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Detection of Antibiotic Resistance Genes in *Escherichia coli* Isolated from Chicken Eggs

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Abstract

Total 150 chicken eggs were randomly collected from farms, households, and markets of Rajshahi and Naogaon districts. Isolation and identification of *E. coli* was done by using cultural and staining characteristics, and biochemical properties. Antibiotic sensitivity assay was also performed for the isolated *E. coli* by using disc diffusion method. Characterization and detection of antibiotic resistance genes in *E. coli* isolates were done through PCR analysis. Prevalence of *E. coli* was 30.00% in chicken eggs in Rajshahi and Naogaon districts. Results of antibiotic sensitivity patterns of the isolated *E. coli* showed 57.78%, 33.33%, 31.11%, 28.89%, 26.67%, 17.78%, 15.56%, 13.33%, 13.33%, and 11.11% resistance to ciprofloxacin, doxycycline, oxytetracycline, ceftriaxone, amoxycillin, erythromycin, levofloxacin, enrofloxacin, gentamycin, and neomycin, respectively. The isolates also showed 94.44%, 82.22%, 80.00%, 77.78%, 66.67%, 60.00%, 55.56%, 48.89%, 46.67%, and 33.33% sensitive to neomycin, enrofloxacin, erythromycin, levofloxacin, gentamycin, ceftriaxone, oxytetracycline, doxycycline, amoxicillin, and ciprofloxacin, respectively. All 45 phenotypic identified isolates were confirmed as *E. coli* by PCR analysis. Genotypic identified isolates of *E. coli* were further characterized by PCR to detect antibiotic resistance genes. Out of 45 *E. coli* isolates 37 (82.22%) showed tetracycline resistance *(tetA*) gene, 32 (71.11%) showed tetracycline resistance *(tetB*) gene, and interestingly all 45 (100%) isolates showed quinolone group resistance (*gyrA*) gene by PCR analysis.

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Introduction

Poultry industry is a promising sector for poverty elevation in Bangladesh. Poultry is a significant agricultural industry that began in the 1980s (Hoque *et al*., 1997). The poultry business in Bangladesh predominantly produces chickens, though duck, pigeon, quail, geese, turkey, and guinea fowl are also available. Bangladesh is now producing 15.52 billion eggs versus a current yearly need of 17.13 billion. In Bangladesh, the poultry sector provides 36% of total protein intake through egg and meat consumption. Chicken eggs and meat are the cheapest source of animal protein in Bangladesh and it is accepted by all religious, economic, social, and demographic groups (Simon 2009). Chicken eggs are one of the most important foods of animal origin which contain many nutrients. High-nutritional-value eggs containing omega-3 fatty acids, which are essential for human vision and brain function (Maki *et al*., 2003). Eggs are also an important source of minerals like phosphorus and iron, and a good source of vitamins like A, D, E, K, and B1, B2, B12, choline, and selenium.

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Despite their nutritional value, chicken eggs can cause health problems when contaminated with pathogenic bacteria. Eggs can be contaminated with pathogenic bacteria such as *Escherichia coli*, *Salmonella* sp., *Proteus* sp., *Listeria* sp., *Staphylococcus* sp., *Streptococcus* sp., *Bacillus* sp. (Lee *et al*., 2016) owing to infected ovaries, and contact with contaminated cages, soil, or water (Svobodova and Tu-mova 2015). Although eggs possess several antibacterial proteins, the level of egg contamination is influenced by several factors such as microbial number in the environment and storage conditions (Scott and Silversides 2000). When the shell cracks, the egg's contents could become contaminated (Neira *et al.*, 2017). It has already been proven that eating contaminated eggs can result in foodborne illnesses (Osimani *et al.*, 2016; Chousalkar and Gole 2018).

Antibiotics are widely used to treat infectious diseases or to promote chicken growth. The usage of antibiotics had facilitated their efficient production and also improved chicken health and well-being by lowering disease incidences. But unfortunately, the unauthorized use of these antibiotics or inappropriate withdrawal period of time before the slaughtering of chickens could lead to the contamination of edible tissues with antibiotics with potential adverse effects on human health (Donoghue 2003). The use of antibiotics to treat bacterial infections without susceptibility testing may lead to the emergence of antibiotic resistance. Antibiotics in poultry are poorly absorbed in the gut and usually excreted without metabolism. Antibiotic resistance continues to be a major issue in human and animal health. Pathogens isolated from food are increasingly showing resistance (Akoachere *et al*., 2009). Food contamination with antibiotic resistant bacteria can therefore be a major threat to public health, as the antibiotic resistance genes can be transferred to other bacteria (Van *et al.*, 2007; Adesiji *et al.*, 2011). Furthermore, the transfer of these resistant bacteria to humans has public health significance by increasing the number of food-borne illnesses and treatment failure (Adesiji *et al.*, 2011). The indiscriminate use and higher doses of antibiotics are common which eventually accumulates antibiotic residues in the edible tissues of poultry (Kabir *et al.*, 2004; Nisha 2008; Goetting *et al.*, 2011). All antibiotic residues produce potential threats of direct toxicity in humans with possible development of resistant strains due to low and continuous exposure to antibiotics (Nisha 2008). In this regard finding out alternatives of antibiotics are crucially important for the production of safe poultry products.

Materials and Methods

The Study Area and Sources of Sample

A baseline survey was conducted at farms and households in Rajshahi and Naogaon districts. The baseline survey of 50 farms (commercial layer chicken) and 50 households (indigenous chicken) was conducted randomly in Rajshahi and Naogaon districts with semi-structures questionnaires. After collection of data from these farms and households, total 150 egg samples were collected randomly from selected farms, households and markets, and brought to the Microbiology Laboratory, Department of Veterinary and Animal Sciences, University of Rajshahi for microbiological analysis.

Isolation and Identification of E. coli

After enrichment in nutrient broth or on nutrient agar, Grams staining was performed on a smear of bacterial culture on a sterile slide for morphological study. The culture was also streaked onto EMB agar, MacConkey agar, SS agar, and brilliant green agar which were incubated at 37ºC for 18-24 hours aerobically for the isolation of bacteria. Following that, microorganisms were identified using a variety of biochemical tests using standard methods as described by Merchant and Packer (1967) and Cheesbrough (1985).

Antibiogram assay of Isolated E. coli

The disc diffusion method (Bauer and Kirby 1966) was used to test the sensitivity patterns of the isolated *E. coli*. In brief, isolated *E. coli* were cultured into the nutrient broth. Then 200 µl of broth culture was taken by micropipette and placed onto a Mueller Hinton agar (MHA) plate and spread evenly over the entire surface with a sterile glass rod spreader. The predetermined battery of antibiotic discs was dispensed onto the surface keeping a distance of about 1 cm apart. The plate was placed in an incubator at 37^0C and after 16-18

hours of incubation and examined. The susceptibility test of the *E. coli* isolates was performed against ten commonly used antibiotic discs including; gentamycin, oxytetracycline, amoxicillin, ciprofloxacin, ceftriaxone, doxycycline, erythromycin, enrofloxacin, levofloxacin, and neomycin (Table 1). Susceptibility zones were measured and interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI 2022).

Genotypic Identification of E. coli Isolates

Extraction and purification of genomic DNA All broth cultures of *E. coli* were incubated at 37°C for 24 hours. Then 1 ml of cultured broth was taken in eppendrof tubes and centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed, and the pellet was resuspended in 1 ml of sterile distilled water. The eppendrof tubes were vortexed and centrifuged again. The supernatant was removed, and the pellet was resuspended in 200 µl of deionized distilled water. The eppendorf tubes were transferred in boiling water bath and boiled for 15 minutes. Immediately after boiling, the eppendorf tubes were placed into ice and kept 10 minutes for cold shock. The tubes were then centrifuged at 10,000 rpm for 10 minutes. Finally, the supernatant was collected as genomic DNAs. The DNAs were analysed by agarose gel electrophoresis on 1% agarose and visualised using the UV transilluminator. DNA concentrations and purity were determined spectrophotometrically.

Confirmation of *E. coli* isolates by PCR Primers were F: GACCTCGGTTTAGTTCACAGA and R: CACACGCTGACG CTGACCA, amplicon size 585 bp for *E. coli* detection was used from the published article. Each PCR assay was carried out with a 25 µl mixture containing 6.5 µl nuclease free water, 12.5 µl Taq master mix (1X), 1.0 µl of each primer (20 nmol/µl) and 3 µl of the DNA template. The PCR assay was done in a thermocycler using the following cycle: after an initial denaturation of five minutes at 95^{o}C, the reaction mixture was subjected to 35 amplification cycles of one minute at 94^{o}C, one minute at 55^{o}C, one minute at 72^{o}C and final extension of ten minutes at 72^{o}C. The PCR products were separated by electrophoresis on 1.5 % agarose gel and visualized using UV transilluminator. A 100 bp DNA molecular marker was used to determine the size of the amplicons.

Detection of antibiotic resistance genes in E. coli isolates by PCR

Primers for antibiotic resistance genes such as gentamicin (*aac3-IV*), tetracycline (*tetA*, *tetB* and *tetC*), betalactams (*blaTEM*, *blaSHV* and *blaCMY*), erythromycin (*ereA*) and Quinolone (*gyrA*) were used from the published article (Table 2). Each PCR assay was carried out with a 25 µl mixture containing 6.5 µl nuclease free water, 12.5 µl Taq master mix (1X), 1.0 µl of each primer (20 nmol/µl) and 3 µl of the DNA template. Then the PCR assay was done in a thermocycler using the following cycle: after an initial denaturation of five minutes at 95^{o}C, the reaction mixture were subjected to 35 amplification cycles of one minute at 94^{o}C, one minute at 55^{o}C, one minutes at 72^{o}C and final extension of ten minutes at 72^{o}C. The PCR products were separated by electrophoresis on 1.5 % agarose gel and visualized using UV transilluminatior. A 100 bp DNA molecular marker was used to determine the size of the amplicons.

Results and Discussion

Isolation and Identification of E. coli

All egg samples were analyzed by different staining, cultural and biochemical methods to determine the prevalence of *E. coli*. On nutrient agar, the development of round, clear, moist, smooth, low convex, and glistening colonies indicated the growth of *E. coli* (Fig.1). On MacConkey agar the *E. coli* isolates produced lactose fermenting bright pink color colonies and on EMB agar produced greenish-black colonies with metallic sheen. The morphology of the isolated *E. coli* showed a Gram-negative, small rod shaped appearance, arranged in single or paired (Fig. 2). In sugar fermentation tests, all the isolates fermented dextrose, sucrose, lactose, maltose, and mannitol with acid and gas production (Fig. 3). Similarly, all the isolates showed positive to MR test, indole test, catalase test, and TSI agar slant reaction but negative to Simmon's citrate reaction and VP test. The results of cultural, staining and biochemical tests of isolated *E. coli* are similar to the findings of Freeman (1985), Buxton and Fraser (1977), and Merchant and Packer (1967).

Table 1. Antibiotics discs with their concentration

Name of used antibiotics	Disc concentration (µg/disc))	Interpretation of results (zone diameter in mm)		
		R	I	S
Gentamicin	10 µg	≤12	13-14	≥15
Oxytetracycline	30 µg	≤14	15-18	≥19
Amoxycillin	30 µg	≤13	14-16	≥18
Ciprofloxacin	5 µg	≤15	16-20	≥21
Ceftriaxone	30 µg	≤12	13-15	≥16
Doxycyclin	30 µg	≤15	16-22	≥23
Erythromycin	15 µg	≤15	16-20	≥21
Enrofloxacin	5 µg	≤16	17-22	≤16
Levofloxacin	5 µg	≤12	13-15	≤16
Neomycin	30 µg	≤22	23-25	≥26

Legends: µg = Microgram, mm = millimetre. S = Sensitive, I = Intermediate sensitive, R = Resistant, ≥ = Greater than or equal to, ≤ = Less than or equal to.

Table 2. Primers list used for the detection of antibiotic resistance genes in *E. coli*

Gentamicin *aac(3)-II*	F	CTTCAGGATGGCAAGTTGGT	286 bp
	R	TCATCTCGTTCTCCGCTCAT	
Tetracycline (*tetA*)	F	GGTTCACTCGAACGACGTCA	577 bp
	R	CTGTCCGACAAGTTGCATGA	
Tetracycline (*tetB*)	F	CCTCAGCTTCTCAACGCGTG	634 bp
	R	GCACCTTGCTGATGACTCTT	
Tetracycline (*tetC*)	F	AAC AAT GCG CTC ATC GT	1138 bp
	R	GGA GGC AGA CAA GGT AT	
Erythromycin (*eraA*)	F	GCCGGTGCTCATGAACTTGAG	419 bp
	R	CGACTCTATTCGATCAGAGGC	
Beta lactams (bla_{TEM})	F	ATA AAA TTC TTG AAG AC	1076 bp
	R	TTA CCA ATG CTT AAT CA	
Beta lactams (bla_{SHV})	F	TCGCCTGTGTATTATCTCCC	768 bp
	R	CGCAGATAAATCACCACAATG	
Beta lactams (bla_{CMY})	F	TGGCCAGAACTGACAGGCAAA	462 bp
	R	TTTCTCCTGAACGTGGCTGGC	
Quinolone (*gyrA*)	F	ATGCTTGAACAAGACAGAATTATAAAG	577 bp
	R	GACTGTCGCCGTCTACAGAACCG	

Prevalence of E. coli in Chicken eggs

The overall prevalence of *E. coli* was 30.00% in chicken eggs in Rajshahi and Naogaon districts of Bangladesh. The prevalence of *E. coli* was 36.00% in commercial layer chicken eggs, 24.00% in indigenous chicken eggs, and 30.00% in market eggs (Table 3). These findings are in agreement with Islam *et al*. (2018) who reported 34.64% of chicken eggs were contaminated with *E. coli*. Similarly, Akond *et al.* (2009) reported that 42% of egg surface was polluted with *E. coli* in poultry and poultry farms environments in Bangladesh.

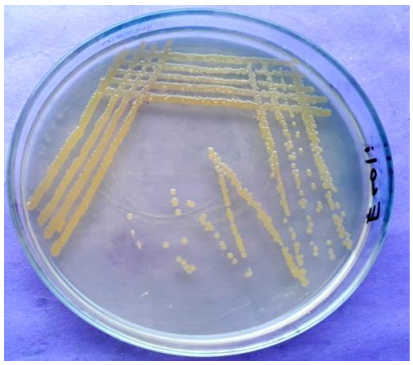

Fig. 1. Growth of *E. coli* on nutrient agar. Growth of *E. coli* on nutrient agar indicated by the development of smooth, circular, white to grayish white colony.

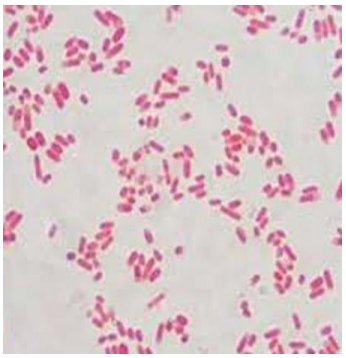

Fig. 2. Gram's stained *E. coli*. *E. coli* appears as Gram negative, pink colored, rod shaped under a light microscope (100X).

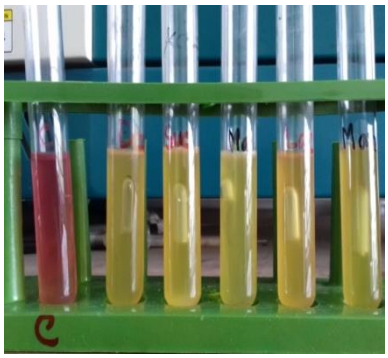

Fig. 3. Sugar fermentation test for the isolated *E. coli*. *E. coli* isolates fermented dextrose, lactose, sucrose, maltose and mannitol with the production of acid and gas.

Table 3. The prevalence of *E. coli* in chicken eggs in Rajshahi and Naogaon districts

Type of chicken eggs	No. of samples tested	Prevalence of *E. coli* (%)
Commercial layer chicken eggs	50	18 (36.00%)
Indigenous chicken eggs	50	12 (24.00%)
Market eggs	50	15 (30.00%)
Total	150	45 (30.00%)

Antibiotic Sensitivity Patterns of the Isolated E. coli

The antibiotic sensitivity profiles of the isolated *E. coli* revealed 57.78%, 33.33%, 31.11%, 28.89%, 26.67%, 17.78%, 15.56%, 13.33%, 13.33%, and 11.11% resistance to ciprofloxacin, doxycycline, oxytetracycline, ceftriaxone, amoxycillin, erythromycin, levofloxacin, enrofloxacin, gentamycin, and neomycin, respectively (Fig. 5). The isolates showed 94.44%, 82.22%, 80.00%, 77.78%, 66.67%, 60.00%, 55.56%, 48.89%, 46.67%, and 33.33% sensitive to neomycin, enrofloxacin, erythromycin, levofloxacin, gentamycin, ceftriaxone, oxytetracycline, doxycycline, amoxycillin, and ciprofloxacin, respectively (Fig. 6). The isolates also showed 26.67%, 20.00%, 17.78%, 13.33%, 11.11%, 8.89%, 6.67%, 4.44%, 4.44%, and 2.22% intermediate sensitive to amoxicillin, gentamycin, doxycycline, oxytetracycline, ceftriaxone, ciprofloxacin, levofloxacin, enrofloxacin, neomycin, and erythromycin, respectively (Table 4). These results are in agreement with the findings of Bantawa *et al.* (2019) who stated that *E. coli* exhibited the highest resistance to amoxycillin (100%), followed by tetracycline (93%), nalidixic acid (25%), and cefotaxime (19%) isolated from meat samples collected from eastern Nepal.

Genotypic Confirmation of E. coli Isolates

Phenotypic identified *E. coli* isolates were confirmed by PCR experiment. The 585 bp amplicon of phenotypic identified *E. coli* was amplified by PCR with the genus specific primers. All 45 isolates (100%) were confirmed as *E. coli* by PCR analysis (Fig. 4).

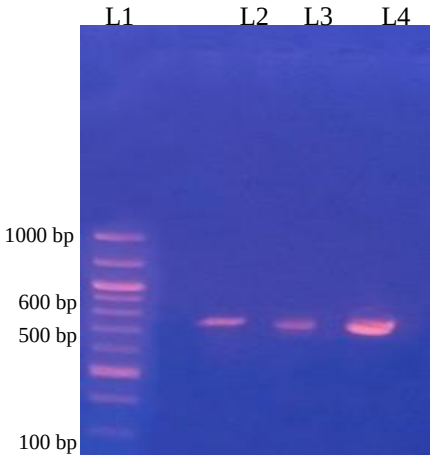

Figure 4. Agarose gel profile of PCR products of *E. coli*. Lane 1 indicated marker and Lane 2-4 indicated PCR products.

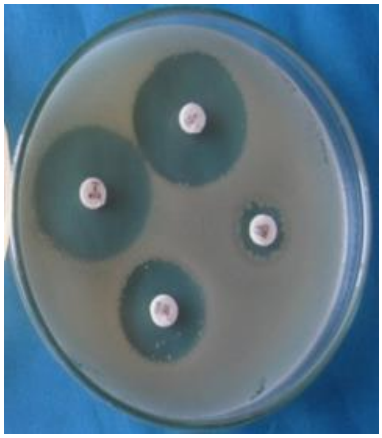

Figure 5. Antibiotic sensitivity and resistant patterns (zone of inhibitions) of isolated *E. coli* on Mueller Hinton agar.

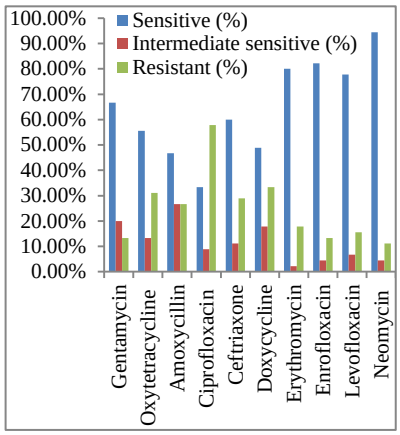

Figure 6. Antibiotic sensitivity and resistant patterns of isolated *E. coli*

Table 4. Antibiotic sensitivity and resistant patterns of isolated *E. coli*

No. of isolates tested	Name of used antibiotics	Sensitivity patterns		
		Sensitive (%)	Intermediate sensitive (%)	Resistant (%)
45	Gentamycin	30 (66.67%)	9 (20.00%)	6 (13.33%)
	Oxytetracycline	25 (55.56%)	6 (13.33%)	14 (31.11%)
	Amoxycillin	21 (46.67%)	12 (26.67%)	12 (26.67%)
	Ciprofloxacin	15 (33.33%)	4 (8.89%)	26 (57.78%)
	Ceftriaxone	27 (60.00%)	5 (11.11%)	13 (28.89%)
	Doxycycline	22 (48.89%)	8 (17.78%)	15 (33.33%)
	Erythromycin	36 (80.00%)	1 (2.22%)	8 (17.78%)
	Enrofloxacin	37 (82.22%)	2 (4.44%)	6 (13.33%)
	Levofloxacin	35 (77.78%)	3 (6.67%)	7 (15.56%)
	Neomycin	38 (94.44%)	2 (4.44%)	5 (11.11%)

Detection of antibiotic resistance genes in E. coli isolates

Genotypic identified isolates of *E. coli* were further characterized by PCR to detect antibiotic resistance genes. The 577 bp amplicon of tetracycline resistance gene *(tetA*) was amplified by PCR with the gene specific primers. Out of 45 isolates 37 (82.22%) showed tetracycline resistance gene *(tetA*) by PCR analysis. The 634 bp amplicon of tetracycline resistance gene *(tetB*) was amplified by PCR with the gene specific primers. Out of 45 isolates 35 (77.77%) showed tetracycline resistance gene *(tetB*) by PCR analysis. Similarly, the 577 bp amplicon of quinolone group resistance gene (*gyrA*) was amplified by PCR with the gene specific primers. Interestingly, all 45 (100%) isolates of *E. coli* showed quinolone group resistance gene (*gyrA*) by PCR analysis. However, we could not detect gentamycin resistance gene (*aac(3)-II*), tetracycline resistance gene (*tetC*), β-lactamases resistance genes (*blaTEM*, *blaSHV* and *blaCMY*), and erythromycin resistance gene *(ereA*) in *E. coli*. Similarly, Momtaz *et al.* (2012) detected tetracycline resistance (*tetA* and *tetB*) genes, erythromycin resistance gene (*eraA*) and sulfonamide resistance gene (*sul1*) in *E. coli* that was isolated in Iran from chicken meat.

Conclusions

The incidence of *E. coli* in chicken eggs and their resistance to antibiotics is obviously important in the context of our investigation. We may draw the conclusion that eating chicken eggs could expose consumers to *E. coli* that is resistant to antibiotics. Therefore, it is important to restrict the use of antibiotics in the poultry industry in order to control of the emergence of resistant *E. coli*.

Acknowledgement

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Conflict of Interest

The author declare no conflict of interest.

Author's Contributions

K. M. Mozaffor Hossain designed research; Tania Sultana and Abdulla Al Mamun Bhuyan performed research; and K. M. Mozaffor Hossain wrote the paper.

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