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Enterotoxin Profiling and Antibiogram of *Escherichia coli* Isolated from Poultry Feces in Dhaka District of Bangladesh

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Bacterial diseases remain a threat to the poultry industry and countermeasures to prevent and control them are needed to prevent production losses. With the continued threat of exotic and emerging diseases and the problem of the use of antibiotics in animal production, there is a serious and urgent need to find safe and practical alternatives to prevent or control pathogens. That's why, our research has addressed in different poultry farms for detection of toxigenic *Escherichia coli* from poultry chicken feces adjacent to the Dhaka city, during the period of January 2009 to June 2009 and characterize their ability to produce enterotoxin and also examined the antibiogram of the isolates. A total of 60 fecal samples were collected and *E. coli* were isolated and identified by conventional cultural, biochemical and motility test.The heat-stable toxins were determined by Suckling Mice Assay (SMA). The overall prevalence of *E. coli* was 100%. Among the isolates of *E. coli*, only 45% and 14% isolates showed β hemolysis and α hemolysis, respectively. Congo red binding has been used as a potential virulence marker and 69 % *E. coli* isolates in the present study showed congo red binder, among which, 59% isolates were hemolytic. A total of 20% and 5% isolates showed the presence of LT and ST enterotoxin in their guts by Suckling Mice assay, respectively. The antibiotic sensitivity pattern shown that the isolates were highly sensitive (100%) to imipenem, pipercillin, neomycin and ceftrixone. Results from this study revealed the high prevalence rate of *E. coli* isolates with variable resistance against a wide range of antimicrobial drug. It may suggest that the high resistance of *E. coli to* antibiotics as well as their toxigenic nature constitutes a threat not only to poultry industry of Bangladesh but also possesses a serious threat to public and animal health.

Poultry is one of the fastest growing industries with a bright future in Bangladesh. Poultry meat contributes substantially to the human diet (1). In Bangladesh, poultry meat is an important, low-cost source of animal protein. This encourages the consumption of poultry products by a large number of consumers. Poultry meat is increasingly used by the growing rural and urban populations. Two kinds of poultry slaughtering are used in Bangladesh. One is an automated poultry slaughtering process established recently, whereby automated systems are used for scalding, plucking, eviscerating, rinsing, and packaging carcasses. The second one is the traditional slaughtering, which is commonly practiced in shops under poor hygienic conditions. More than 90% of poultry slaughtering in Bangladesh is done by traditional procedures. In these shops, the conditions are favorable for potential contamination by pathogens, which may originate from the animal itself and from the environmental factors (water, litter, air). Thus, controlling microbial contamination in poultry meat during slaughtering, processing, storage, handling, and preparation becomes a great challenge (2, 3).

It is well documented that the contamination of food with pathogens is a major public health concern worldwide (4). Because of the relatively high frequency of contamination of poultry with pathogenic bacteria, raw poultry and products are reported to be responsible for a significant number of cases of human food poisoning (5). In the absence of hygienic conditions, birds may be highly exposed to the wide range of bacterial pathogens such as *Listeria monocytogenes, Campylobacter,* and other enteric bacteria (6). Among these, this poultry industries are most vulnerable to attack by *Escherichia coli* that increased mortality of poultry chickens. *E. coli* is one of the common microbial flora of gastrointestinal tract of poultry and human being including other animals but may become pathogenic to both (7, 8). 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 (9). *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) (10). 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* LT-II is found primarily in animal *E. coli* isolates and rarely in human isolates but in neither animals nor in humans has it been associated with disease. Thus the term LT refers to the LT-I form (11).

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Heat stable enterotoxin has two subtypes-STa and STb. STa toxins are produced by ETEC and several other Gram-negative bacteria including *Yersinia enterocolitica* and *V. cholerae* non-O1, while STb has been found only in ETEC (8). In Bangladesh, for many years, antibiotic is randomly used for treatment purpose.

Antibiotics are extensively used as growth promoters in poultry production or to control infectious disease. Due to enormous exploitation of antibiotics in the field of veterinary medicine, an increased number of resistant bacterial strains were developed in recent years. Moreover, antibiotic treatment is considered the most important issue that promotes the emergence, selection and spreading of antibiotic-resistant microorganisms in both veterinary and human medicine (12, 13). It was stated by well established evidence that antibiotics can lead to the emergence and dissemination of resistant *E. coli* which can then be passed into people via food or direct contact with infected animals. These resistant microbes may function as a potential source in the transportation of antimicrobial resistance to human pathogens (14, 15). Acquired multi drug resistance to antimicrobial agents creates an extensive trouble in case of the management of intra and extra intestinal infections caused by *E. coli*, which is a major source of illness, death, and increased Health care costs (16). Therefore, present study, focused on the detection of toxigenic *E. coli* and their antibiogram profiling.

MATERIALS AND METHODS

Sample collection. A total of 250 fecal samples from poultry chickens were collected from different poultry farms adjacent to Dhaka city, using sterile cotton buds and transferring the buds immediately to sterile nutrient broth. The samples were wrapped with ice, kept in box, and transferred within 30 minutes.

Identification of *E. coli*. The isolates of *E. coli* were identified by observing gross colony morphology using MacConkey agar, Eosin Methylene Blue (EMB) agar, Salmonella-Shigella (SS) agar, Gram staining properties and motility as described by Merchant and Packer in 1967 (17). In the basal level, these isolates were subjected to different biochemical tests such as sugar fermentation test, Indole production test, Methyl-Red and Voges-Proskauer (MR-VP) test described by Cowan in 1985 (18). Pure culture of *E. coli* was obtained using EMB agar.

Maintenance of stock culture. Nutrient agar slants were used to maintain the stock culture for each of the *E. coli* isolates. The *E. coli* isolates were introduced in the slant by streaking and were incubated at 37 °C for 24 hours. Finally, glycerol was overlaid and the culture was kept at room temperature.

Hemolytic Property. The hemolytic activity of *E. coli* strain isolates was tested on a blood agar plate containing 5% defebrinated sheep blood. Initially, the strains were subcultured onto a nutrient agar (NA) plate and incubated at 37 °C overnight. The lower surface of the blood agar plate was marked into several squares of 0.7 cm each and labeled with the test strain number. A portion of the colony from the overnight *E. coli* culture on the NA plate was transferred into one chamber having a corresponding isolate number. The inoculated plates were incubated aerobically at 37 °C overnight. Following incubation, hemolytic strains were identified according to their types/extent of hemolysis by each colony onto blood agar plate (19).

Congo red Binding. To evaluate the Congo red binding, bacteria were grown at 37º C for 18h on tryptic soy agar supplemented with 0.02% congo red and 0.15% bile salt. Positive colonies appeared red, whereas negative colonies were pale. Based on the intensity of red color, the binding was scored as +, ++, and +++.

Toxin profile. Overnight broth cultures were centrifuged at 1500 rpm for 20 minutes and supernatants were collected and transferred into new vials, then gentamycin (5ug/ml) was added and kept at room temperature for overnight, following the method described by Gianella (20). The purity of the toxins were tested by streaking the supernatants on EMB agar and incubated at 37 °C for 18 hours. Absence of any colony leads the supernatant to be used for detection of heat-stable (ST) toxin by Infant Suckling Mice Assay (SMA).

Enterotoxigenic effect in suckling mice and determination of ST toxin by SMA. SMA was used for the detection of heat-stable toxin. Sixty Swiss Albino suckling mice of 1-3 days old were separated from their mother immediately before used and were divided into two groups A and B consisting of three mice in each group. An amount of 2.5µl crude culture supernatant containing enterotoxin were administered to the mice of group A through oral route with micropipette. Group B mice were kept as control. Mice were incubated at 37 °C for 18 hours to observe toxic effects. The weight of the gut and the remaining carcass were taken and the ratio was calculated for each mouse. The average ratio of less than 0.070 was considered negative while 0.070«0.085 was considered positive for ST toxin (20).

Drug sensitivity test. Single disc diffusion method (21) was used to examine bacterial susceptibility to antimicrobial agents. A total of 21 antibiotic discs (Becton Dickinson, U.S.A.) with Erythromycin 15µg, Tetracycline 30µg, Norfloxacin 10µg, Cefixine 5µg, Chloramphenicol 30µg, Ampicillin 10µg, Gentamycin 10µg, Neomycin 30µg Riphampicin 15µg, Kanamycin 20µg, Penicillin 10µg, Streptomycin 10µg, Ciprofloxacin 5µg, Nalidixic Acid 30µg, Ceftriaxone 30µg, Nitrofurantoin 25 µg, Bacitracin 30µg, Piperacillin 20µg, Amikacin 15µg, Trimethoprim/sulfamethoxazole 30ug and Imipenem 20µg were used. A single colony was introduced into 2 ml of Mueller-Hinton broth. The broth culture was then allowed to incubate at 37 °C for 4 hours. The turbidity of the growing cultures was then adjusted to a 0.5 McFarland standard. A sterile cotton swab was dipped into the adjusted suspension within 15 min and excess broth was purged by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The swab was then spread evenly over the entire surface of the plate of LB agar to obtain uniform inoculums. The plates were then allowed to dry for 3-5 min. Antibiotics impregnated discs were then applied to the surface of the inoculated plates with sterile forceps. Each disc was gently pressed down onto the agar to ensure complete contact with the agar surface. Even distribution of discs and minimum distance of 24 mm from center to center were ensured. Five discs (four antibiotics discs and one blank disc as control) were placed in each petri dish. Within 15 min of the application of the discs, the plates were inverted and incubated at 37 °C. After 16-18 h of incubation, the plates were examined, and the diameters of the zones of complete inhibition were measured in millimeter scale. The zone diameter for individual antimicrobial agents was then translated into susceptible, intermediate and resistant categories according to the interpretation table of the Becton Dickinson Microbiology Company, USA.

RESULTS AND DISCUSSION

Isolation and identification of *E. coli*. The results of gross colony morphology on EMB, MacConkey and Salmonella-Shigella (SS) agar followed by Gram staining and motility tests are summarized in Table 1. For biochemical characterization, a series of biochemical tests, selective for *E. coli* were performed with the suspected Gram-negative rod shaped bacteria. All the isolates fermented five basic sugars producing acid and gas.

TABLE 1. Identifying characteristics of *E. coli*

	Motility	Colony Characteristics			Morphology & arrangement	Staining Properties
		EMB agar	MacConkey agar	SS agar		
Poultry chicken Feces (n=60)	+ve[a]	Green metallic sheen	Circular pink	Light pink	Short rod, single, pair or in short chain	Gram negative

[a] Results based on motility test . Plus(+) indicates positive reaction.

All the isolates were methyl red positive, Voges-Proskauer test negative and indole test positive (Table 2). All poultry feces samples (n=60), were found to be positive for *E. coli* isolates. In this study, the prevalence of *E. coli* in the fecal sample was 100.0% (Table 2). Bhattacharjee et al. (22) reported 40.82% prevalence of *E.coli* in chicken from Bangladesh but Nazir (23) stated the overall prevalence was 62.5% from chicken, however, the present study showed 100% prevalence of *E. coli* from chicken feces.

TABLE 2. Biochemical tests used for identification of *E. coli*

No. of Isolates	Biochemical Test	Reaction[a]
250	Catalase	Positive
	TSI	
	Indole Production	
	Nitrate Reduction	
	Methyl Red	
	Gelatin liquefaction	Negative
	Urease	
	Voges Proskaur	
	Simmon's citrate	
	H_2S	

[a] Results based on types of substrate utilization

Congo red and Hemolytic profile. The hemolytic activity, more widely known as the Kanagawa phenomenon, is one of the important virulence markers. Red blood cell of the host organism is lysed due to the presence of hemolysin gene which in turn helps in the spread of the pathogen in the host blood. The hemolytic activity of *E. coli* is related to the presence of hemolysin genes (24-26). In this study, alpha hemolytic activity was detected in 45 % isolates and β hemolytic activity was detected in 14 % isolates out of 250 isolates of *E. coli* on blood agar plates (Table 3). Gamma hemolytic or non hemolytic strain detected in 41% of 250 isolates. 69% *E. coli* isolates in the present study were positive for congo red binder and among them 59% isolates were hemolytic (Table 4). Congo red binding has been used as a potential virulence marker (27), which indicated that the isolates were pathogenic.

Toxin profile. To determine the presence of heat labile toxin, enterotoxigenic *E. coli* species (ETEC) were detected based on mortality and survivability of 1-4 day old mice within 24 hours of inoculation. Out of 166 isolates, 33 (20%) *E. coli* isolates were found positive for enterotoxigenicity. It can be speculated that the toxic effects could be due to heat-labile (LT) toxin which supports previous data by another research group (28). Positive results of ETEC denoted in the following Table 5. The results showed that only 8 isolates of *E. coli* from 166 *E. coli* isolates induced fluid accumulation in SMA (Table 6).

TABLE 3. Hemolytic identification of *E. coli*

Isolates	Colony characteristics	Types of hemolysis	Percentage
n=250	Hemolytic with small zone or partial zone, grey white moist, glistening, opaque, circular, convex colonies with entire edge	Alpha hemolysis	45%
	Hemolytic with large zone , circular, convex colonies with entire edge	Beta hemolysis	14%
	Non hemolytic, grey white moist, glistening, opaque, circular, convex colonies with entire edge	Gamma hemolysis	41%

Therefore, these results suggest that the toxic effects could be due to the presence of stable toxin. From this study, it is evident that frequency of heat stable toxin (ST) is lower than Labile toxin (LT), similar report has also been reported from Guerrant et al. (29) and Echeverria et al. (30), where they found only 4% ST isolates on their study.

TABLE 4**.** Correlation between Hemolysis and Congo red binding

Types of Hemolysis	Hemoysis (Percentage)	Congo red positive n (%)
Alpha (n=112)	45%	108 (45%)
Beta (n=37)	14%	34 (14%)
Gamma (n=101)	41%	24 (10%)
n=250		166 (69%)

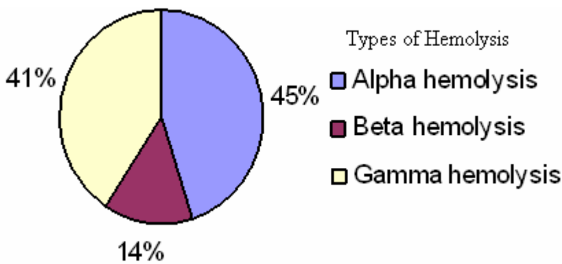

FIG. 1. Hemolytic Pattern of *E. coli*

Antibiotic resistance profile. The antibiotic susceptibility pattern of *E. coli* isolates from the poultry chicken feces samples has been outlined in Table 7. Resistance spectrum of *E. coli* for 21 antibiotics tested in descending order was respectively trimethoprim/sulfamethoxazole, nalidixic acid, bacitracin, ampicillin, streptomycin, riphampicin, penicillin, ciprofloxacin erythromycin, kanamycin, cefixine, tetracycline, chloramphenicol, nitrofurantoin, gentamicin and neomycin. It was shown that 20% strain were found resistant against neomycin and gentamicin and 100% strains were found resistant to trimethoprim/sulfamethoxazole, nalidixic acid, bacitracin and ampicillin (Table 7). No strain was found sensitive to erythromycin, riphampicin, kanamycin, cefixine, penicillin. All these findings were almost similar to the previous data published by several research groups (31-33). The highest sensitivity (100%) was recorded for the antibiotic ceftriaxone, piperacillin, amikacin and imipenem and it was followed by norfloxacin (86%), gentamicin (80%), neomycin (60%), chloramphenicol (55%), nitrofurantoin (45%), tetracycline (36%) and streptomycin (10%). In a previous study, Tricia et al. (34) reported that 43% isolates of *E. coli* were resistant to ampicillin but no isolate was found resistant to gentamicin, but in our study, 80% strain of the *E. coli* was found sensitive to gentamicin. This is not consistent with the previous study. All 250 isolates examined in this study showed multiple resistances to at least 14 to 16 antibiotics. Similar findings on multiple drug resistance of *E. coli* strains has been reported from Bangladesh and other parts of the world (35-37).

TABLE 5**.** Determination of heat-labile (LT) toxin by SMA

Source	Crude culture supernatant			Positive Effect	No. of isolates tested	Positive for ETEC n (%)
	Quantity	Route	Incubation			
Poultry chicken Feces	25ul	Oral	24 Hrs	Death (3/3)	108	24
					34	9
					24	0
		Total			166	33 (20)

TABLE 6. Determination of heat-stable (ST) toxin by SMA

Source	Crude culture supernatant			Gut weight and body weight ratio		No. of isolates tested	Positive (%)
	Quantity	Route	Incubation	Ranges of obtained value	Ranges of standard value		
Poultry chicken Feces	0.1 ml	Stomach	4 Hrs	0.092 to 0.103	Positive if it is ≥ 0.089	108	7
						34	1
						24	0
Total						166	8 (5%)

TABLE 7. Antibiogram profiling of *E. coli* against different antibiotics

Antibiotics	Sensitivity groups of *Escherichia coli* isolates					
	Resistant		Intermediate		Sensitive	
	% of strains	Inhibition zone (mm)	% of strains	Inhibition zone (mm)	% of strains	Inhibition zone (mm)
Chloramphenicol (30µg)	45.00	<25	--	26-28	55.00	>29
Erythromycin (15µg)	80.00	<15	20.00	16-20	--	>21
Ampicillin (10µg)	100.00	<13	00.00	14-15	00.00	>17
Gentamicin (10µg)	20.00	<06	00.00	7-9	80.00	>10
Riphampicin (5µg)	90.00	<15	10.00	17-19	--	>20
Neomycin (30µg)	20.00	<12	20.00	13-14	60.00	>15
Kanamycin (20µg)	76.00	<13	24.00	14-17	--	>18
Cefixine (5µg)	68.00	<14	32.00	14-15	--	>18
Penicillin (10µg)	88.00	<25	12.00	26-27	--	>29
Tetracycline (30µg)	52.00	<25	12.00	26-28	36.00	>29
Streptomycin (10µg)	90.00	<06	00.00	7-9	10.00	>09
Norfloxacin (10 µg)	--	<12	14.00	13-15	86.00	>07
Ciprofloxacin (5 µg)	82.00	<30	18.00	30-33	--	>33
Ceftriaxone(30 µg)	--	<15	--	17-19	100.00	>16
Nalidixic acid(30µg)	100.00	<10	--	11-15	--	>16
Pipercillin(10µg)	--	<06	--	7-9	100.00	>10
Nitrofurantoin (300 µg)	25	<14	--	11-15	--	>07
Bacitracin (30 µg)	100.00	<12	--	13-14	--	>15
Amikacin (20µg)	--	<14	--	16-17	100.00	>18
Imipenum(20µg)	--	<14	--	16-17	100.00	>18
Trimethoprim/sulfamethoxazole(30 µg)	100.00	<10	--	11-15	--	>10

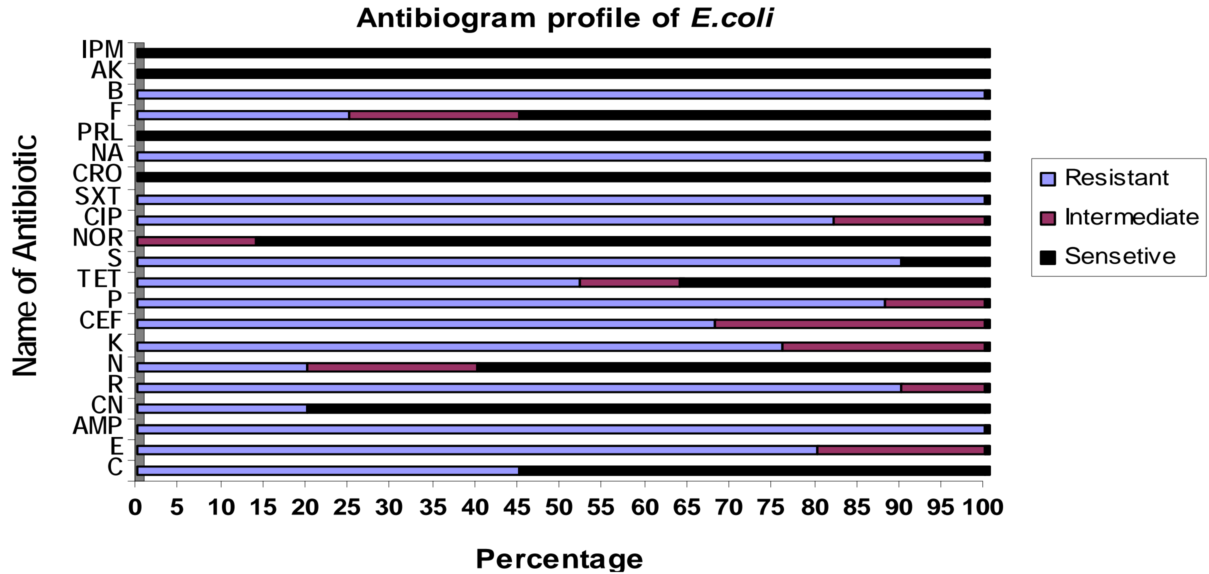

FIG. 2. Graphical representation of Antibiogram profiling by poultry *E. coli*.

In fine, our study reveals that, the poultry borne bacteria could be a good source as vectors in transmitting drug resistance. Attention is to be paid for personal hygiene in processing and handling of poultry and poultry products and the excess use or abuse of antibiotics should be reduced or stopped by the judicious application of antibiotics for the safety of public health in Bangladesh.

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