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Original research

Molecular Profiling of Multidrug-Resistant *Escherichia coli* Harboring tetA and eaeV3 Genes in Poultry Production System

Mahe Afroz[1], Nazmi Ara Rumi[1], Md. Aoulad Hosen[1], Md. Shajedur Rahman[2], Mst. Deloara Begum[1], Md. Shiblee Sadik Sabuj[1], Nasrin Sultana Tonu[3], Md. Hasanul Baker Tamlikha[1]

[1]*Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science & Technology University, Dinajpur-5200, Bangladesh* |[2]*Department of Medicine, Surgery and Obstetrics, Facuulty of Veterinary and Animal Science, Hajee Mohammad Danesh Science & Technology University, Dinajpur-5200, Bangladesh*|[3] *Upazila Livestock Office and Veterinary Hospital, Barura, Cumilla, Bangladesh*

Correspondence should be addressed to Md. Aoulad Hosen; aoulad1992@gmail.com

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ABSTRACT

Escherichia coli, a common gastrointestinal pathogen in warm-blooded animals, poses a significant challenge to poultry health. This study investigated the prevalence, serogroup distribution, and molecular characteristics of multidrug-resistant (MDR) *E. coli* in dead chickens from Dinajpur, Bangladesh. A total of 131 organ samples (intestine, liver, heart, lungs, oviduct) were analyzed using cultural, biochemical (Indole, MR-VP, TSI, citrate), and molecular (PCR) methods. *E. coli* was detected in 41.22% (n=54) of samples, with the highest prevalence in intestines (90%) from Basherhat (p=0.021). Molecular identification of the isolates was confirmed by 16S rRNA gene sequencing (585 bp). The resistance genes *TetA* and *EAEV3* were detected in 29.6% and 14.8% of isolates, respectively. Serogroup D was the most prevalent (61.11%). All *E. coli* showing 100% resistance to several classes, including penicillins, cephalosporins, macrolides, and tetracyclines. In contrast, they remained 100% susceptible to fluoroquinolones (ciprofloxacin, norfloxacin) and aminoglycosides (gentamicin, streptomycin). Among the isolates, 34 (68.51%) were classified as MDR. The findings underscore the escalating threat of antimicrobial resistance (AMR), which poses significant challenges to poultry health management. The results also raise public health concerns due to the potential transmission of resistant *E. coli* through the consumption of contaminated poultry products. Therefore, implementing measures such as restricting antibiotic misuse, strengthening surveillance, and improving farm biosecurity is critical to combat the AMR crisis in this poultry sector.

1. Introduction

Bangladesh's favorable climate supports poultry farming, and over time, various poultry species have been domesticated. Since its emergence in the 1980s, the poultry industry has evolved into a significant agribusiness sector. By 2017, investments reached approximately 300 billion BDT, encompassing around 150,000 farms. Despite this growth, poultry health and consumer safety remain major concerns, particularly in small-scale operations (Nayem, 2020).

The estimated poultry population in Bangladesh includes more than 350 million chickens (Hussain et al., 2021) and 68.261 million ducks (DLS, 2024). Approximately 50,000 chicken farms and 26,000 duck farms have been established in the private sector, alongside the government-operated farms. The poultry sector in Bangladesh faces several challenges, with diseases identified as a primary issue, leading to an annual mortality rate of 30% in chickens (Das et al., 2004). *Escherichia coli* strains are commonly commensal, and avian pathogenic *E. coli* (APEC) strains can cause severe systemic infections in broilers, resulting in significant economic losses (Dziva & Stevens, 2008). APEC is the primary causative agent of colibacillosis, a prevalent disease characterized by respiratory infections, septicemia, cellulitis, pericarditis, perihepatitis, and airsacculitis (Nolan et al., 2020). The pathogenesis of avian colibacillosis begins with colonization of the respiratory tract, often involving predisposing factors such as environmental stress, mycoplasma infections, or viral diseases (e.g., Infectious Bronchitis Virus) that compromise the host's immune system. The bacteria disseminate hematogenously, leading to septicemia, multiple organ infections, and mortality in severe cases (Mellata, 2013). Among bacterial diseases, reports indicate that E. coli is among the most prevalent pathogenic bacteria affecting chicken production. (Biswas et al., 2006).

E. coli is generally considered a benign inhabitant of the gut; however, certain strains possess virulence factors that can lead to various illnesses, including diarrhea, hemorrhagic colitis, urinary tract infections, and meningitis (Pouillot et al., 2012). Over the past two decades, reports of increasing antibiotic resistance have emerged in various countries, including Bangladesh (Kapil, 2004). Antibiotics have long been used in poultry for disease treatment. As growth promoters to improve feed efficiency (Dibner & Richards, 2005), elevated levels of antimicrobial resistance (AMR) in bacteria sourced from poultry samples have been documented (Van Boeckel et al., 2019). However, growing concerns over antimicrobial resistance and public health risks have prompted many countries to restrict or ban their use in animal feed (Van Boeckel et al., 2015). The use of antibiotics in livestock has been restricted globally, with the EU banning growth promoters in 2006 and the US limiting medically important antibiotics (Castanon, 2007; FDA, 2017). Although antibiotics help control outbreaks like *E. coli*, their misuse in poultry

has driven the emergence of multidrug-resistant strains, reducing effectiveness in both human and veterinary medicine (Hasina, 2006). In Bangladesh, previously published studies reported that multidrug-resistant E. coli cause significant economic losses and public health risks (Aknod et al., 2009). Antibiotics used in poultry farms for therapeutic purposes can easily transfer resistant bacteria to human consumers, carrying resistant genes and creating health threats (Sultana et al., 2023)

Serogrouping identifies specific APEC serotypes commonly associated with collibacillosis and poultry disease outbreaks, while virulence gene detection determines disease severity, aiding in understanding disease control and vaccine development. Serogrouping is a fundamental method for E. coli classification and shows a strong correlation with bacterial pathogenicity (Goudarztalejerdi et al., 2020). Among the serogroups implicated in avian colibacillosis, O1, O2, O18, and O78 have been identified as the most prevalent in previous studies. However, the distribution and prevalence of avian pathogenic *E. coli* (APEC) serogroups vary across farms and geographical regions (Goudarztalejerdi et al., 2020). In their study (Goudarztalejerdi et al., 2020), they reported that APEC and avian fecal *E. coli* (AFEC) predominantly comprised serogroups O78, O1, and O18, with 54% of APEC strains and 23% of AFEC strains identified within these groups. The pathogenicity of APEC is closely associated with various virulence-related genes that contribute to bacterial colonization, adhesion, invasion, and toxin production, thereby facilitating immune evasion and host infection (Dou et al., 2016).

The core gap lines in the lack of geographically specific, integrated (phenotypic/genotypic), and comprehensive surveillance in Dinajpur. This highlights the study's direct contribution to understanding *E. coli* epidemiology in poultry farm environments and in the broader poultry sector of Bangladesh. PCR-based ARG detection is common, but detailed WGS-based analyses of the resistome and plasmids, longitudinal transmission studies, quantified farm-level antimicrobial use data, and pragmatic intervention trials remain scarce. Together, these gaps hinder robust inference about local transmission pathways, the role of mobile genetic elements, and effective, evidence-based stewardship strategies for Dinajpur's poultry sector.

To begin addressing these gaps, our study focused on the isolation and identification of *E. coli*, serogrouping of *E. coli*, and the assessment of virulence genes associated with antibiotic resistance in *E. coli* that lead to infections in poultry birds, along with their implications for human consumers and the resulting economic losses.

This current research hypothesis will be following assumption, *E. coli* isolated from dead chickens will exhibit resistance to multiple antibiotics, specific serogroups of *E. coli* will be identified, some of which may be associated with avian pathogenic *E. coli* (APEC) strains, molecular analysis will reveal the presence of genes responsible for antibiotic resistance and virulence, and poultry farms in Dinajpur may serve as a reservoir for MDR *E. coli*, posing a risk to both animal and human health.

2. Materials and Methods

2.1. Ethical approval

The ethical committee of Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh, approved this research methodology. This research experiment was approved by the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh, and also received approval from the assigned poultry farm owners. During the collection of internal organs from dead chickens, strict biosafety precautions were followed, including the use of personal protective equipment (gloves, mask, lab coat), aseptic handling of tissues with sterilized instruments, and proper disposal of carcasses and contaminated materials through incineration or deep burial with disinfectants.

2.2. Study design and area selection

A total of 131 different internal organs (intestine: 34 samples, liver: 24 samples, heart: 25 samples, lungs: 20 samples, and oviduct: 28 samples) of dead birds were randomly collected from 5 different poultry farms in Dinajpur Sadar (Basherhat, Mohabalipur, Gobindapur) of Bangladesh and brought to the laboratory for microbiological analysis. Sterile containers were used for sample collection. All selected organs were removed from clinically signs of colibacillosis in dead chickens within 2-3 hours of death with the necroscopy method. First, sterilized necropsy tools (scalpel, scissors, forceps) were autoclaved, and then small (1–2 cm) tissue samples were collected from each organ (Figure 1). Additionally, causes of death were recorded for colibacillosis disease. The age was found to be 32-35 days during sampling from different categories of poultry farms. The present research work was conducted between July, 2017 and December, 2018.

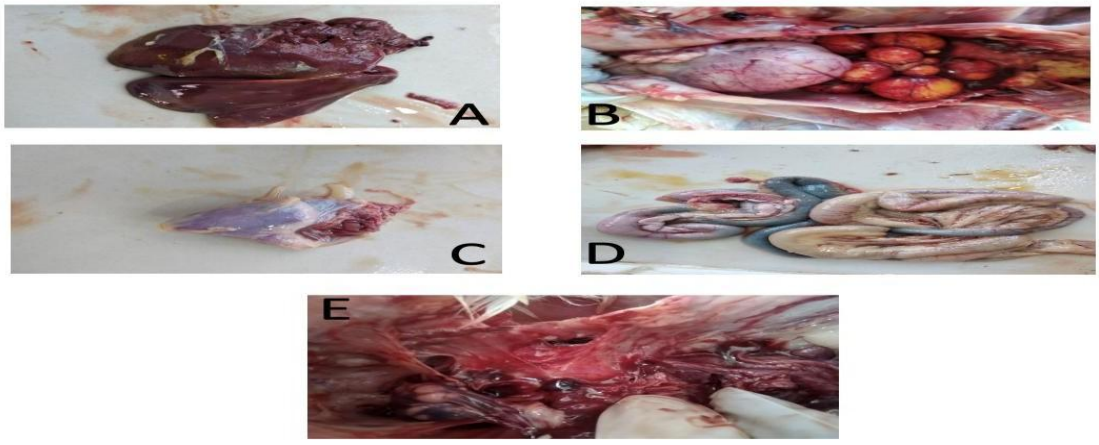

Figure 1: A: Sample from liver; B: Sample from oviduct; C: Sample from heart; D: Sample from intestine; E: Sample from lungs

2.3. Sample selection criteria

The specific samples, like liver, oviduct, heart, intestine, and lungs, were selected because higher bacterial load was found in these organs. In colibacillosis, *E. coli* spreads through the bloodstream to the primary site of infection, then colonizes other organs. Another reason for choosing these samples from the liver is that the liver is the key organ where bacterial manipulation occurs. Intestinal organs were chosen because they are the initial sites where bacteria colonize before spreading, and because researchers can easily detect both commensal and pathogenic strains of *E. coli* in these organs. All other selected samples have different reasons for selection.

2.4. Phenotypic characterization of *E. coli*

All the samples were taken aseptically with transparent zipper lock poly (thickness 30-100mic; size 175mm*100 mm) and transferred to the bacteriology laboratory for microbiological analysis. After sample collection, samples were primarily inoculated into Nutrient broth for primary isolation. Subsequently, Nutrient agar, MacConkey agar, Eosin Methylene Blue agar (EMB), and Blood agar (Hi Media Private Ltd., India) were employed for cultural identification under microscopic preparation. A group of standard biochemical tests, including Oxidase, Catalase, Indole, Methyl red (MR), Voges-Proskauer (VP), Simmon's citrate, Triple Sugar Iron (TSI), and Motility Indole Urease (MIU), was performed to identify the desired bacteria. All biochemical test media were purchased from Oxoid Private Ltd., UK. According to Azam et al. (2023), all cultural and biochemical tests were performed. A reference strain of *E. coli* ATCC 25922 (Thermo Fisher Scientific and Microbiologics) was used as a positive control.

2.5. Serotyping of *E. coli*

According to the protocol by Goudarztalejerdi et al. (2020), serogrouping of *E. coli* was determined. For serotyping, a specific *E. coli* culture was first selected. A volume of 40 µL of normal saline (0.85%) was placed on a clean glass slide, followed by the addition of a loopful of *E. coli-positive culture obtained* from EMB agar (Hi Media Pvt. Ltd.). The mixture was thoroughly homogenized to ensure uniformity. Subsequently, 5–10 µL of the selected polyvalent antisera was added to the mixture, which was then thoroughly remixed. The slide was gently rocked for 1 minute, and agglutination was observed under a microscope or to the naked eye. A dark background was used to enhance visibility and facilitate the detection of agglutination when observed directly. Polyvalent antisera, including Poly A–I, group B (O:8, 19, 84), and group D (O:2, O: 55, O:78), were procured from Thermo Fisher Scientific, USA, ensuring high-quality reagents for accurate serotyping. To ensure accuracy and avoid cross-reactivity during slide agglutination for *E. coli* serogrouping, the following quality control measures were implemented: use specific antisera; use positive and negative controls; ensure colony purity; avoid autoagglutination; and maintain strict aseptic techniques.

2.6. Detection of Antibiotic resistance and virulence genes of *E. coli*

2.6.1 DNA extraction and purity analysis

The double-boiling centrifugation method for DNA extraction of the desired isolates was applied according to the protocol established by De Medici et al. (2003). A single colony of *E. coli* isolated from selective EMB Agar was transferred into a microcentrifuge tube containing 200 µL of DNAse-RNAse-free distilled water. The microcentrifuge tube was incubated at 100°C for 15 minutes, then promptly cooled on ice for 5 minutes. The tube was centrifuged at 13,500 rpm for 10 minutes, after which the supernatant was carefully transferred to a new microcentrifuge tube. Ultimately, 5 µL of supernatant was used as the DNA template in the PCR reaction. The PCR reaction was conducted at a scale of 25 µl. The reaction included 12.5 µl of 2x master mix (Go Taq green master mix, Promega, Dane County, WI, USA), 2 µl of the sample (samples were diluted to 50 ng/µl), along with 0.2 µl of Taq DNA polymerase, 0.5 µl of the forward primer, and 0.5 µl of the reverse primer. Furthermore, 9.5 µl of molecular-grade water was added to achieve a final volume of 25 µl for the adjusted PCR assays. For the *tetA* gene of *E. coli* the thermocycler (Thermal Cycler Analytik Biometra TOne 96G, Germany) reaction parameters are maintained as initial denaturation at 95°C for 2 min, denaturation 95°C for 1 min, annealing at 53°C for 40s, extension at 72°C for 1 min and final extension at 72°C for 5 min with *TetA* forward and *TetA* reverse primers. For the EAE gene of *E. coli*, the thermocycler reaction parameters are maintained as initial denaturation at 95°C for 2 min, denaturation at 95°C for 1 min, annealing at 54°C for 40s, extension at 72°C for 1 min, and final extension at 72°C for 5 min with EAE*V3* forward and *EAEMB* reverse primers. (Table 1a and Table 1b).

The purity of the PCR product's DNA was assessed using the Thermo Scientific NanoDrop 2000 spectrophotometer, employing the A260/A280 ratio; a typical range of 1.8-2.0 was considered acceptable (Figure 2). Following electrophoresis, the gel was carefully removed from the electrophoresis chamber and placed on a UV transilluminator (WUV-L50, Korea) for an initial examination of the DNA bands. It was then transferred to a high-performance gel documentation chamber (UVD1-254) for additional analysis and image storage. The PCR band sizes of products were measured using 2% (w/v) agarose gel electrophoresis with ethidium bromide (0.5µg/ml) at 70-100 V and 500 mA for a duration of 30 to 70 minutes (Aklilu et al., 2016). A 100 bp DNA ladder (Thermo Scientific, USA) served as the reference marker in this study. The PCR band image was captured using a high-resolution camera with Vtech software and a TV zoom lens from Japan.

2.6.2 Nucleotide sequence, BLAST, and phylogenetic tree analysis

The nucleotide sequence data from the 16S rRNA gene region were submitted to the NCBI Nucleotide Sequence Database. Using the BLAST tool and a phylogenetic tree, primer pairs were designed from the NCBI database (Sanger sequencing, ABI 3130 Genetic Analyzer, Foster City, CA, USA). The phylogenetic tree was constructed and analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) software, based on edited and processed sequences, following the methodology outlined by Kumar *et al.* (2016). The neighbor-joining algorithm, as described by Saito (1987), was used to construct the tree. Sequence comparison and alignment were performed using the BLASTN algorithm (http://www.ncbi.nih.gov/BLAST). Additionally, the finalized gene sequence was uploaded to the GenBank database for public access and reference.

2.7. Antibiotic Sensitivity Tests (AST)

The antimicrobial sensitivity test was conducted according to the Kirby-Bauer disk diffusion susceptibility test protocol, as recommended by CLSI 2021. A total of 16 commercially available antibiotics, such as Amoxicillin (10 µg), Ampicillin (10µg), Penicillin (10µg), Colistin (10 µg) (Beta-lactams class), Erythromycin (15 µg) and Azithromycin (30 µg) (Macrolides class), Neomycin (30 µg), Streptomycin (10 µg), Kanamycin (30 µg), Gentamycin (10 µg), Amikacin (30µg) (Aminoglycosides class), Chloramphenicol (30 µg) (Amphenicol class), Ciprofloxacin (5µg), Norfloxacin (10 µg) (Fluroquinolones class), Tetracycline (30 µg) (Tetracyclines class), Cephalexin (30 µg) (Cephalosporins class) were applied for antimicrobial sensitivity tests. On a Mueller-Hinton agar plate, a single bacterial colony (0.1 mL) was spread, antibiotic discs were placed, and the plates were incubated overnight at 37°C. After incubation, the zone of inhibition was measured to the nearest millimeter as per the manufacturer's guidelines. All antibiotic sensitivity tests were

performed 3 times to ensure accurate results. All antibiotic discs were purchased from Hi Media Private Ltd., India. A reference strain of *E. coli* ATCC 25922 (Thermofisher Scientific and Microbiologics) was used for susceptibility validation.

2.8. Multi-drug Resistance Profile (MDR) of *E. coli*

According to the methodology established by Paul et al. (2022) and Munim et al. (2024), MDR is defined as the ability of an isolate (e.g., a bacterial strain) to exhibit resistance to three or more antibiotic classes. Results are interpreted according to international standards, such as those of the Clinical and Laboratory Standards Institute (CLSI). MDR rates are calculated to evaluate the prevalence and spread of resistance. Ezekiel et al.'s definition of MDR as resistance to at least three classes of antibiotics provides a clear benchmark for identifying and monitoring resistant isolates in various settings, including hospitals, community health centers, and environmental studies.

Table 1a: Properties of primers used in the present study

Primer name	Target gene	Primer sequence	Annealing temperature	Expected band size (bp)	Reference
16E1	16S rRNA	F: 5'-GGG AGT AAA GTT AAT ACC TTT GCT C-3'	56 °C	585 bp	Tsen et al. (1998)
16E2		R: 5'-TTC CCG AAG GCA CAT TCT-3'			
TetA (F)	TetA	F: 5'-GGTTACTEGAACGACGICA-3'	53 °C	577 bp	Abdelgader et al., 2018
TetA (R)		R: 5'-CTGTCCGACAAGTTGCATGA-3'			
EAEV3	EAE gene	F: 5'-CATTGATCAGGATTTTTCTG-3'	54°C	510 bp	Mora et al., 2010
EAEMB		R: 5'-TCCAGAATAATATIGITATTACG-3'			

Table 1b: The thermocycler parameters for the PCR reaction of *E.coli*

Thermocycle	Universal primer	tetA	EAEV3 (F)EAEMB (R)
Initial denaturation	95°C	95 °C	95°C
	2 min	2 min	2 min
Denaturation	95°C	95°C	95°C
	1 min	1 min	1 min
Annealing	56 °C	53 °C	54°C
	40 sec	40 sec	40 sec
Extension	72°C	72 °C	72 °C
	1 min	1 min	1 min
Final extension	72°C	72 °C	72 °C
	5 min	5 min	5 min
Cycle	35X Step 2		

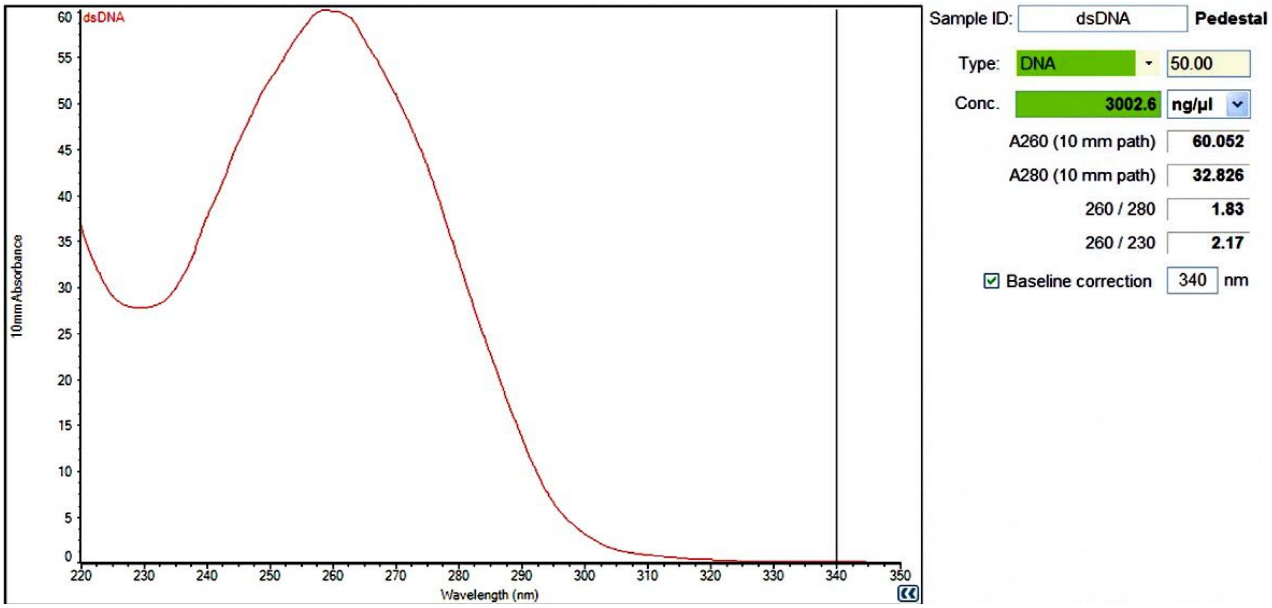

Figure 2: Software output, showing both spectra and numerical data.

2.9. Correlation between phenotypic and genotypic characters

Phenotypic characters were detected using antibiotic susceptibility tests of *E. coli* by the disk diffusion method and virulence factor assessment on blood agar with hemolysis (Badr et al., 2022; Joseph et al., 2024). Additionally, genotypic identification of antibiotic resistance and virulence genes was performed using PCR (Lemlem et al., 2023).

2.10. Statistical analysis

All raw data were entered into an Excel spreadsheet (version 2020, USA) and analyzed using built-in Excel formulas. The analysis used various categorical variables to summarize the prevalence of the isolates effectively. The SPSS software (version 25.00, IBM, Chicago, IL, USA) was used for descriptive analysis, and the chi-square test and 95% CI were used. A p-value (p-value < 0.05) was considered statistically significant, and a p-value (p-value < 0.001) was considered highly significant.

3. Results

The current research focused mainly on antimicrobial resistance, the virulence genes of *E. coli* isolates from dead chickens in Dinajpur district, Bangladesh, and the significant public health hazard posed by resistance-carrying E. coli strains. Our study's central hypothesis was to identify the prevalence of MDR E. coli in different organs of poultry, with implications for public health.

3.1. Phenotypic characteristics of the recovered *E. coli* strain

A total of 131 samples were collected from five distinct internal organs, including the chicken liver, heart, lungs, oviduct, and intestine, for this study. Among the 131 samples analyzed, 54 (41.22%) tested positive for *E. coli*, while 77 (58.78%) were negative. In this study, 54 *E. coli* were morphologically isolated using cultural tests, which included nutrient agar resulting in white colonies, rose pink colonies observed after prolonged incubation on MacConkey agar, colonies with a black centre and blue-green metallic sheen on Eosin methylene blue agar, hemolysis colonies on blood agar (Beta -hemolysin 20/54: 38%; alpha hemolysin 10/54: 19%) and small white colonies on Tryptic soy agar. Following cultural confirmation, we conducted biochemical tests for secondary validation. The observed *E. coli* exhibited positive reactions for catalase, indole, methyl red, and motility tests, but negative responses to the oxidase, Simmons citrate, and Voges-Proskauer tests (Table 2).

Table 2: Results of different biochemical tests

Name of isolate	OX	CT	IN	MR	VP	SC	TSI		MIU	
							Slant	Butt	M	I
E. coli	-	+	+	+	-	-	A(yellow)	A(yellow)	+	+

Legends: + = Positive, - = Negative, A=Acid, OX= Oxidase, CT=Catalase, IN= Indole, MR= Methyl-red, VP= Voges-proskauer, SC= Simmon's citrate, TSI= Triple sugar iron, MIU= Motility indole urease.

The prevalence of isolated *E. coli* by sampling source is detailed in Table 3. In the present study, a total of 47 samples were collected from five poultry farms located in Basherhat, Dinajpur, and 17 samples tested positive for *E. coli* (Table 3). Similarly, E. coli was detected in chicken intestine (9/90 %), chicken liver (3/30 %), chicken heart (2/25 %), chicken lungs (1/12.5 %), and chicken oviduct (2/18.18 %), respectively (Table 3). Statistical analysis revealed that in Basherhat, the highest prevalence of *E. coli* was significantly recorded in chicken intestine with a p-value (0.021) than other samples (95% CI, p-value = 0.021).

A total of 41 samples were collected from eight farms in Mohabalipur, Sadar, Dinajpur district, of which 16 tested positive for *E. coli* (Table 3). The occurrence of *E. coli* in the samples of chicken, including the

intestine, liver, heart, lungs, and oviduct, was found to be 10 (83.33%), 2 (20%), 1 (20%), 2 (40%), and 1 (11.11%), respectively (Table 3). A significantly higher prevalence of *E. coli* was found in chicken intestine (p=0.038) and chicken oviduct (p=0.039) than in other samples (95% CI, p=0.038).

In this study, a total of 43 samples were collected from seven chicken farms in Gobindapur, Sadar, Dinajpur. Out of these, 21 samples tested positive for *E. coli*, with 11 (91.66%) found in chicken intestines, 1 (25%) in chicken liver, 4 (33.33%) in chicken heart, 2 (28.57%) in chicken lungs, and 3 (37.5%) in chicken oviduct, respectively (Table 3). The extremely significantly higher prevalence of E. coli was observed in the chicken intestine with p-value (0.006) than in the chicken oviduct (37.5%), chicken heart (33.33%), chicken lungs (28.57%), and chicken liver (25%) in Gobindapur, respectively (95% CI, p<0.001).

A total of 131 samples were analyzed, including 34 chicken intestines, 24 chicken livers, 25 chicken hearts, 20 chicken lungs, and 28 chicken oviducts, for the presence of *E. coli* (Table 3). Among the 34 chicken intestine samples, 30 (88.23%) tested positive. In the analysis of 24 chicken liver samples, 6 (25%) were positive. For the 25 chicken heart samples, 7 (28%) yielded positive results. Out of 20 chicken lung samples, 5 (25%) were found to be positive, and all six chicken oviduct samples (21.42%) tested positive for *E. coli* (Table 3). The collected chicken intestinal samples from Gobindapur, Sadar, Dinajpur district showed the highest percentage of E. coli, with 11 samples (91.66%) testing positive (Table 3).

The sampling source indicated that the highest number of positive isolates was found in Gobindapur (21/43; 48.33%), followed by Mohabalipur (16/41; 39.02%). In contrast, the lowest prevalence was observed in Basherhat, 17/47 (36.17%) (Figure 3). In this study, we selected heart, liver, and oviduct samples from dead chickens with colibacillosis due to systemic infection with Avian *E. coli* (APEC). *E. coli* often enters the bloodstream (bacteremia/septicemia) and colonizes the heart, especially the pericardium. The liver filters blood, making it a common site for bacterial colonization during sepsis. In laying hens, *E. coli* can ascend the reproductive tract and cause salpingitis (oviduct infection), leading to decreased egg production or internal laying.

Table 3: Summary of isolated *E. coli* from internal organs of dead chicken

\SL. No	Sampling location	Sample source	Number of samples	Number of E.coli positive (%)	95% CI		p-value
					Lower limit	Upper limit	
1	Basherhat	CI	10	9 (90%)	59.58	99.48	0.021
		CL	10	3 (30%)	10.78	60.32	0.343
		CH	8	2 (25%)	4.44	59.07	0.283
		CLN	8	1 (12.5%)	0.64	47.08	0.07
		CO	11	2 (18.18%)	3.23	74.69	0.065
		Total	47	17(36.17%)			
2	Mohabalipur	CI	12	10 (83.33%)	55.19	97.03	0.038
		CL	10	2 (20%)	3.55	50.99	0.109
		CH	5	1 (20%)	1.02	62.44	0.375
		CLN	5	2 (40%)	7.10	76.92	1
		CO	9	1 (11.11%)	0.56	43.49	0.039
		Total	41	16(39.02%)			
3	Gobindapur	CI	12	11 (91.66%)	64.61	99.57	0.006
		CL	4	1 (25%)	1.28	69.93	0.625
		CH	12	4 (33.33%)	13.81	60.93	0.387
		CLN	7	2 (28.57%)	5.07	64.1	0.453
		CO	8	3 (37.5%)	13.68	69.42	0.726
		Total	43	21(48.83%)			
Total			131	54(41.22%)			

Legends: CI for Chicken intestine, CL for Chicken liver, CH for Chicken heart, CLN for Chicken lungs, CO for Chicken oviduct.

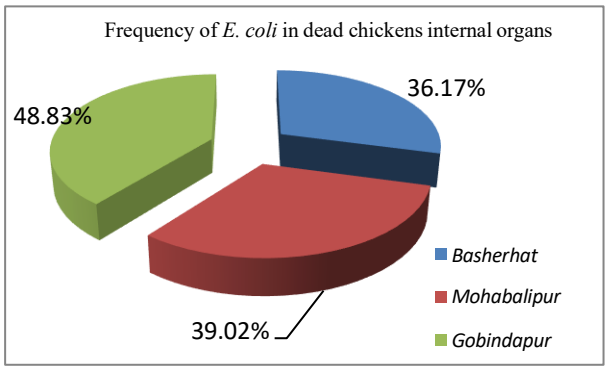

Figure 3: Frequency of *E. coli* in dead chickens' internal organs

3.2. PCR amplification and phylogenetic tree analysis of *E. coli*

After confirmation of E. coli by cultural and biochemical tests, PCR amplification targeting the tetA and eae genes was performed with forward and reverse primers. A 577 bp band for the tetA gene and a 221 bp band for the eae gene were detected in isolates (Figure 4: A, B). Out of 54 isolates, we recorded 50/54 (92.60%) 16S rRNA gene by PCR targeting genes, 16/54 (29.62%) TetA gene, and 8/54 (14.81%) EAEV3 gene, respectively. The phylogenetic tree revealed neighbour-joining with other relevant isolates (Figure 5). Four isolates did not yield a 16S rRNA gene band, suggesting potential DNA degradation or PCR inhibition. The BLAST query ID is IcI Iquery_4173191, with a query length of 569. We identified the E. coli strain WBS1 and our gene bank submission ID: SUB15343201, and accession ID: PV686899.

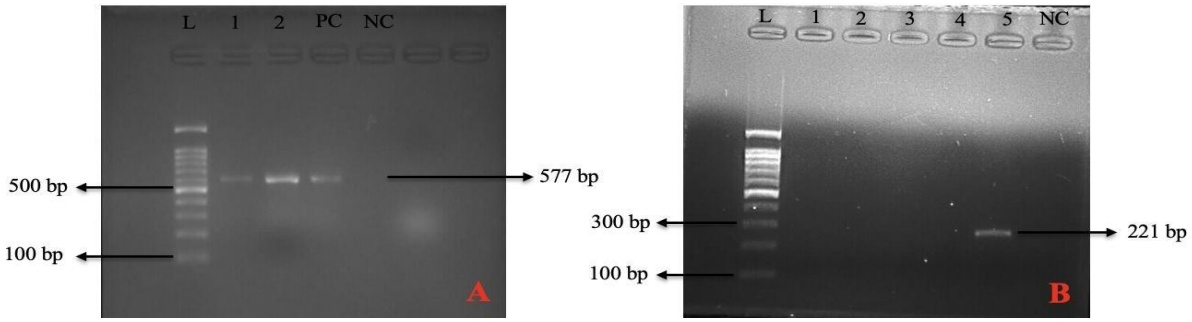

Figure 4: Resistance genes detected by the PCR technique from poultry internal organ samples; (A): tetA gene of *E. coli* detected by tetA-F and tetA-R primers design, confirming 577 bp. L:100 bp DNA Ladder, Lanes 1: intestine sample, Lane 2: liver sample, PC: Positive control band, NC: Negative control band; **(B):** *eae* gene of *E. coli* detected by EAEV3-F and EAEMB-R primers design confirming 221 bp. L:100 bp DNA Ladder, NC: Negative control, Lanes 1-5: poultry internal organ samples.

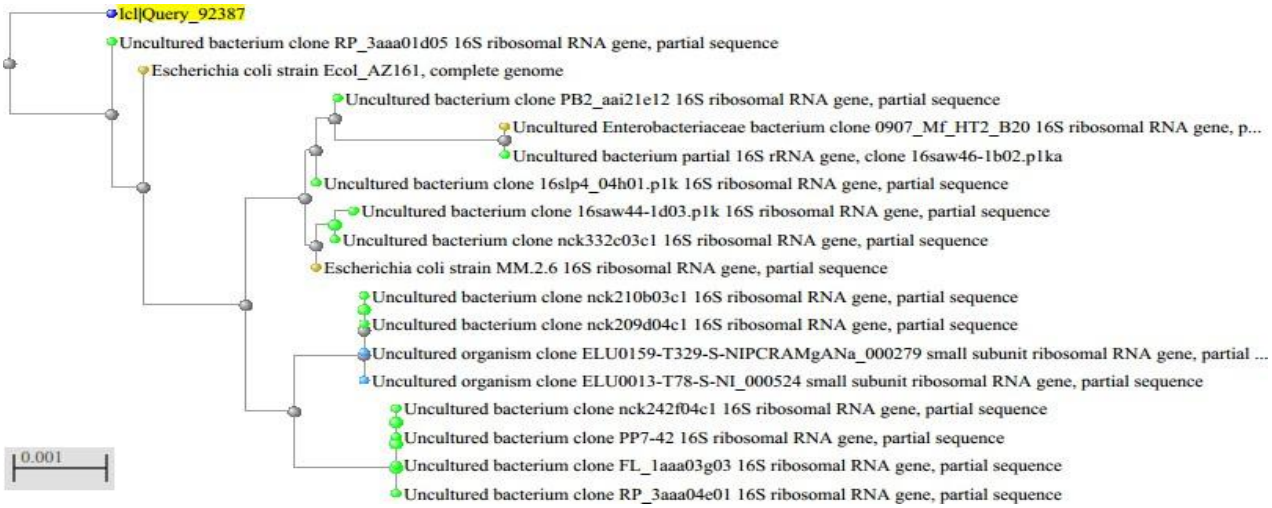

Figure 5: Phylogenetic tree of multidrug-resistant *E. coli* isolates based on partial *16S rRNA* sequences. The tree was constructed using the Neighbour-Joining method in MEGA11. Bootstrap values (>70%) based on 1000 replications are shown at the branch nodes. The scale bar indicates the number of substitutions per nucleotide position. Our Isolated bacteria were closely related to *E. coli* strain Ecol_AZ161, with a complete genome, and were very far from the uncultured bacterium clone RP_3aaa04e01, *16S* ribosomal RNA gene, partial sequence.

3.3. Result of *E. coli* serogrouping

Sero grouping of *E. coli* isolates was carried out using the slide agglutination test with commercial *E. coli*- specific polyvalent O (A-I) antisera, *E. coli* O group B (Factor O: 8, 19, 84) antisera, and *E. coli* O group D (Factor O: 2, 55, 78) antisera, procured from S & A Reagent Lab. The test was conducted according to the manufacturer's protocol. All isolates tested positive for *E. coli* Poly A-I antisera 54(100%), with some showing positive reactions to *E. coli* O group B antisera 21(38.88%) and others to *E. coli* O group D antisera 33 (61.11%).

3.4. Results of antimicrobial susceptibility of *E. coli*

The heatmap illustrates the antibiotic resistance patterns of *E. coli* isolates across a panel of commonly used antimicrobials. A pronounced resistance trend is evident for several first-line antibiotics, with amikacin, ampicillin, cephalexin, erythromycin, penicillin, and tetracycline showing 100% resistance, suggesting their ineffectiveness for therapeutic use. Moderate resistance was observed for azithromycin (38.9%), chloramphenicol (25.9%), and kanamycin (74.1%), indicating partial loss of efficacy (Table 4, Figure 6).

Conversely, ciprofloxacin, gentamicin, and norfloxacin exhibited complete susceptibility (100%), indicating they are potentially reliable treatment options. Colistin showed mixed responses, with a majority of isolates (57.4%) falling into the intermediate category, reflecting reduced but incomplete resistance. Neomycin showed comparatively better activity, with 79.6% susceptibility.

Overall, the data underscore a critical multidrug resistance (MDR) problem, particularly against β-lactams and macrolides, while certain aminoglycosides and fluoroquinolones remain effective. This pattern aligns with global reports of rising resistance in poultry-associated *E. coli*, emphasizing the urgent need for rational antibiotic use and continuous surveillance. The detailed results are presented in Table 4 and Figure 6.

Table 4: Antimicrobial susceptibility pattern of *E. coli* by the disk diffusion method

Antimicrobial agents	No. (%) of *E. coli* isolates		
	S	I	R
Amoxicillin	33 (61.11%)	9 (16.66%)	12 (22.22%)
Azithromycin	15 (27.77%)	18 (33.33%)	21 (38.88%)
Ciprofloxacin	54 (100%)	0 (0%)	0 (0%)
Erythromycin	0 (0.0%)	0 (0%)	54 (100%)
Gentamicin	54 (100%)	0 (0%)	0 (0.0%)
Norfloxacin	54 (100%)	0 (0%)	0 (0.0%)
Streptomycin	54 (100%)	0 (0%)	0 (0%)
Tetracycline	0 (0.0%)	0 (0%)	54 (100%)
Chloramphenicol	29 (53.70%)	11 (20.37%)	14 (25.92%)
Ampicillin	0 (0.0%)	0 (0%)	54 (100%)
Amikacin	0 (0.0%)	0 (0%)	54 (100%)
Cephalexin	0 (0.0%)	0 (0%)	54 (100%)
Colistin	13 (24.07%)	31 (57.40%)	10 (18.51%)
Penicillin	0 (0.0%)	0 (0%)	54 (100%)
Neomycin	43 (79.62%)	6 (11.11%)	5 (9.25%)
Kanamycin	14 (25.92%)	0 (0%)	40 (74.07%)

Legends: S= Susceptible; I= Intermediate; R= Resistance

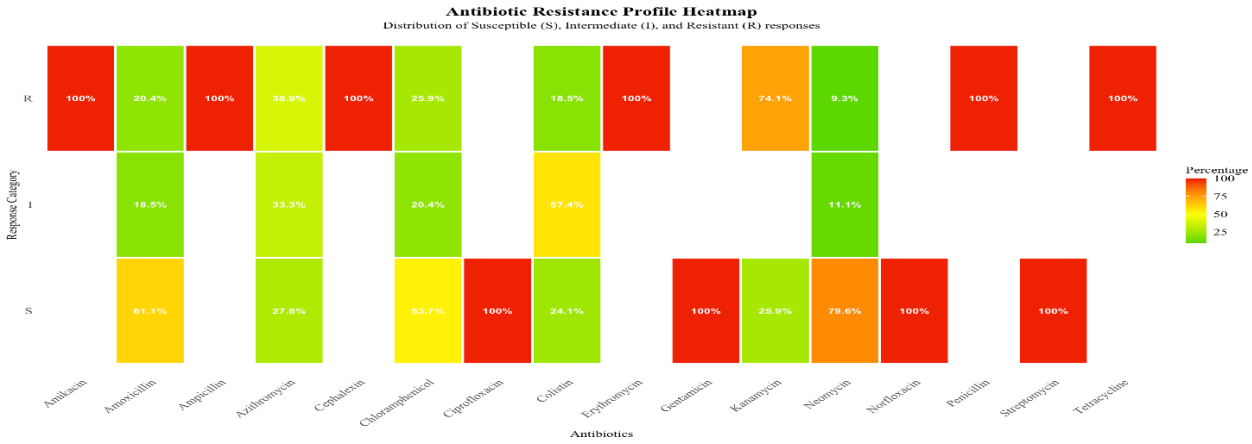

Figure 6: Antibiotic Resistance Profile Heatmap

3.5. MDR pattern of *E. coli*

In this study, 37 out of 54 *E. coli* isolates (68.51%) were classified as multidrug-resistant (MDR). Among the isolates, 17 (31.48%) exhibited resistance to two antibiotics, specifically erythromycin (E) and tetracycline (TE). Resistance to three antibiotics, including erythromycin (E), amoxicillin (AMX), and tetracycline (TE), was observed in 10 isolates (18.51%). Additionally, 12 isolates (22.22%) demonstrated resistance to four antibiotics, such as erythromycin (E), azithromycin (AZM), and tetracycline (TE). Furthermore, the highest number of isolates (15, 27.77%) exhibited resistance to five antibiotics, including amoxicillin (AMX), azithromycin (AZM), erythromycin (E), tetracycline (TE), and penicillin (PE). These findings are summarized in Table 5.

Table 5: Results of antimicrobial resistance pattern of *E. coli* by disc diffusion method (*E. coli*; n= 54)

SL. No	Resistance Patterns	No. of isolates with resistance (%)	No. of isolates with MDR(%)
1	AMX, AZM, E, TE, P	15(27.27%)	15(27.27%)
2	AMX, AZM, E, TE	12 (22.22%)	12 (22.22%)
3	E. AMX. TE	10 (18.51%)	10 (18.51%)
4	E, TE	17 (31.48%)	
Total		54(100%)	37(68.51%)

Legends: AMX=Amoxicillin, AZM=Azithromycin, E=Erythromycin, TE=Tetracycline, P=Penecillin.

Out of 54 positive *E. coli* isolates, 54 were phenotypically identified with morphology, cultural, and biochemical tests, whereas genotypically, 92. 60% detected *16S rRNA* gene, 29.62% antibiotic resistance *TetA* gene, and 14.81% virulence gene *EAEV3* gene (Table 6, Figure 7).

Table 6: Correlation between phenotypic and genotypic characters of *E. coli*

Isolates	Phenotypic positive	Genotypic positive		
		Universal gene	Antibiotic resistance gene	Virulence gene
		16S rRNA gene	*TetA* gene	*EAE* V3
E. coli	54	50/54 (92.60%)	16/54 (29.62%)	8/54 (14.81%)

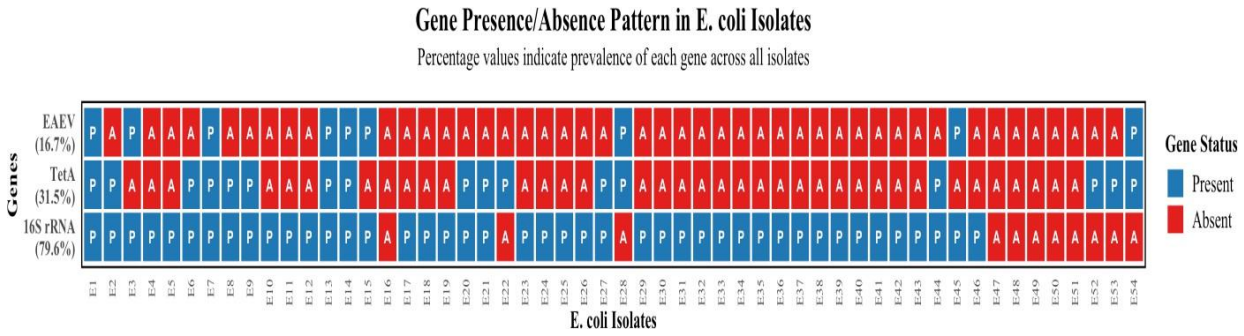

Figure 7: Heatmap diagram of resistance genes of *E. coli*

4. Discussion

Dead poultry infected organs, including the liver, heart, oviduct, and intestine, carry pathogenic bacteria that cause several infectious diseases in poultry (colibacillosis) and human consumers by spreading in the environment. In this research, we attempted to identify pathogenic *E. coli* (APEC) using both cultural and molecular techniques. This study used biochemical test methods for *E. coli* identification, which were also employed in previous investigations (Azam et al., 2023).

This study involved molecular characterization using PCR, specifically amplifying the 16S rRNA gene (585 bp), the tetA gene (577 bp), and the EAE gene (510 bp) to detect isolated *E. coli.* All conditions and results observed in the PCR were correlated with the findings of several authors (Ahmed et al., 2024; Srinivas, 2023).

In this study, 88.23% of *E. coli* isolates were obtained from intestinal samples collected from apparently healthy chickens, consistent with previous reports in Bangladesh (Hossain et al., 2015; Sudershan et al., 2012; Ahmed et al., 2009). Previously published studies in Bangladesh (Khaton et al., 2008) reported that *E. coli* was detected in 87% of liver samples, 73% of lung samples, and 96% of intestinal samples. In another study in Dhaka city, Bangladesh, the highest prevalence of *E. coli* was observed in liver (45.33%) and heart (44%) samples, which are higher than our findings (Islam, 2020; Khalid et al., 1990; Younis et al., 2017). Consequently, we detected 21.42% *E. coli* in oviduct samples from dead broiler chickens, which supported the research of Mukhopadhyaya et al. (1992) in India. In Bangladesh, another study by Khaton et al. (2021) reported the prevalence of dead broiler chicken internal organs but did not provide detailed percentages for liver, heart, and intestine. However, in our novelty, we observed the prevalence of details and also detected antibiotic resistance genes (tetA) and a virulence gene (EAE) using band size and phylogenetic tree analyses.

The highest intestinal prevalence, 91.66% (n=11), was recorded in Gobindapur, Sadar, Dinajpur district. These results align with previous studies (Kabir et al., 2010; Sarker et al., 2012) and reinforce the evidence of persistent intestinal colonization by *E. coli* in poultry (Nolan et al., 2013). Previously published studies by Kabir (2010) supported our findings, which showed E. coli at 36.17% (n=17) in Basherhat, 34.04% (n=16) in Mohabalipur, and 48.83% (n=21) in Gobindapur. Among the 54 *E. coli* isolates, serogroup D was predominant at 61.11% (n=33), followed by serogroup B at 38.88% (n=21), which exhibited motility, consistent with serogroup characteristics and aligning with findings reported by Shuchismita et al. (2007).

This study revealed that *E. coli* isolates were fully susceptible to ciprofloxacin, gentamicin, norfloxacin, and streptomycin, consistent with prior reports (Jakaria et al., 2012; Chen et al., 2014). Globally, day by day, the fluoroquinolone class of antibiotics is increasingly resistant. In the southern United States, tetracycline resistance was detected in E. coli, contradicting our findings (Feng et al., 2023). In contrast, universal resistance to tetracycline was observed, corroborating the findings of Islam et al. (2025), who reported 52.34% resistance. Ahmed et al. (2013) and Jiang et al. (2011) also reported similar findings. Notably, 27.77% (n=15) of isolates exhibited multidrug resistance (MDR) to four antibiotics—amoxicillin, azithromycin, erythromycin, and penicillin—aligning with earlier studies in Bangladesh (Jakaria et al., 2012; Olarinmoye et al., 2013; Sahoo et al., 2012). The data further indicated widespread resistance to first-, second-, and third-generation antibiotics, particularly within the β-lactam, macrolide, cephalosporin, and aminoglycoside classes. These findings underscore the urgent need for implementing advanced-generation antimicrobials in poultry management. This research provides valuable guidance for veterinarians and poultry producers in selecting effective therapeutic agents against pathogenic *E. coli.* The virulence of APEC is determined by the presence of specific virulence-associated genes (VAGs) that encode Type 1 fimbriae (fimH) and P fimbriae (papC), which facilitate bacterial attachment to the respiratory and intestinal epithelium (Schouler et al., 2012) and subsequent invasion. Hemolysin is associated with APEC and virulence factors, as supported by previously published reports in the Southern United States (Freng et al., 2023). Iron acquisition systems, namely aerobactin (*iutA*) and enterobactin, enable efficient iron uptake in iron-limited environments, such as the host bloodstream (Mellata, 2013; Feng et al., 2023), and support host survival.

Our research significantly reflects Public Health Impact. Multidrug-resistant *E. coli* poses a serious threat to both animal and human health. Investigating its prevalence in dead chickens in Dinajpur, Bangladesh, provides crucial insights into the risk of zoonotic transmission and the spread of antibiotic resistance. Our findings state that the dominance of Serogroup D and the identified resistance genes (*tetA*, *eae*) are of particular concern for zoonotic transmission. Antimicrobial resistance (AMR) surveillance is a novelty in the poultry sector in Bangladesh, and AMR in *E. coli* could severely impact poultry production, food safety, and public health. Understanding MDR *E. coli* in dead chickens helps assess the role of poultry as a reservoir of resistant bacteria. Furthermore, identifying specific *E. coli* serogroups provides insights

into their pathogenic potential. Some serogroups are associated with severe infections in both birds and humans, making this study important for disease control and prevention. Regional studies on AMR in Bangladesh remain limited, particularly in Dinajpur. This research fills a gap in the surveillance of MDR *E. coli* in poultry and can help update local and national policies on antibiotic use. The study aligns with the One Health approach, which recognizes the interconnection between human, animal, and environmental health. Findings could contribute to global efforts in combating AMR. Our study strongly suggested that Ciprofloxacin and Gentamicin are treatment options for colibacillosis diseases caused by *E. coli* (APEC), and farmers should be aware of antibiotic resistance.

In a limited attempt, samples were collected from just three locations in Sadar, Dinajpur district, for the isolation and identification of *E. coli.* Due to economic constraints, we did not collect additional samples or perform whole-genome sequencing, which would have confirmed the resistance genes of *E. coli* transferred from broiler chickens to human consumers and causing disease. Therefore, investigations in other areas of Dinajpur district and other districts will be required to identify the *E. coli* associated with commercial poultry production and to control the economic losses of farm owners. Furthermore, future genomic studies could examine different genes responsible for *E. coli* pathogenicity and drug resistance, which affect poultry farms and economic growth.

5. Conclusion

This study revealed a high prevalence of multidrug-resistant (MDR) *E. coli* isolates in poultry samples from Dinajpur, Bangladesh, with a notable presence of the *tetA* (29.6%) and *eaeV3* (14.81%) genes, as well as virulence-associated serogroups (such as O2 and O78) that can be transmitted zoonotically to human consumers. The widespread contamination of poultry with MDR *E. coli* poses a significant public health threat, as these resistant and virulent strains can be transmitted to humans through the food chain, direct contact, or environmental dissemination. To mitigate this emerging risk, a One Health approach-integrating animal, human, and environmental health sectors-is essential. Key priorities include the rational use of antibiotics in poultry, routine antimicrobial resistance monitoring, improved biosecurity, and public awareness programs. Future research should focus on molecular characterization of resistance determinants and surveillance of zoonotic transmission pathways to ensure the safeguarding of both poultry productivity and human health.

Declarations

Ethics approval and consent to participate

Not applicable

Ethical consideration

This research methodology was approved by ethical committee of Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Author's Contribution

Conceptualization, original draft writing, reviewing, and editing: Nazmi Ara Rumi, Md. Aoulad Hosen, Mahe Afroz. Formal analysis, investigations, funding acquisition, reviewing, and editing: Md. Aoulad Hosen, Nazmi Ara Rumi, Md. Shiblee Sadik Sabuj, Md. Shajedur Rahman. Tables and figures formating: Md. Hasabul Baker Tamlikha, Nasrin Sultana Tonu. Resources, data validation, data curation, and supervision: Nazmi Ara Rumi, Md. Aoulad Hosen. Before final submission, all authors gave their approval to publish this manuscript.

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Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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