

## Determination of lethal effect of *Escherichia coli* toxin in suckling mouse

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### ABSTRACT

The study was conducted for determination of lethal *Escherichia coli* toxin of isolates from two selective strains of Novogen brown (layer) and Hubbard classic (broiler) of Paragon Grand Parent layer farm and Ahana broiler farm. The whole study was performed in two steps of which the first step include isolation and identification of the *E. coli* while the second one included toxin extraction and lethality analysis. A total number of 60 fecal samples, 30 from Novogen brown and 30 from Hubbard classic were aseptically collected using stratified random sampling and brought to the Microbiology laboratory of the Department of Microbiology and Hygiene, Sylhet Agricultural University, Tilagor, Sylhet. For extraction of toxin *E. coli* organisms were isolated and identified from chicken. Out of 60 samples 42 were found to be positive to *E. coli*. The overall prevalence of *E. coli* was 70%. Among the positive samples 22 (73.33%) samples were found to be positive from broilers while 20 (66.67%) were found positive from layers. Toxin was extracted from *E. coli* field isolates and subjected to lethality test in suckling mouse. The minimum lethal dose that causes 33.33 % mortality was 12.5 µl of toxin when administered orally. In this study it was noted that considerable duration needed for production of toxin from dead bacteria to produce lethal effect in suckling mouse.

**Key words:** *E. coli*, chicken faeces, lethal effect, mouse.

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### INTRODUCTION

Poultry rearing is now a rising sector in Bangladesh, because an almost assured and quick good return could be achieved in relatively short period of time by a moderate amount of investment. *Escherichia coli*, a major pathogen of world-wide importance in commercially produced poultry is responsible for major losses to the poultry industry. *E. coli* causing colibacillosis with manifestations of airsacculitis, pericarditis, septicemia, and death of the birds (about 28% death in Sonali birds) (Biswas et al., 2006). The enteric *E. coli* are divided into six groups on the basis of their virulence properties such as enterotoxigenic (ETEC which is the causative agent of diarrhea in humans, pigs, sheeps, goats, cattle, dogs and horses), enteropathogenic (EPEC, causative agent of diarrhoea in humans, rabbits, dogs, cats and horses), enteroinvasive (EIEC,

found only in humans), verotoxigenic (VTEC, found in pigs, cattle, dogs and cats), enterohaemorrhagic (EHEC, found in human, cattle, and goats) and enteroaggregative *E. coli* (EAaggEC, found only in human). *E. coli* is considered as the normal bowel flora of different species of mammals and birds but some strains of *E. coli* possess pathogenic character. Microbial characteristics associated with virulent avian *E. coli* include production of enterotoxin, verotoxin, colins and siderophores, type 1 pill and motility, resistance to lactic action of host complement and antibiotics (Dho and Lalont, 1984 and wooley et al., 1992).

*E. coli* a member of the family *Enterobacteriaceae* is a short Gram negative, non-spore forming and usually peritrichous and fimbriate bacillus. *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). 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 cholerae*. These enterotoxins have been serogrouped 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 in their antigenic specificity (Holmes *et al.*, 1996). Two types of heat stable enterotoxins (STs) have been described, based on their methanol solubilities: a methanol-soluble molecule with biologic activity in suckling mice, rats and piglets (referred to as STa) and a methanol-insoluble molecule with biological activity in piglets (referred to as STb) (Greenberg and Guerrant, 1986). So, it is necessary to emphasize the detection of *E. coli* from poultry that may cause severe illness in chicken as well as in human being. Therefore, determination of lethal effect of *E. coli* toxin isolated from layer and broiler birds is important to reveal the distribution of different strains of *E. coli* organism in the environment of poultry. The present research work was undertaken to detect the lethal effects of toxin extracted from isolated *E. coli* from broiler and layer chicken.

## MATERIALS AND METHODS

### Field samples

A total number of 60 fecal samples (30 from Novogen brown and 30 from Hubbard classic) were aseptically collected from Paragon Grand Parent layer farm and Ahana broiler farm of Moulvibazar district and brought to the Department of Microbiology and Hygiene in Sylhet Agricultural University.

### Experimental design

The whole study was performed in two steps. The first step includes isolation of *E. coli* culture and the second step leads to toxin extraction and lethality analysis.

### Isolation and identification of *E. coli*

### Culture

At first samples were inoculated in nutrient broth with the help of sterile inoculating loop and incubated at 37° C for 24 hours. With the help of sterile inoculating loop culture from nutrient broth were inoculated into nutrient agar and incubated at 37° C for 24 hours. The incubated media were then examined for the growth of bacteria. Colonies from nutrient agar were subcultured into MacConkey agar, SS agar and incubated at 37° C for 24 hours. The incubated media were then examined for the growth of bacteria.

From Mac Conkey agar bright pink coloured colonies were sub cultured onto EMB agar and from SS agar pink to red coloured colonies were sub cultured on EMB agar. The cultures showing characteristic colony morphology of *E. coli* was repeatedly sub cultured onto EMB agar until the pure culture with homogenous colonies were obtained.

### Identification of the isolates

Morphological characteristics (shape, size, surface texture, edge, elevation, colour, opacity etc.) developed after 24 h of incubation were carefully studied as described by Merchant and Packer (1967) and recorded.

### Biochemical test

The suspected isolated organism were subjected to different biochemical tests, such as sugar fermentation test for acid or acid and gas production, Indole production test, Methyl-red and Voges-proskauer (VP) test. Standard methods were followed for conducting these tests as described by Cowan (1985) during the experiment.

### Toxin extraction and purity test

Pure *E. coli* culture was allowed to grow in nutrient broth. *E. coli* were grown at peak. The bacterial cells were disintegrated by ultrasonic sound produced by sonicator. Toxin were liberated from died bacterial cell. Centrifugation was done at 4000 rpm for 20 minutes and supernatant fluid was taken in another test tube. The supernatant

fluid was filtered by a millipore filter paper (0.45µm) for purity. The purity of the toxins were tested by streaking the supernatants on EMB agar and incubated at 37<sup>o</sup> C for 24 hours.

### **Determination of lethal effect of *E. coli* enterotoxin**

Swiss Albino suckling mice of 1-4 days old were separated from their mother immediately before use and divided into groups and marked on their head A, B, C, and D. Every group contains 3 mice. 50 µl of toxin was administered orally in Group D. Then two fold dilutions were made. 25µl Toxin+25µl Distilled water given to Group C, 12.5µl Toxin+37.5 µl Distilled water given to Group B and Group A kept as control. The mice were kept with their mother at normal feeding condition and observed for 24 hours for any mortality. Again toxin was prepared from 48 hours broth and procedure same as step 1 was done and observed for 24 hours. The minimum dose that causes mortality was identified from first two steps. Again toxin was prepared from 72 hours of broth and was administered orally in another group E at the minimum dose that causes mortality in the first 2 steps and group A kept as control and observed for 24 hours. Lethal effect was calculated according to mortality of mice after 24 hours of incubation.

## **RESULTS AND DISCUSSION**

### **Isolation and identification of *E.coli* from chicken**

*E.coli* isolates produced turbidity in nutrient broth and on Nutrient agar (NA) the isolates produced translucent, opaque, smooth colonies. Samples inoculated onto MacConkey agar plates produced bright pink or red colonies after overnight incubation (Image 1). In S-S agar the isolates produced pinkish colony after 24 hours of incubation. In EMB agar, the fecal isolates produced large, smooth and sticky colony with yellow green metallic sheen was suspected for *E. coli* (Image 2). The above colony characteristics of the isolates confirm the isolate as *E. coli*. In addition the thin smears prepared with the colony from EMB agar for Gram's staining revealed

Gram-negative, pink colored, small rod shaped appearance, arranged in single or paired or in short chain under the microscopic examination, which further confirm the organism as *E. coli*.

In biochemical test 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. All *E. coli* isolates were Indole test positive, motility, indole and urea were positive in MIU medium. In the MR test the appearance of the red colour in the media after the addition of 3 ml methyl red with the cultural growth was observed and thus indicating positive MR test. In the Voges-Proskauer (V-P) test, no change of colour of the media was observed after the addition of 3 ml of 3% KOH to 3 ml V-P broth media with the cultural growth of the isolated *E. coli* and thus indicated that the isolated *E. coli* from chicken was negative for V-P test. In TSI medium butt and slant were yellow indicating production of acid.

For toxin extraction *E. coli* organisms were isolated and identified from chicken. Out of 60 samples 42 were found to be positive to *E. coli*. Among the positive samples, 22 were from Paragon grand parent layer farm, Moulvibazar and 20 were from Ahana broiler farm, Moulvibazar. The overall prevalence of *E. coli* was 70% which is in agreement with the result of Masuder *et al.*, (2008). The highest recovery was found from layer faeces (73.33%) which were 66.67% in broiler. Nazir (2004) observed 65% prevalence of *E. coli* in faecal samples of broiler, 60% in layer. The present findings were higher than the report of Nazir (2004).

Different media were used to isolate *E. coli* from faeces. The greenish-black colonies with metallic sheen on EMB agar were presumptively identified as *E. coli* (Pelczar *et al.*, 1998). Several biochemical tests were performed for confirmation of *E. coli*. They were characterized by their ability to ferment glucose, sucrose, lactose, maltose, mannitol and sorbitol to produce gas (CO<sub>2</sub>), positive for indole test and MR test and negative for VP and Citrate utilization test which are in agreement with the

reports of Buxton and Fraser (1977) and Honda et al. (1982)

### Lethal effect of *E. coli* enterotoxin

No mortality was found after 24 hours with 24 hours broth (data not shown). For 48 hours broth, 3 mice were found dead from group D and C (Table 1) after 24 hours of oral administration. One mouse was found dead from group B which

indicated minimum lethal dose as group B contained lowest dose of toxin.

Toxin was also prepared from 72 hours broth and administered orally in group E at minimum lethal dose of 12.5  $\mu$ l (Table 2). Group A was kept as control. The mice were observed at room temperature at normal feeding condition. After 24 hours, 1 mouse was found dead from group E.

Table 1

Lethal effect of toxin extracted from *E. coli* isolates (48 hours broth).

Group	No. of suckling mice	Dose	No of dead mice after 24 h	Average toxicity (%)
A	3	Control	0	0
B	3	12.5 $\mu$ l	1	33.33
C	3	25 $\mu$ l	3	100
D	3	50 $\mu$ l	3	100

Table 2

Lethal effect of toxin extracted from *E. coli* isolates (72 hours broth).

Group	No. of suckling mice	Dose	No of dead mice after 24 h	Average toxicity (%)
A	3	Control	0	0
E	3	12.5 $\mu$ l	1	33.33



Figure 1  
Growth of *E. coli* on Mac Conkey agar showing bright pink color colonies.

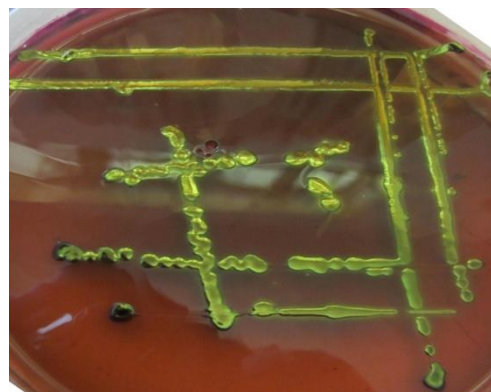


Figure 2  
Growth of *E. coli* on EMB agar showing greenish black colonies with metallic sheen.

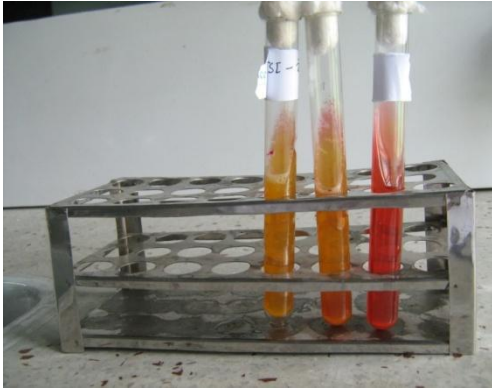


Figure 3  
*E. coli* in TSI medium showing yellow color Butt and Slant (left two test tubes) and control (right).



Figure 4  
Mice of Group E before (upper) and after (lower) inoculation of minimum lethal dose of toxin.

In this study, the minimum lethal dose of suckling mouse was 12.5  $\mu$ l. It can cause 100 % mortality with at least 25  $\mu$ l dose of toxin. The study indicated that the presence of *E. coli* in chick faeces is alarming and mortality due to the toxic effect of this organism can cause economic loss in poultry industry.

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