

Isolation and identification of *Escherichia Coli* from apparently healthy chicken of selected areas of Bangladesh

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ABSTRACT

The study was conducted for Isolation and identification of *Escherichia Coli* from apparently healthy broiler, layer, sonali and indigenous breeds of chicken from Bogra, Gazipur and Joypurhat districts of Bangladesh. The study was performed using cultural, morphological, biochemical, molecular characteristics and pathogenicity for identification of isolates. A total of 100 cloacal swab samples were collected (25 from each breed group). Of them 67 were found positive for *E. coli* isolated in EMB agar and MacConkey agar media. The rates of isolation of *E. coli* were 80%, 68%, 64% 56% from layer, sonali, indigenous and broiler respectively. Over-all prevalence of *E. coli* was 67%. Area basis prevalence was found 64%, 71% and 65% at Bogra, Gazipur and Joypurhat, respectively. For molecular detection, PCR was done by using the primers EC16srRNA/F (sequences 5'-GAC CTC GGT TTA GTT CAC AGA-3') and EC16srRNA/R of (sequence 5'-CAC ACG CTG ACG CTG ACC A-3') of 585 bp. Among the four representative isolates of *E. coli* from four different breeds of chicken, only two of them were confirmed as *E. coli* by PCR reaction. The genome of *E. coli* was detected by conventional primers which were not specific for *stx*, *stx*₂, *hlyA* etc. The DNA of two other isolates which were failed to detect by the primer of this study, indicated that the two isolates might be of different strains of *E. coli*. In case of toxin profile bacterial products (Bacteria, Toxin and Bacteria + Toxin) of multidrug sensitive and resistant *E. coli* isolates of four breeds of three different areas were inoculated orally to a total of 120 day-old broiler chicks. No mortality found indicating that the isolates were non-pathogenic. It can be concluded that non-pathogenic *E. coli* were prevailing in the study areas.

INTRODUCTION

There are many types of microorganisms which reside as commensal, in layer, broiler, sonali and indigenous poultry. *Escherichia coli* are one of them. It may be found both in pathogenic and non pathogenic forms, causing major losses of commercially produced poultry as a major pathogen of world-wide importance. The organism was first isolated by Escherich in 1885 from the feces of infants. Later on the organism was found in the intestinal tract of all the vertebrates. Major species of *E. coli* encounter in the lower portion of the intestine of human, warm blooded animals and birds, where they are mostly responsible for gastroenteritis (Pelczar et al., 1986).

Microbial characteristics associated with virulent avian *E. coli* include production of enterotoxin, verotoxin, colicins, and siderophores, type 1 pili and motility, resistance to the lytic action of host complement and antibiotics (Dho and Lafont, 1984; Chulasiri and Suthienkul, 1989; Wooley et al., 1992). *E. coli* produces two distinct enterotoxins : a high-molecular weight, immunogenic, heat-labile toxin (LT) and/or a low-molecular weight, non-immunogenic, heat stable toxin (ST) (Greenberg and Guerrant, 1986). These enterotoxins are responsible for diarrhea in human and neonatal animals. The LTs of *E. coli* from human and porcine origin have been shown to share a common structure that activates adenylate cyclase and cross reacts immunologically with the heat-labile enterotoxin of *Vibrio cholera*. These

enterotoxins have been sergrouped as LT-1 (Pickett et al., 1986). LT-11 a variant of LT-1 has recently been isolated from some isolates of *E. coli*. The LT-11 has characteristics that are similar to those of LT-1 but that are different are different in their antigenic specificity (Holmes et al., 1996).

Strains that cause enteric infections are designated diarrheagenic *E. coli*, a group that includes emergent pathogens with public health relevance worldwide (Nataro and Kaper, 1998). Five categories of *Escherichia coli* have been well associated with diarrhoea in several epidemiological studies (Nataro and Kaper, 1998): enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and Shiga toxin-producing *E. coli* (STEC).

Food animal's viz. cattle, sheep, goats, pigs and poultry are the principal reservoirs of STEC harboring in the intestine and zoonotic transmission occurs through consumption of undercooked meat, unpasteurized dairy products or water contaminated by the faeces. Person-to-person transmission has also been documented (Blanco et al. 2003; Chapman et al. 1997).

In Bangladesh, Isolation and characterization of *E. coli* performed from normal flora of milk, water samples, calf, sheep etc (Choudhury et al., 1967; Nazir et al., 2005 and Hasina, 2006). The study on poultry *E. coli* generally has been performed at BAU campus and its surroundings. There is no study on indigenous and sonali poultry related with *E. coli*. As well as there is no study about *E. coli* in poultry farms of Gazipur, Bogra and Joypurhat region. Keeping in mind all the above facts, the present study was undertaken for Isolation and characterization of *E. coli* from apparently healthy hybrid (broiler and layer), crossbreed (sonali) and indigenous chicken.

MATERIALS AND METHODS

Study area

This research work was conducted at the laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh. Samples were collected

from three different districts of Bangladesh named Bogra, Gazipur and Joypurhat.

Breeds of chicken

Hybrid chicken (Broiler and layer from farms of Bogra Sadar and Kapasia upazila of Gazipur districts), Crossbred chicken (Sonali - Sonali farms of Bogra Sadar and Khetlal upzila of Joypurhat districts) and indigenous chicken (Backyard poultry of Sreepur upazila of Gazipur and Khetlal upazila of Joypurhat districts) were collected.

Collection of samples

Cloacal swab sample were collected from apparently healthy broiler, layer, sonali and indigenous poultry from different poultry farm and back yard poultry of Bogra, Gazipur and Joypurhat districts of Bangladesh. Total number of samples was 100. Of them 25 samples were collected from each group like broiler, layer, sonali and indigenous poultry. All samples were collected with the help of sterile cotton buds and transferring the cotton buds immediately to sterile nutrient broth. At each time of collection, precaution was taken to prevent or minimize cross-contamination of samples. After collection of the samples, they were transported to the laboratory as soon as possible in an insulated foam box with ice to maintain a temperature ranging from 4⁰C to 6⁰C. Microbiological examinations were done immediately to avoid undesirable changes.

Isolation of Bacteria

Primary growth of all kinds of bacteria is performed in nutrient broth. The sample was given in the nutrient broth and incubated for overnight at 37°C. Red hot iron sterilized platinum loop is used to streaking the nutrient broth culture on EMB agar or MacConkey agar for isolated colony. The media containing streaked culture was kept at 37°C for overnight in incubator. The colony of pink/red and greenish black with metallic sheen appears on MacConkey and EMB agar plates respectively.

Identification of the isolates

Colony morphology

Morphological characteristics (shape, size, surface texture, edge, elevation, colour, opacity etc.) developed after 24 hrs of incubation were carefully studied and recorded.

Gram's Staining Method

Gram's staining was performed to determine the size, shape, arrangement and Gram reaction of the isolated strains as described by Merchant and Packer, (1967).

Motility test of E. coli isolates

The motility test was performed according to the method described by Cowan, (1985) to differentiate motile bacteria from the non-motile one.

Biochemical studies

Several biochemical tests were performed for confirmation of the culture. The biochemical tests are sugar tests, indole test, methyl red (MR) test, Voges-Proskauer (VP) test etc.

Indole test

Two ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours. 0.5 ml of Kovac's reagent was added, shaken well and examined after 1 minute. A red colour in the reagent layer indicated indole. In negative case there is no development of red colour.

Catalase test

For this study, a small colony from the pure culture was smeared on a slide. Then one drop of catalase reagent (3% H₂O₂) was added on the smear. The slide was observed for bubble formation. Formation of bubble within few seconds was the indication of positive test while the absence of bubble formation indicates negative result.

Oxidase test

A colony of the test organism was taken by using inoculating loop and smeared across the surface of the impregnated paper. Positive reaction is

recorded by the development of dark purple colour within 10 seconds.

Molecular detection of *E. coli*

Out of 67 *E. coli* isolates of chicken 4 representative (1 from each breed groups) isolates were prepared for PCR examination.

Genomic DNA extraction

DNA from *E. coli* present in prospective sample was extracted using Wizard genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions. Extracted DNA was stored at 4° C until use.

Polymerase chain reaction (PCR)

PCR was performed by targeting the EC16srRNA gene of *E. coli* for species specific detection. A PCR product of 585bp from target gene was amplified by forward (5'-GAC CTC GGT TTA GTT CAC AGA-3') and reverse primer (5'-CAC ACG CTG ACG CTG ACC A-3'). PCR reaction was performed by thermocycler (MJ Mini thermocycler, BIO-RAD, USA) in a total 50 µl reaction volume containing PCR Master mix (Promega, USA), 200 nM of each forward and reverse primer and 2 µl DNA sample. A total of 35 cycles were carried out, each consisting of initial denaturation at 95°C for 5 min, Denaturation at 95 °C for 30 sec, annealing at 50°C for 2 min, elongation at 65°C for 2 min and final extension at 65°C for 10 min.

Agarose gel electrophoresis PCR products were analyzed in 1.5% agarose gel, stained with ethidium bromide and examined against UV light using an image documentation system. The positive sample was recorded based on the appearance of expected size of band in the gel.

Maintenance of stock culture

After inoculation and incubation of the pure culture, stored in sterilized 40% glycerin and were used as stock culture. The equal volume of 40% glycerin and bacterial culture were mixed and was capped tightly and stored at -20°C. The isolated

organisms were given code name for convenience (Merchant and Packer, 1967).

Total viable count of the sample (TVC)

The collected samples were grown in NB for overnight. Then 10 fold dilutions of broth culture of samples were prepared using sterilized PBS and 0.5 ml of each dilution was inoculated to the PCA plates using a fresh pipette for each dilution. The diluted samples were spread on the plate with a sterile L-shaped glass spreader. One sterile glass spreader was used for each plate. The plates were then incubated at 37°C for 24 hours. Following incubation, plates those exhibited 30-300 colonies were counted. For each dilution three plates were used and the mean of the three plates were calculated. The number of bacteria per ml of original sample was obtained by multiplying the number of colonies by diluting factor. The result of TVC was expressed as the CFU per ml of inoculums

Toxin profile of *E. coli*

Production of toxin

Culture filtrate was prepared for the production of toxin. For this, *E. coli* cultures were inoculated into nutrient broth and incubated at 37°C for 24 hours. In the next morning, the overnight cultures were centrifuged for 15-20 minutes at 4000 rpm. The supernatants were collected and transferred into new vials. Then, Gentamycin was added to those vials at a concentration of 5 µg per ml and stored at room temperature for overnight. Crude toxin was prepared by 0.22 µm Millipore filters (Satorius Stedium, Germany). Overnight grown culture was allowed to pellet down by centrifugation at 4000 rpm for 30 minutes and washed with the Tryptose Soy Broth (TSB) then the pellet was re-suspended and diluted with TSB.

For detection of purity of toxin, free from *E. coli*, the supernatants were streaked on EMB agar media with a red hot iron loop and incubated at 37°C for 24 hours. After incubation if no colony was observed the supernatant was used for detection of heat-stable (ST) toxin by oral inoculation to Day-old broiler chicks.

Determination of toxigenic effect on day-old chicks

Each bacterial isolates was divided into three categories which are bacteria, bacteria + toxin and toxin only. A total of 125 broiler chicks were used. Day-old chicks were divided into 25 experimental groups; each group consisted of 5 chicks. The product was inoculated orally by using micropipette (Table 1). They were observed for 10 days.

Table 1
Oral inoculation of *E. coli* and their products.

Bacterial products	<i>E. coli</i> isolated from breeds of birds								Control
	Broiler		Layer		Sonali		Indigenus		
	S	R	S	R	S	R	S	R	
Bacteria	5	5	5	5	5	5	5	5	
Bacteria +Toxin	5	5	5	5	5	5	5	5	5
Toxin	5	5	5	5	5	5	5	5	

S= Multidrug sensitive, R= Multidrug resistant

RESULTS AND DISCUSSION

Isolation and identification of *E. coli*

A total of 100 cloacal swab samples were cultured on Nutrient broth and produced turbidity (Table 2, Figure 1A). The organisms produced circular, smooth, colorless colony on NA (Table 2, Figure 1B). Among them 67 were isolated by producing greenish-black colonies with metallic sheen on EMB agar which is very much characteristic to *E. coli* (Table 2, Figure 1C). They also produced bright pink or red colonies on MC agar (Table 2, Figure 1D). The organisms produced hemolysis on BA with discoloration of the media around the growth of the organisms (Table 2, Figure 1E). On S-S agar suspected isolates produced pinkish colony after 24 hours of incubation (Table 2, Figure 1E). In Gram's staining the organism revealed Gram-negative, pink colour, small rod shaped and they were as single or paired under microscope (Table 2, Figure 2). The strains of suspected *E. coli* isolates were found to be mobile in hanging drop slide preparation. All the isolates fermented the five basic sugars producing acid and gas. Acid production was indicated by the color change from reddish to yellow and the gas production was noted by the appearance of gas

bubbles in the inverted Durham's tubes (Table 3). All the isolates were MR Catalase, Oxidase and

Indole tests positive but VP negative (Table 3, Figure 3).

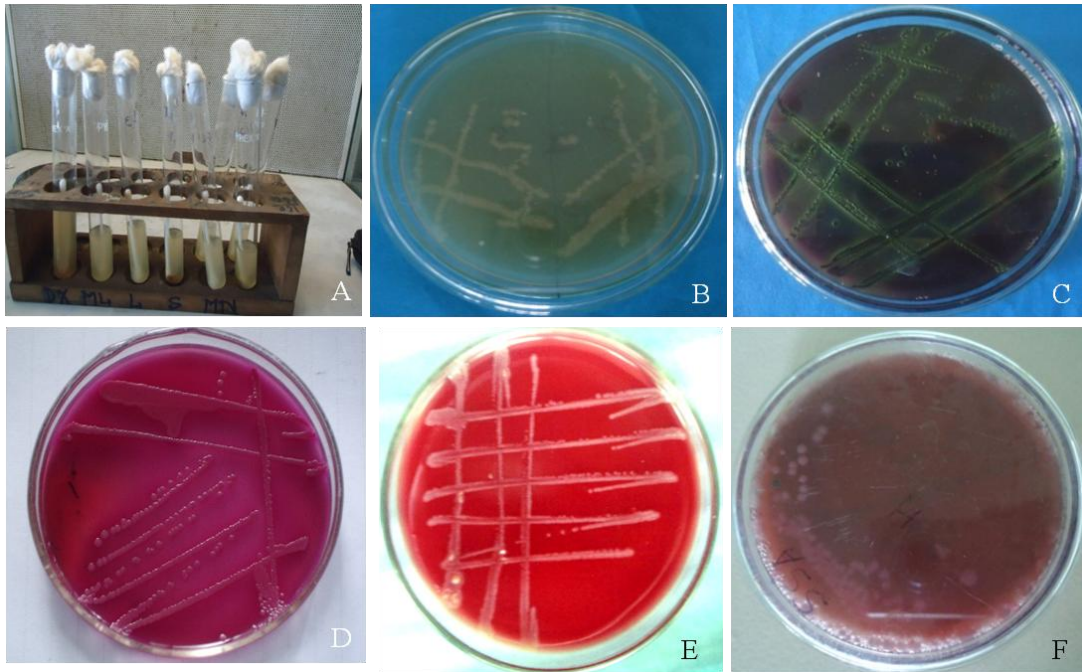


Figure 1

A: Growth of *E. coli* on Nutrient broth, B: Colony of *E. coli* showing smooth, circular, white colony on Nutrient agar. C: Colony of *E. coli* showing metallic sheen on Eosin Methylene blue agar. D: Growth of isolated *E. coli* in Mac Conkey agar showing pink. E: Colorless colony of *E. coli* with hemolysis on Blood agar. F: Pinkish, circular small colonies of *E. coli* on SS agar.

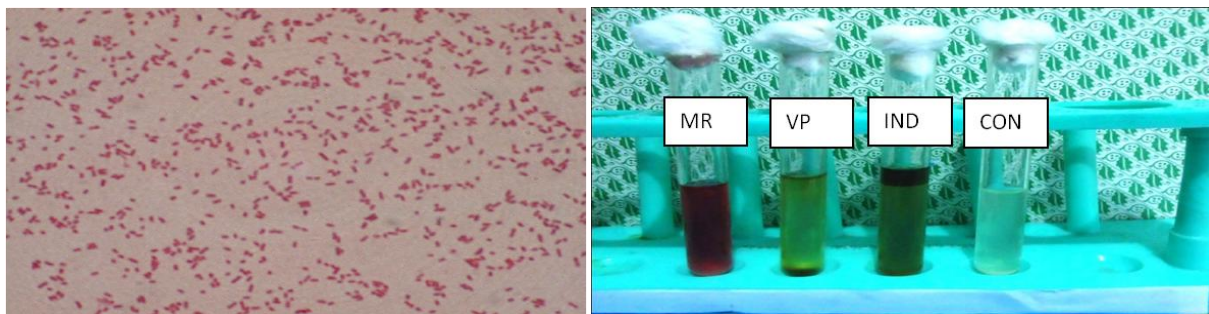


Figure 2 (Left)

Grams staining of *E. coli* isolates showing gram negative, pink coloured, small rod-shaped, single or paired organisms (X1000).

Figure 3 (Right)

MR positive, VP negative, Indole positive of *E. coli*. (MR= Methylene-red, VP= Voges-Proskauer, IND= Indole, CON= Control)

Table 2
Identifying characteristics of *E. coli*.

Breeds of chicken	Motility	Media used	Change in broth and colony character				Gram's staining
			Nutrient broth	Colony on EMB	McConkey agar	S-S agar	
		Nutrient broth, McConkey agar, EMB agar, S-S agar	Turbidity	Black centered, Greenish-black colony with metallic sheen	Bright pink or red colonies	Pinkish colony	Pink, short rod, Gram-negative, single or pair or in short chain
Broiler	+	+	+	+	+	+	+
Layer	+	+	+	+	+	+	+
Sonali	+	+	+	+	+	+	+
Indigenous	+	+	+	+	+	+	+

Table 3
Biochemical characteristics of *E. coli*.

Indole	MR	VP	Oxidase	Catalase	Sugar fermentation test				
					Dextrose	Sucrose	Fructose	Maltose	Mannitol
+	+	-	+	+	+	+	+	+	+

+ = Positive reaction, - = Negative reaction

PCR detection of *E. coli*

Among the four isolates of *E. coli*, two isolates shown positive (Figure 4).

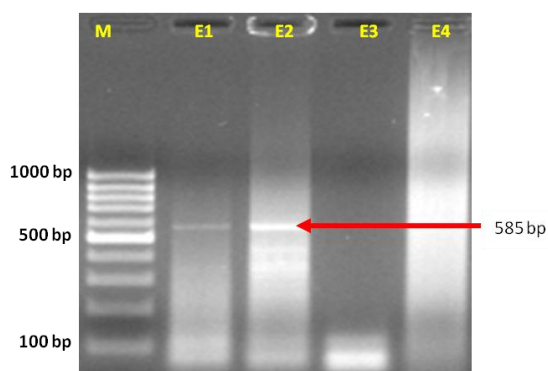


Figure 4
The PCR of *E. coli*. Lane M: 100 bp DNA Marker; Lanes E1-E2: Positive; E3-E4: Negative PCR Products of *E. coli* (585 bp)

Prevalence of *E. coli* in chicken

Out of 100 samples, 67 samples were found to be positive for *E. coli* isolates. The prevalence of *E. coli* in the cloacal sample was 67% (Table 4 and Figure 5).

Table 4
Prevalence of *E. coli* in broiler, layer, sonali and indigenous chicken.

Breeds of chicken	Total samples examined	Samples positive for <i>E. coli</i>	Prevalence (%) of <i>E. coli</i>
Broiler	25	14	56%
Layer	25	20	80%
Sonali	25	17	68%
Indigenous	25	16	64%
Total	100	67	67%

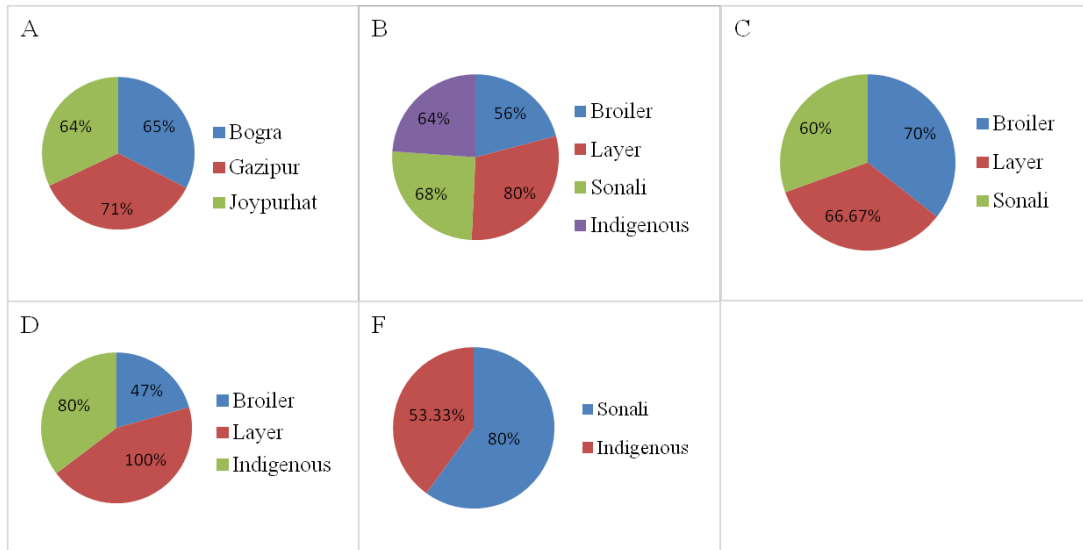


Figure 5

A: Area basis prevalence of *E. coli* in cloacal sample of chicken, B: Breed basis prevalence of *E. coli* in cloacal sample of chicken, C: Prevalence of *E. coli* in cloacal sample of chicken at Bogra, D: Prevalence of *E. coli* in cloacal sample of chicken at Gazipur, E: Prevalence of *E. coli* in cloacal sample of chicken at Joypurhat.

Total viable count

Table 5
Colony morphology and number/ml of organisms found in TVC.

Name of organism	Colony on PCA	Morphology	No/ml
<i>E. coli</i> ,	Smooth, circular, white to grayish white colony.	Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain.	55X10 ⁸ /ml
<i>Salmonella spp</i>	Circular, smooth, white to grayish or white colony	Gram- negative, pink color, small rod shaped organisms arranged in single or paired.	32X10 ⁸ /ml
<i>Staphylococcus spp</i>	Gray, white or yellowish colony	Gram positive, cocci arranged in grapes like clusters.	47X10 ⁸ /ml
<i>Bacillus spp</i>	Thick, grayish-white or cream colored colonies were produced.	Gram-positive, large rod shaped organisms arranged in chain.	5X 10 ⁸ /ml

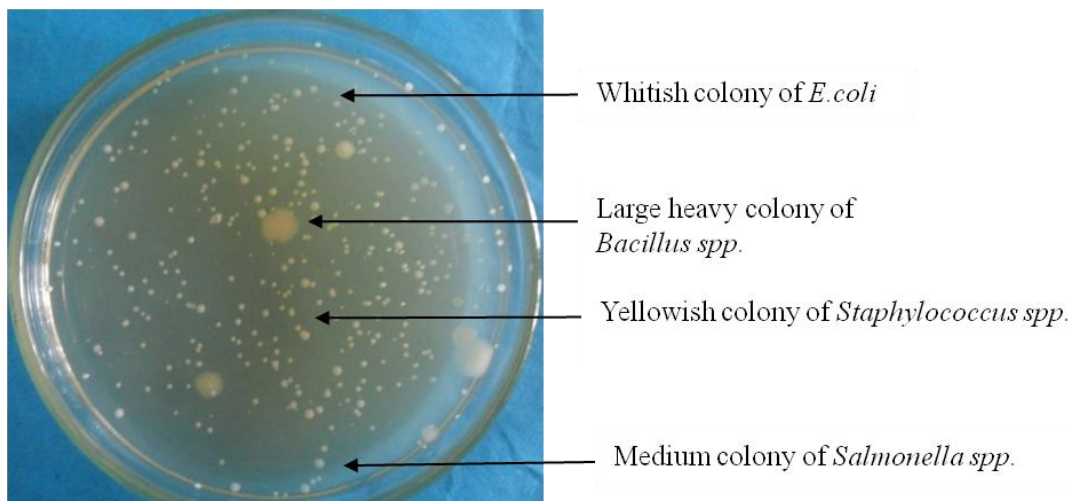


Figure 6
Total viable count

Table 6
Results oral inoculation of *E. coli* isolates and their products in Day-old-chicks.

Products of <i>E. coli</i>	Results of multidrug sensitive isolates of <i>E. coli</i>			Results of multidrug resistant isolates of <i>E. coli</i>		
	No. of inoculation	Clinical signs	Mortality	No. of inoculation	Clinical signs	Mortality
Bacteria	20	0 (0%)	0 (0%)	20	0 (0%)	0 (0%)
Toxin	20	0 (0%)	0 (0%)	20	2 (5%)	0 (0%)
Bacteria +Toxin	20	2 (10%)	0 (0%)	20	8 (20%)	0 (0%)
Total	60	2 (3.33%)	0 (0%)	60	9 (15%)	0 (0%)

Toxin profile

Day-old-chicks

Only some clinical signs with diarrhea, loss of appetite and drowsiness were observed. No mortality found within they seven days of observation. The chicks recovered normally. Summary of results of Toxin profile was shown in Table 6.

Prevalence of *E. coli* in this study was 67%. This was shared with the findings of Hashem *et al.*, 2012 (54.55%); Sampa 2012 (52%) and Jakaria 2011 (78.86%). Slight variations in prevalence rate of *E. coli* in this study were found with those studies. In those study samples were collected

from limited number of breeds of chicken and area. But in our study samples were collected from broiler, layer, sonali and indigenous breeds of chicken of Bogra, Gazipur and Joypurhat districts. Seasonal variation of sample collection also may affect the prevalence rate (Lambie *et al.*, 2000). The prevalence rate of *E. coli* in this study was 56% in broiler, 80% in layer, 68% in sonali and 64% in indigenous breeds of chicken. Area basis prevalence was found at Bogra 64%, Gazipur 71% and Joypurhat 65%. In case of inter area and breed basis prevalence rate of *E. coli* was in broiler of Bogra 70% and Gazipur 47%; in layer of Bogra 67% and Gazipur 100%; in sonali of Bogra 60% and Joypurhat 80% and in indigenous chicken of Gazipur 80% and Joypurhat 53%. In case of breed basis prevalence of *E. coli* was the highest in layer

and the lowest in broiler. In case of area basis prevalence of *E. coli* was the highest at Gazipur and the lowest at Bogra. In case of inter area and breed basis prevalence rate of *E. coli* was the highest in and also the lowest layer of Gazipur. Such variation in prevalence of *E. coli* might be due to farm practice, use of drugs and faulty transportation of samples.

In case of Toxin profile bacterial products (Bacteria, Toxin and Bacteria + Toxin) of multidrug sensitive and resistant *E. coli* isolates of four breeds of three different areas were inoculated orally to a total of 120 day-old broiler chicks. Among them bacteria was inoculated to 40, toxin to 40 and bacteria + toxin to 40 chicks. No clinical signs or mortality found in the chicks inoculated with bacteria. Only 2 (5%) chicks inoculated with toxin shown some clinical signs with diarrhea, loss of appetite and drowsiness but no mortality were found. 8 (20%) chicks inoculated with bacteria + toxin shown some clinical signs with diarrhea, loss of appetite and drowsiness but no mortality were found. All these observation indicated that the *E. coli* isolates of chicken were non pathogenic, which disagreed with the result of Jakaria (2011).

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