



PCR Based Detection of Shiga Toxin Producing *E. coli* in Commercial Poultry and Related Environments

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ABSTRACT

Shiga toxin (Stx)-producing *E. coli* (STEC) is the most important foodborne pathogen which is the causal agent of mild diarrhea, bloody diarrhea, hemolytic-uremic syndrome (HUS) in human. The present study was designed to determine the prevalence and identification of Shiga toxin (Stx)-producing *E. coli* in poultry, detection of its source of infection in poultry and transmission pattern to human. For this purpose a total of 150 samples (cloacal swab-60, feed -15, water-15 and egg -60) were collected and analyzed in bacteriology laboratory by cultured in different bacteriological media followed by gram's staining, biochemical tests and Polymerase Chain reaction (PCR). The PCR was performed by targeting 16s rRNA gene and shiga toxin producing gene in *E. coli*. Out of 150 collected samples, *E. coli* was found in 81 (54%) samples. Presence of *E. coli* was 100% in both feed (n=15) and egg (n=60), whereas 10% in cloacal swab (n=6). Water samples were totally free of *E. coli*. The *stx2* gene was detected in all samples whether all samples were negative for *stx1* gene. The study revealed that, poultry feed acts as a source of *E. coli* infection in poultry, which may be transmitted to environment and human via meat or eggs. Antibiotic sensitivity test revealed that isolated bacteria were highly sensitive to Ciprofloxacin.

Introduction

Escherichia coli commonly called *E. coli* are the best-studied gram-negative bacterium in the world. It is a rod-shaped facultative anaerobe propelled by long, rapidly rotating flagella. It is part of the normal flora of the mouth and gut and helps in preventing colonization of pathogenic bacteria in the intestine (Hudault, 2001), aids in digestion, and can benefit their hosts by producing small amounts of vitamins B₁₂ and K₂. Though it is a part of normal enteric microflora, but it is capable of producing serious diarrheal diseases, as well as other systemic diseases, especially infection of the urinary tract. *E. coli* and other facultative anaerobes constitute about 0.1% of gut flora (Eckburg, 2005) and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. In Bangladesh, poultry rearing is considered superior to the other sector in agriculture because of an almost assured and quick return in a relatively short period of time. The poultry industry comprising of commercial poultry with broiler and layers plays an important role in the economy of Bangladesh. There are approximately 163.50 million of poultry including ducks reared throughout Bangladesh (Kamaruddin and Giasuddin, 2003). The sector has already proved itself as a potential income generation and poverty alleviation, as well as improving human nutrition

through the supply of meat and eggs to their daily life and also contributes 2.79% GDP (BBS, 2008). Shiga toxins are a family of related toxins with two major groups, Stx1 and Stx2, expressed by genes considered to be part of the genome of lambdoid prophages (Friedman, 2001). *E. coli* that produces shiga toxin (Stx) (Shiga toxin producing *E. coli*, STEC) is characterized by the production of cytotoxins that disrupt protein synthesis within host cells. These toxins are synonymously either called verocytotoxins (VT), because of their activity on Vero cells, or Shiga toxins (Stx) because of their similarity with the toxin produced by *Shigelladysenteriae*. Present study was undertaken to detect the prevalence of shigatoxin producing *E. coli* in commercial poultry and to identify the source of the organism in poultry and routes of transmission from poultry to human.

Materials and Methods

Sample collection

To detect shiga toxin producing *E. coli*, 60 cloacal swab, 15 poultry feed sample, 15 water samples and 60 egg samples were collected from BAU poultry farm, poultry shed of the department of Microbiology and hygiene, BAU, Mymensingh. The cloacal swab samples were collected using sterile cotton bud. The feed samples,

water samples and eggs were collected in sterile tube. After collection, the samples were carried to the bacteriology laboratory for analysis.

Bacteriological analysis

The nutrient broth containing the swab sample was incubated for overnight at 37°C for primary growth. Growth of the organism is indicated by the development of turbidity of the broth. The feed and eggs were washed with PBS. The water sample and PBS used for feed and egg wash was directly used for culture. EMB agar (Hi-media, India) and MacConkey agar media (Hi-media, India) were streaked aseptically with primary culture and incubated at 37°C for overnight. Appearance of the colony of pink/red and greenish black with metallic sheen appears on MC and EMB agar plates respectively was considered positive for *E. coli* and stained with Gram's stain. Then examined under microscope with high power objectives (100X) using immersion oil. *E. coli* were characterized by their ability to ferment glucose, sucrose, lactose, maltose and mannitol to produce gas (CO₂), positive for indole test and MR test, and negative for VP test.

Molecular Characterization by PCR

Bacterial DNA was extracted using Wizard® Genomic DNA purification kit, (Promega, USA). The extraction procedure was done according to protocol provided by kit. All samples were examined by three pairs of primers (Table 1) to detect *16S RNA* gene and shiga toxin producing gene, *stx1* and *stx2*. Thermal profiles used in PCR are discussed below (Table 2, 3 and Fig. 1, 2).

The thermal conditions to amplify *stx2* gene was same as *stx1* except the annealing temperature was at 59°C for 1 min. PCR amplification was performed on a thermo cycler (Eppendorf Personal, Germany). PCR products

were separated on 1.5% agarose gel, stained with ethidium bromide and photographed using a Gel documentation system (BioRad). *E. coli* reference strain EDL933 was used as positive control. In vitro antibiotic sensitivity test was performed according to the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2012). Ciprofloxacin, Levofloxacin, Nalidixic Acid and Gatifloxacin were used in this study. The concentration of each disc was 5µg.

Data analysis

The results were compared statistically by chi-square test. Statistical analysis was performed by using SPSS software version 11.5 (SPSS Inc., Chicago, IL, USA).

Result

After 2 hours culture in nutrient broth, the clear transparent broth were changed to turbid, which indicates bacterial growth. EMB agar plates streaked with the organism and incubated at 37°C for 24 hrs. The growth of *E. coli* was indicated smooth, circular, greenish black color colonies with metallic sheen. MC agar plates streaked separately with the organism and revealed the growth of *E. coli* after 24 hours of incubation at 37°C aerobically and were indicated by the growth of bright pink or red colored colony. The microscopic examination of Gram's stained smears from NB, MC agar and EMB revealed Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain. A series of biochemical tests especially selective for *E. coli* were performed. *E. coli* can ferment all the five basic sugars (dextrose, sucrose, lactose, maltose and mannitol) and produce acid and gas. *E. coli* also showed positive reaction in MR and Indole test but negative to VP reaction.

Table 1 Primers used in this study

Primers	Sequence (5'-3')	Target gene	Product size	References
ECO-1	GACCTCGGTTTAGTTCACAGA	<i>16s RNA</i>	585 bp	Hassan et al, 2014
ECO-2	CACACGCTGACGCTGACCA			
STX1-F	CACAATCAGGCGTCGCCAGCGCACTTGCT	<i>stx1</i>	606 bp	Talukdar et al, 2013
STX1-R	TGTTGCAGGGATCAGTCGTACGGGGATGC			
STX2-F	CCACATCGGTGTCTGTTATTAACCACACC	<i>stx2</i>	372 bp	
STX2-R	GCAGAACTGCTCTGGATGCATCTCTTGTC			

Table 2 Thermal profile used to amplify 16s RNA gene in *E. coli*

PCR condition	Temperature (°C)	Time	Cycle
Initial denaturation	95	3 min.	1
Denaturation	94	45 sec.	
Annealing	58	45 sec.	30
Extension	72	1 min.	
Final extension	72	7 min	1

Table 3 Thermal profile used to amplify *stx1* gene in *E. coli*

PCR condition	Temperature (°C)	Time	Cycle
Initial denaturation	94	5 min.	1
Denaturation	94	1 min	
Annealing	58	1 min.	30
Extension	72	2 min.	
Final extension	72	5 min	1

Table 4 Summary of the result

Sample	Total	Positive	P value (Chi-square test)
Cloacal swab	60	6	P < 0.001(0)
Egg	60	60	
Feed	15	15	P < 0.001 (0.000796)
Water	15	0	
Total	150	81	

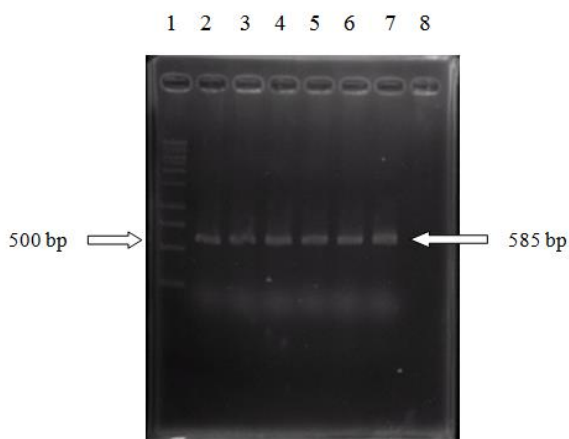


Fig. 1 Detection of *16s rRNA* gene in *E. coli*. Lane 1: 1 kb DNA ladder, Lane 2-6: Tested sample (*E. coli* positive), Lane 7: Positive control, Lane 8: Negative control.

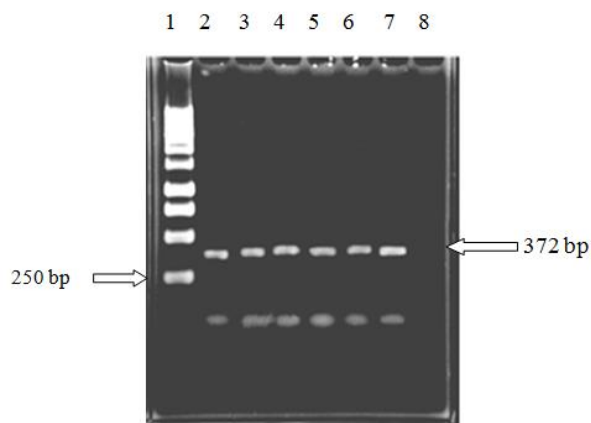


Fig 2 Detection of *stx2* gene in *E. coli*. Lane 1: 1 kb DNA ladder, Lane 2: Positive control, Lane 3 - 7: Tested samples (*stx2* gene positive) Lane 8: Negative control.

Detection of 16s RNA and shiga toxin producing gene in E. coli

All 81 isolates were positive for *16SrRNA* of *E. coli*. The shiga toxin producing *stx2* gene was present in all isolated *E. coli*. But all samples were negative for the presence of *stx1* gene. Among four antibiotics, *E. coli* were found 92.60% and 74.10% sensitive to Ciprofloxacin and Levofloxacin respectively.

Among 120 samples of cloacal swab and egg, 66 were *E. coli* positive. Which was about 55% of the total sample and presence of *E. coli* was significantly higher ($P < 0.001$) in egg than feed. On the other hand, *E. coli* were positively observed in 15 feed and water samples of total 30. The prevalence is 50% and was statistically highly significant at $P < 0.001$. (Table 4)

Discussion

STEC can cause diarrhea, bloody diarrhea and hemorrhagic colitis in humans. STEC infections also frequently result in hemolytic-uremic syndrome (HUS), a life-threatening condition characterized by hemolytic anemia, thrombocytopenia and renal failure (Tarr et al, 2005). 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 (Karmali, 2004). Transmission of STEC occurs through contaminated foods, such as ground beef, through contaminated water and by person-to-person spread (Steinmuller et al, 2006). *E. coli* were found in

cloacal swab 10%, egg 100%, feed 100% and water 00%). The growth of *E. coli* was indicated smooth, circular, greenish black color colonies with metallic sheen on EMB agar and bright pink or red colored colony on MC agar (Kalin et al, 2012, Hasina, 2006), Derakhshantar and Ghanbarpour, 2002). For the confirmation of the presence of *E. coli* in the samples ECO -1 and ECO-2 primer (Hassan et al, 2014) was used in the PCR. The shiga toxin producing *stx2* gene was detected by PCR. The isolates were sensitive to Ciprofloxacin and Levofloxacin. The possession of such factors by the *E. coli* isolates signifies the fact that the intermediate resistance organisms may gain resistance property due to the indiscriminate use of antibiotics. The *E. coli* should be considered as hazardous to health and advocate the preventing risk factors. However, in the present study Ciprofloxacin were proved to be the best antibiotics to treat *E. coli* infection since they were highly effective.

Conclusion

Feed is the main source of shiga toxin producing *E. coli* in poultry. The environmental condition of the farm may be a reason for the highest prevalence of the bacterium in poultry feed, which have been transmitted to human via egg if the egg is not washed properly before cook. The environment inside and outside of the farm should be kept clean and the feed supply should be carefully monitored. Pathogenic *E. coli* may produce disease in poultry if the immunity of poultry is reduced due to various reasons. Ciprofloxacin would be the choice

of drug against *E. coli*. The dose of antibiotic to poultry should be carefully controlled as the intermediate sensitive bacterium can gain resistance to antibiotics.

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