 200µl of distilled water followed by boiling for 10 minutes. After boiling the samples were immediately kept on ice for few minutes. Finally centrifugation was done at 10000 rpm for 10 minutes. The supernatant were collected and used as DNA template for PCR. To detect 16S RNA gene and shiga toxin producing gene, *stx*1 and *stx*2, all samples were examined individually. The thermal profile of 16S rRNA, *stx*1 and *stx*2 gene specific primers are given in table **(1)**. PCR products were analyzed by 1.5% Agarose gel electrophoresis. After electrophoresis the gel was stained with ethidium bromide (EtBr) solution for 20 minutes. After washing the gel by distilled water for 5 minutes, The EtBr stained PCR products were visualized by UV trans-illuminator (Biometra, Germany).

Table 1. Primers used in this study with sequences

Primer Name	Gene Targeted	Primer Sequence (5´-3´)	Amplicon size (bp)	Reference
EC16SrRNA F	16SrRNA	5´GACCTCGGTTTAGTTCACAGA3´	585	Hassan et al. [19]
EC16SrRNA R		5´CACACGCTGACGCTGACCA3´		
EC *Stx*-1 F	*Stx*-1	5´CACAATCAGGCGTCGCCAGCGCACTTGCT3´	606	Talukdar et al. [20]
EC *Stx*-1R		5´TGTTGCAGGGATCAGTCGTACGGGGATGC3´		

EC *Stx*-2 F	*Stx*-2	5´CCACATCGGTGTCTGTTATTAACCACACC3´	372	Talukdar et al. [20]
EC *Stx*-2 R		5´GCAGAACTGCTCTGGATGCATCTCTGGTC3´		

Table 2. Thermal profile for 16sRNA, *Stx*-1 and *Stx*-2 gene specific primer

PCR steps	Temperature (℃) and time (min) 16sRNA	Temperature (℃) and time (min) *Stx*-1 and *Stx*-2	Cycle
Initial denaturation	95, 5	95, 5	
Denaturation	94, 0.5	94, 0.5	30
Annealing	58, 1	56, 1	
Elongation	72, 1	72, 1	
Final extension	72, 10	72, 10	
Holding	4	4	Until use

2.6 Statistical Analysis: Finally Chi-square test was done to determine the level of significance.

3. RESULTS AND DISCUSSION

Shiga-toxin producing *E. coli* infections are of significantly important as of public health concern. STEC infections also frequently result in hemolytic-uremic syndrome (HUS), a life-threatening condition characterized by hemolytic anaemia, thrombocytopenia and renal failure [6]. Humans most frequently become infected with STEC by ingestion of contaminated food or water or by direct contact with animals, resulting in sporadic cases of disease or outbreaks, involving up to several thousand individuals [21].

There are many studies conducted for the detection of *Stx*-1 and *Stx*-2 genes of *E. coli* from different animal such as cattle, goat, fish and poultry but in duck in the context of Bangladesh, it is still unknown. There is no previous report on prevalence of STEC in duck in Bangladesh. The present study was undertaken for the prevalence study, isolation, identification and molecular characterization of *E. coli* from apparently healthy and diarrheic duck of BAU poultry farm and Shamvuganj. A total number of 60 cloacal swab samples were collected using sterile cotton buds and transported in NB maintaining cool chain. The culture media used in this study were selected considering the experience of the past researchers worked in various fields relevant to the present study by Nazir et al. [22] and Hasina [23].

Previously Shiga toxin producing *E. coli* were isolated from poultry meat shown the positive result for *Stx*-1 and *Stx*-2 gene in PCR similar isolation done by several workers [24,25]. The amplification of specific gene like *Stx*-1 and *Stx*-2 gene represent that the pathogenic form of *E. coli* that's have a public health importance where threat like bloody diarrhea, hemorrhagic colitis and a life-threatening hemolytic-uremic syndrome (HUS) already established by Fratamico and Bagi [26].

A few studies of the occurrence of STEC have been done, and most of the studies were done in India and Thailand [27,28]. Recently, a study was done in central Vietnam that found a prevalence of STEC were 27%, 23%, and 38.5% in buffaloes, cattle and goat respectively [29]. In India, the prevalence of STEC O157 in fecal samples from slaughtered cattle and diarrheic calves was 2.0% and 7.6%, respectively [28]. STEC O157 has also been isolated in India from foods of cattle origin; namely, beef surface swabs (3.7%; $n = 27$), and milk samples (2.4%; $n = 81$) [28]. In China, STEC O157:H7 was isolated from 10% to 20% of the animals in the villages, including pigs, cattle, goats, and chick [30-32]. By observing the prevalence rate of STEC in different animals, we collected cloacal swab sample from duck for the detection of *Stx*-1 and *Stx*-2 genes. The samples were placed into nutrient broth and incubated at 37℃ for 2 hours. The clear transparent broth was changed to turbid, which indicates bacterial growth. MC agar plates were streaked with the samples which were collected from the nutrient broth and bright pink or red colored colored colonies were observed after 24 hours of incubation at 37℃ which indicates the growth of *E. coli.* Then the bright pink colonies were streaked into EMB agar media and incubated at 37℃ for 24 hours. Smooth, circular, greenish black color colonies with metallic sheen were found which was indicated

confirm growth of *E. coli.* After that Gram's stain was performed for microscopic examination by collecting sample from NB, MC agar and EMB agar which revealed Gram negative, rod shaped, pink colored organisms arranged in single, pairs or short chain. Biochemical tests with five basic sugars (dextrose, sucrose, lactose, maltose and mannitol) were performed and it was identified that *E. coli* can ferment all those sugars and produce acid and gas. Positive reaction was found in MR and Indole test and negative reaction was found in VP test.

All the isolates of *E. coli* which were presumptively identified on the basis of cultural, Gram's staining and biochemical tests were confirmed by Polymerase chain reaction using genus specific 16s rRNA primers. A total of 26 isolates were confirmed as *E. coli* by amplifying genus specific 16S rRNA primers.

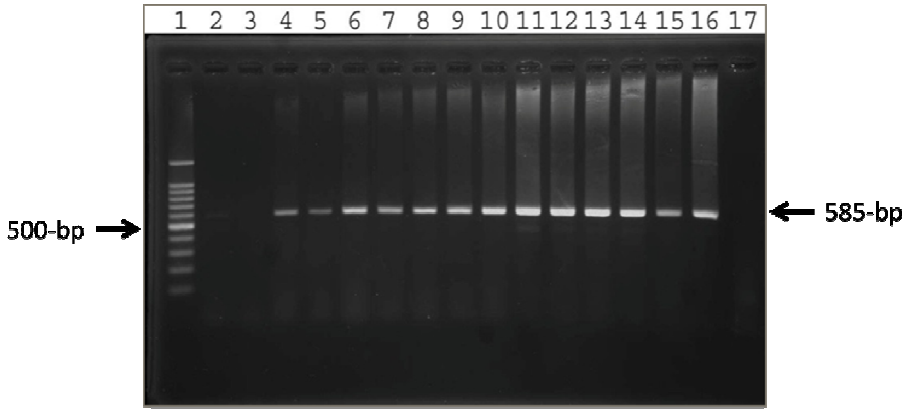

Fig.1. Amplification of 16s rRNA (585 bp) specific genomic primer; Lane 1: 100 bp DNA ladder, Lane 4-15: positive for 16s rRNA; Lane 16: Positive control; Lane 17: Negative control

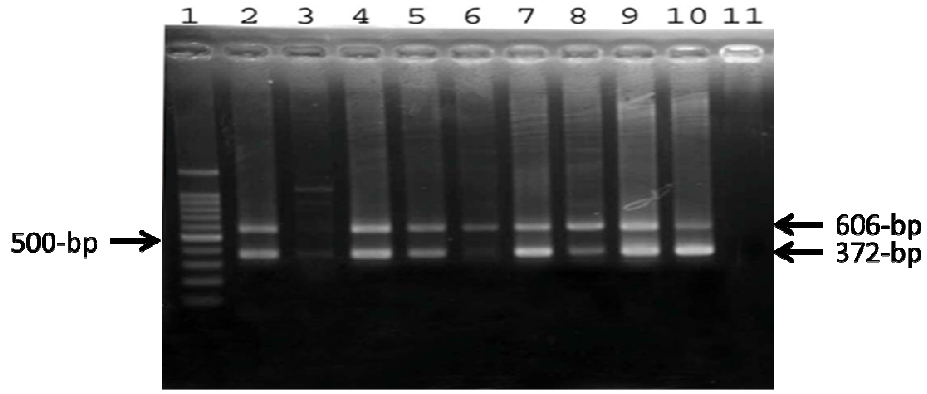

Fig.2. Amplification of *Stx*-1 (606 bp) and *Stx*-2 (372 bp) genes; Lane 1: 100 bp DNA ladder, Lane 2-9: amplified *Stx*-1 and *Stx*-2 positive genes from *E. coli*; Lane 6: *Stx*-1 positive; Lane 10: Positive control; Lane 11: Negative control

To identify shiga toxin producing *E. coli* at genomic level a multiplex PCR was also performed using *Stx*-1 and *Stx*-2 gene specific primers and the results are furnished in Fig.(1) and Fig.(2) respectively. Fig.(1) shows the amplicon size 585 bp in case 16s rRNA specific primer and Fig. (2) shows 606 and 372 bp amplicon size in relation to *Stx*-1 and *Stx*-2 gene specific primers respectively.

Out of 26, 12 isolates were found to be positive for both *Stx*-1 and *Stx*-2 genes. So overall prevalence of *E. coli* was 43.33% and among *E. coli* positive isolates 12 (46.15%) samples were found to be positive for *Stx*-1 and 11 (43.31%) were *Stx*-2.

Table 3. Cultural characteristics and overall prevalence of 16s rRNA, *Stx*-1 and *Stx*-2

Source of samples	**No of samples**	**No. of *E. coli* positive samples on the basis of cultural properties**	**No. of 16srRNA Positive samples**	***Stx*-1 Positive**	***Stx*-2 positive**	**No. (%) of 16srRNA**	**No. (%) of *Stx*-1**	**No. (%) of *Stx*-2**
BAU poultry farm	**30**	**12**	**12**	**5**	**5**	**12 (40%)**	**5 (41.66%)**	**5 (41.66%)**
Shamvugonj	**30**	**14**	**14**	**7**	**6**	**14 (46.66%)**	**7 (50%)**	**6 (42.86%)**
Total	**60**	**26**	**26**	**12**	11	**26 (43.33%)**	**12 (46.15%)**	**11 (42.31%)**
P value						**0.0010**	**0.0054**	**0.0410**
Level of significance						**	**	*

** means sig. at 1% level ($p<0.01$)

* means sig. at 1% level ($p<0.05$)

The result of the present study showed that the STEC strains isolated from duck may be readily transmit to human by consumption of eggs and meat of duck or via its environmental samples, specially by water. Our result showed that 42.31% *E. coli* possesses both virulent genes *Stx*-1 and *Stx*-2.

Prevalence of *E. coli* in these studies was 43.33% and also on the basis of virulence, the prevalence was 46.15% and 42.31% with *Stx*-1 and *Stx*-2 respectively. In case of BAU poultry farm the prevalence of 16s rRNA was 40% and *Stx*-1 and *Stx*-2 were 41.66%. In case of Shamvuganj the prevalence of 16s rRNA was 46.66% and *Stx*-1 and *Stx*-2 were 50% and 42.86% respectively.

4. CONCLUSION

Escherichia coli is a bacterium, which releases shiga toxin that causes watery diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome (HUS) in humans. Different kinds of animal, birds and food act as an important source for the growth of *E. coli.* The study was conducted to investigate the cultural, biochemical character and to study the genomic DNA confirmation for 16S rRNA, *Stx*-1 and *Stx*-2 gene by molecular technique (PCR).

CONSENT (WHERE EVER APPLICABLE)

Not applicable

ETHICAL APPROVAL

Not applicable

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