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RESEARCH ARTICLE

Identification of Virulence Genes and Multidrug Resistance in Shiga-Toxin Producing *Escherichia coli* (STEC) from Migratory and Captive Wild Birds

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ABSTRACT

Multi-drug resistant-Shiga toxin producing *Escherichia* coli (MDR-STEC), poses considerable health risks for human, animals and birds. Migratory and captive wild birds are known carriers of this pathogen. This study aimed to investigate prevalence of MDR-STEC along with its associated virulence genes from migratory and captive wild birds in Bangladesh. A total of 247 fecal specimens were obtained from both migratory (119) and captive wild birds (128) for the isolation and characterization of *E. coli*. Standard microbiological and biochemical methods were used for identification and Polymerase Chain Reaction (PCR) was employed for confirmation of *E. coli* isolates followed by disc diffusion method to determine antibiotic susceptibility. The overall *E. coli* prevalence was 80.97% (200/247; 95% CI: 75.51-85.67), with a prevalence of 77.31% (92/119) in migratory birds and 84.38% (108/128) in captive wild birds. Among the 200 *E. coli* isolates, 53 (26.5%; 95% CI: 20.5-33.2) were identified as multidrug-resistant (MDR), with 21.7% (20/92) of MDR isolates originating from migratory birds and 30.6% (33/108) from captive wild birds. Only 24 (12%) isolates were positive for virulent gene *stx2* whereas 167 (83.50%) isolates were positive for *fimC*. Among the β-lactamase resistant genes, bla_{TEM} (91.50%; 183/200) was found significantly ($p<0.0001$) higher than bla_{SHV} (9.00%; 18/200). Among the antimicrobial resistant genes, 175 (87.50%) isolates were found positive for *qnrS* resistant gene. *E. coli* isolates of birds exhibited diverse phenotypic AMR patterns, with complete (100%) resistance to several antibiotics (ampicillin, ceftazidime, cefuroxime, and tetracycline) while being entirely sensitive to others (ceftriaxone, amikacin and aztreonam). This research underscores the concerning prevalence of *E. coli* strains having various virulent genes and resistant to multiple drugs among the wild birds. It emphasizes the immediate requirement for bridging wildlife and public health domains to address the threats posed by the antibiotic-resistant pathogens.

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INTRODUCTION

There are approximately 10,000 distinct bird species globally (Orubu *et al*., 2020). Birds have the ability to fly enormous distances from country to country. Mostly, the migratory birds come to temperate countries in winter and take harbor in haor, ponds, lakes and rivers. It has been observed that migratory birds can spread disease causing

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organisms to people and animals (Hird *et al*., 2015; Shah *et al*., 2022). Numerous studies have revealed bacterial disease transmission pathways from migrating birds to aquatic habitats (Guenther *et al*., 2010). Pathogenic bacteria from polluted aquatic environments can be disseminated to humans, livestock, and poultry through anseriformes species, including ducks and other waterfowl. Individuals living near migratory bird resting areas might find themselves exposed to water contamination, which they may attempt to utilize for residential or agricultural needs. Additionally, using contaminated water for the production of dairy or poultry might spread bacterial infections to humans and other animals. One of the microorganisms that migrating birds carry that have been found in their gut microbiomes is *Escherichia coli*, which is frequently found in both humans and avian hosts (Rahman *et al*., 2020). Fostering stronger connections among wildlife, domestic animals, and humans is crucial for improving veterinary and public health. Emerging research has shown that the intestinal microbiome of wild birds, particularly multidrug-resistant (MDR) *Escherichia coli (E. coli)*, plays a pivotal role in this intricate network (Guenther *et al*., 2010; Hopkins et al., 2005). Multidrug resistant avian pathogenic *E. coli* (APEC) have been found to inhabit in wild birds, and these birds can act as mechanical vectors to spread the infection to people and commercial poultry (Joseph *et al*., 2023). This complex relationship emphasizes the importance of understanding and controlling the dynamics existed between various animal populations and people in order to reduce the risks related to antibiotic resistance. According to Pokharel *et al*. (2023), pathogenic strains of *E. coli* pose a risk to the respiratory, urinary, and circulatory systems in both human and animals. In particular, avian colibacillosis, a condition affecting chicken, is attributed to avian pathogenic *E. coli* (APEC). This infectious disease holds substantial importance within the poultry industry (Foxman, 2010). Beyond its effects on avian health, APEC has a significant impact on human and animal health by highlighting the connection between pathogenic *E. coli* infections and the importance of holistic approaches to controlling and preventing such diseases. This disease induces inflammation in the bone marrow, pericardium, peritoneum, joints, fallopian tube, and the yolk sac in poultry (Joseph *et al*., 2023). Numerous virulence factors, including *fimC, stx1, stx2, fimH, cvi, iss, papC, tss,* and *iucD,* have been linked to its pathogenesis (Ievy *et al*., 2020; Islam *et al*., 2021). STEC strains exhibit one or both of the Shiga toxins, *Stx1* and *Stx2*. An elevated risk of hemolytic uremic syndrome is specifically linked to the *Stx2* gene (Nada *et al*., 2023; Ylinen *et al*., 2020). The number and arrangement of virulence genes in APEC determines the pathogenic potential of the bacteria (Johar *et al*., 2021).

The virulence genes (*fimC, iucD, stx1 and stx2*) play crucial roles in the pathogenicity of STEC. The *fimC* gene encodes a component of type 1 fimbriae, which is involved in bacterial adhesion to host cells, facilitating colonization and infection, while *iucD* gene is part of the aerobactin operon involved in iron acquisition, essential for bacterial growth in iron-limited environments (Rubab and Oh, 2020). The *stx1* and *stx2* genes encode Shiga toxins, which inhibit protein synthesis in host cells, leading to severe conditions like hemorrhagic colitis and hemolytic uremic syndrome (Naidoo and Zishiri, 2023). Regarding the issue of antibiotic resistance, bla_{TEM}, bla_{OXA}, and bla_{SHV} genes encode beta-lactamases that break down beta-lactam antibiotics (Van Hoek *et al*., 2023), *tetA* pumps out tetracycline, *qnrS* protects against quinolones, *sul1* confers resistance to sulfonamides, *strA* inactivates streptomycin, and *mefA* expels macrolides, reducing their efficacy. These genes collectively contribute to the multidrug resistance observed in STEC strains (Amézquita-López *et al*., 2024; Rubab and Oh, 2020). The *iucD* gene enhances iron acquisition, promoting bacterial growth and increasing the severity of systemic infections (Ievy *et al*., 2020). APEC infections, recognized as zoonotic diseases, are linked to *E. coli* infection in newborn meninges, leading to infant meningitis, as well as pathogenic infections in the urinary tract in human (Hird *et al*., 2015; Mellata, 2013).

A critical global issue threatening One Health is "antimicrobial resistance (AMR)". Failure to address AMR effectively by 2050 is projected to lead to 100 million human fatalities, alongside considerable economic losses and reduced productivity (Ahmed *et al*., 2024). Drug-resistant *E. coli* bacteria have been detected in both wild and migratory bird species (Naseer *et al*., 2017; Orubu *et al*., 2020) emphasizing the critical role that these birds play as reservoirs and significant vectors in the dissemination of antimicrobial resistance (AMR) within the environment (Bonnedahl and Järhult, 2014). *E. coli* strains exhibiting resistance to multiple treatments have been identified in wild birds held in captivity and during migration across various regions of the world (Sano *et al*., 2023). This study thoroughly examined the frequency, as well as the phenotypic and genotypic characteristics of AMR traits in Shiga toxin-producing *E. coli* (STEC) isolated from captive wild and migratory bird species.

MATERIALS AND METHODS

Ethical approval: The Animal Experimentation and Ethics Committee (AEEC), Sylhet Agricultural University, Bangladesh assessed and approved this work with the goal of improving animal welfare and minimizing the use of animals in vital research. The Divisional Forest Office and Wildlife Conservation Center, Sylhet, Bangladesh, as well as Animal Use Protocol No. [#AUP2022035] approved this inquiry. No specific authorization was required to perform the fieldwork because no sensitive or endangered species were harmed in any of the field investigations for this study.

Research framework and sampling approach: A cross-sectional study was carried out at a number of places in Sylhet division of Bangladesh from January 2022 to February 2023. The wild birds' (Black Crowned Heron, Dove, Eagle, Golden Pigeon, Grey Parrot, Grey Parrot, Grey Heron, Horn bill, Kalim, Kite, Macaw, Mathura, Myna, Parrot, Peacock, Purple Heron, Red Jungle Fowl, Silver Pigeon, Sun Conure, Vulture) fecal samples were collected from Tilagor Eco Park, Sylhet and Bangladesh Bannyaprani Sheba Foundation (BBSF), Sreemangal whereas migratory birds' (Red-crested Pochard, Glossy Ibis, Kingfisher and mixed flocks of duck species) fecal

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samples were collected from Horipur, Tanguar Haor and Tahirpur, Sunamganj (Fig. 1). Convenience sampling strategy was employed to collect the desired samples from the study area.

Determining the sample size: The sample size needed to estimate the prevalence was determined using a specific equation (Asha *et al*., 2024):

$$\boldsymbol{n = \frac{Z^2 P_{exp}(1 - P_{exp})}{d^2}}$$

Where, P_{exp}= expected prevalence, n = required sample size, d= desired absolute precision and Z = 1.96 for 95% confidence interval level. Utilizing prior data indicating a prevalence rate of 83.33% for *E. coli* in migratory birds (Islam *et al*., 2021) denoted as P_{exp} = 0.83, from a previous study, was employed to optimize the sample size. Using this P_{exp} with a desired absolute precision *d=0.05,* a required sample size of at least 214 birds were determined. We collected a total 247 fecal samples from both migratory (119) and captive wild birds (128). Fecal material was obtained using sterile collection techniques through sterile cotton swab stick and immediately transferred into sterile tubes containing buffered peptone water (BPW) to maintain the viability of the bacteria. The samples were kept in a thermal ice cooler box, ensuring a temperature between 2°C and 8°C during transportation to the laboratory. Upon arrival, the samples were promptly incubated at 37°C to ensure the optimal growth conditions for *E. coli* isolation.

***E. coli* Isolation and Identification:** *E. coli* was isolated and characterized using conventional culturing methods as described by Emon *et al*., (2024). The fecal specimens underwent an initial pre-enrichment phase in Buffered Peptone Water (BPW; Oxoid, UK), followed by incubation at 37°C for 24 ± 2 hours. Isolation procedures were then performed using selective media, including Eosin Methylene Blue (EMB; Oxoid, UK) and MacConkey agar (Oxoid, UK). Samples yielding positive cultures were further sub-cultured on EMB agar plates to obtain pure colonies, which were distinguished by their characteristic green metallic sheen. Confirmation of *E. coli* purity was enhanced through Gram staining and a series of biochemical assays, including sugar fermentation, MRVP (Methyl Red-Voges Proskauer), citrate utilization, motility, indole, and urease tests. Finally, the isolated *E. coli* strains were preserved in Brain Heart Infusion (BHI) broth with 10% glycerol at -20°C for future analysis.

Extraction and Amplification of Genomic DNA: Following the manufacturer's instructions for PCR analysis, genomic DNA was extracted from the isolated *E. coli* strains using a DNA extraction kit (AddBio Inc. Ltd., Daejeon, Korea). A specific primer set, *phoA* (SFC Probes Ltd., Gyeonggi, Korea), was used to identify *E. coli*; Table 1 contains the primer sequences. Each PCR reaction was prepared with a final volume of 20 µl, consisting of 5 µl of DNA sample, 10 µl of 2x master mix (Add Bio Inc., South Korea), 1 µl of each primer (10 pmol/µl), and 3 µl of DNase-free water. Following the guidelines set forth by Shome *et al*., (2011), electrophoresis was performed using a 1.5% agarose gel following a successful amplification.

Identification of virulence genes: To identify virulence genes, each *E. coli* isolate was subjected to uniplex PCR screening, following a specific protocol described by Al-Kandari and Woodward, 2019**.** The results of the PCRs, conducted in separate reactions using primers to detect the two virulence genes, *fimC* and *iucD*, are presented in Table 1.

Multiplex PCR for detection of STEC: The analysis of each *E. coli* isolates for the presence of *stx* genes (*stx1* and/or *stx2*) was performed using a multiplex polymerase chain reaction (PCR) method, following the protocols outlined by Al-Kandari and Woodward, (2019)**.** This multiplex PCR utilized targeted primers specifically engineered to detect two distinct variants of STEC (Table 1). A total volume of 25 µL was used for the PCR, which included 4.5 µL of nuclease-free water, 12.5 µL of 2x master mix (Add Bio Inc, South Korea), 1 µL of forward and reverse primers (10 pmol/gL), and 5 µL of genomic DNA. Cycling conditions were optimized at initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 20 sec, annealing at 58°C for 40 sec, and 90 sec of extension at 72°C, followed by 5 minutes of 72°C extension.

Antimicrobial susceptibility assessment: Using disk diffusion techniques on Mueller-Hinton agar (Oxoid, UK), antimicrobial- susceptibility testing (AST) for *E. coli* isolates was carried out in accordance with the guidelines outlined by Hindler and Munro, (2024). Following that, the diameter of the inhibitory zones (mm) was measured using the Clinical and Laboratory Standards Institute's (CLSI, 2020, M100, 30th Edition) recommendations. Thirteen distinct antimicrobial disks in all, comprising the beta-lactam combination agent: amoxicillin + clavulanate (AMC, 20/10 µg); Penicillin: ampicillin (AMP, 10 µg); Tetracyclines: tetracycline (TE, 30 µg); Cephems: cefuroxime (CXM, 30 µg), ceftriaxone (CTR, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg); Quinolones: ciprofloxacin (CIP, 5 µg); Macrolides: aztreonam (AT, 30 µg); Folate Pathway Antagonists: trimethoprim-sulfamethoxazole (COT, 1.25/23.75 µg); Aminoglycosides: gentamicin (GEN, 10 µg), amikacin (AK, 30 µg), and monobactams: aztreonam (AT, 30 µg) were used to perform AST. In this investigation, isolates that were resistant to at least one agent from each of three distinct antibiotic classes were classified as multidrug-resistant (MDR) isolates (Magiorakos *et al.,* 2012; Sweeney *et al.,* 2019). In this study, *Pseudomonas aeruginosa* ATCC 27853 was employed for the quality control (QC) test. The Multiple Antibiotic Resistance (MAR) index was determined and assessed using the approach and formula described by Rahman *et al*., (2024).

Identification of Antibiotic Resistance Genes: The ß-lactamase genes were checked in all of the isolated samples (bla_{TEM}, $bla_{OXA,}$ bla_{SHV}), tetracycline resistant genes (*tetA*), ciprofloxcin resistant gene (*qnrS*), sulfonamide resistant genes (*sul1*), streptomycin resistant gene (*strA)* and azithromycin resistant gene (*mefA*) using uniplex PCR. The primers used in this investigation for PCR are shown in Table 1.

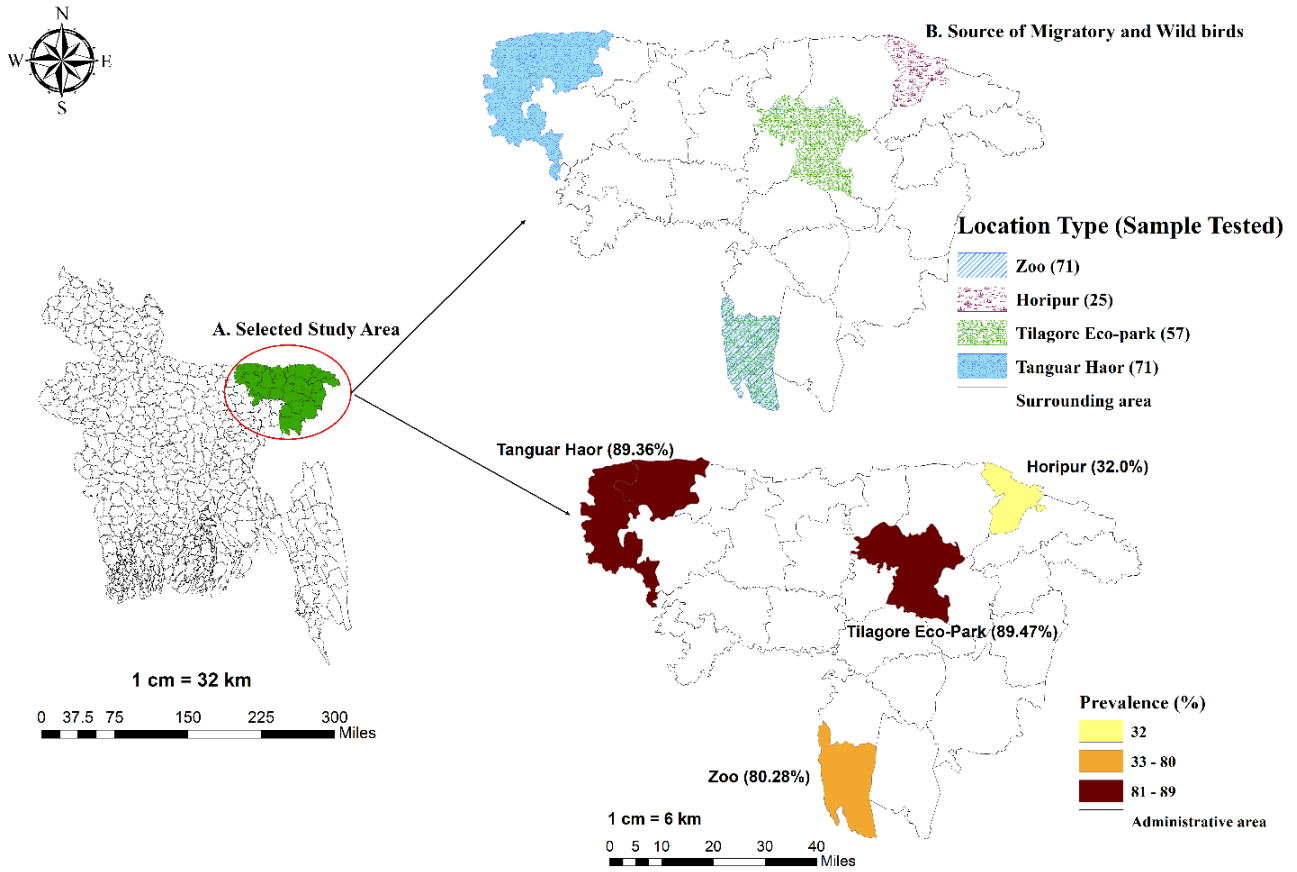

Fig. 1: Study area showing the different location where samples were collected and Prevalence was shown in choropleth map. Shape file was extracted from DIVA-GIS using Geographical information system (GIS) to develop the map with ArcMap 10.7 (ArcGIS, ESRI, USA).

Table 1: The primer sequences, amplicon sizes and target genes for the detection of *E. coli*, STEC, ESBL resistant and Virulence gene.

Target Genes	Primer Sequence (5'–3')	Amplicon Size (bp)	AT (°C)	References
PhoA	F: GGTAACGTTTCTACCGCAGAGTTG R: CAGGGTTGGTACACITGTCATTACG	464	59	(Shome *et al*., 2011)
fimC	F: GGTAGAAAATGCCGATGGTG R: CGTCATTTTGGGGGTAAGTGC	496	59	(Islam *et al*., 2021)
iucD	F: ACAAAAAGTTCTATCGCTTCC R: CCTGATCCAGCTGATGCTC	692	58	(Janßen *et al*., 2001)
Stx1	F: ACACTGGATGATCTCAGTGG R: CTGAATCCCCCTCCATTATG	614	58	(Dipineto *et al*., 2006)
Stx2	F: CCATGACAACGGACAGCAGTT R: CCTGTCAACTGAGCAGCACTTT	779	58	(Dipineto *et al*., 2006)
bla_{TEM}	F: CATTTCCGTGTCGCCCTTATTC R: CGTTCATCCATAGTTGCCTGAC	800	62	(Ibrahim *et al*., 2021)
bla_{SHV}	F: AGCCGCTTGAGCAAATTAAAC R: ATCCCGCAGATAAATCACCAC	713	62	(Ibrahim *et al*., 2021)
bla_{OXA}	F: GGCACCAGATTCAACTTTCAAG R: GACCCCAAGTTTCCTGTAAGTG	564	62	(Ibrahim *et al*., 2021)
tetA	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	502	63	(Karczmarczyk *et al*., 2011)
qnrS	F: ACGACATTCGTCAACTGCAA R: TAAATTGGCACCCTGTAGGC	417	53	(Gay *et al*., 2006)
SulI	F: CGGCATCGTCAACATAACCT R: TGTGCGGATGAAGTCAGCTC	433	66	(Lanz, Kuhnert, and Boerlin, 2003)
strA	F: ATGGTGGACCCTAAAACTCT R: CGTCTAGGATCGAGACAAAG	893	63	(Kozak *et al*., 2009)
mef(A)	F: AGTATCATTAATCACTAGTGC R: TTCTTCTGGTACTAAAAGTGG	345	54	(Nguyen *et al*., 2009)

F: Forward; R: Reverse; AT: Annealing Temperature.

Statistical analysis: Data were input, structured, and organized into Excel spreadsheets. Disease prevalence was determined using the standard formula followed by Mahen *et al*., (2024)**.** A univariate analysis was performed to investigate relationships among different variables using the Chi-square test. Fisher's Exact Test was applied when the expected frequency in a cell was below 5 and this condition was met in at least 20% of the cells (Hoque *et al*., 2023)**.** The Binomial exact test was used to compute confidence intervals, and a threshold of less than 0.05 was used to demonstrate statistical significance. All the statistical analysis was carried out using R version 4.3.3.

Geo-spatial mapping and plot: Heat maps and Venn diagrams were created using Origin-Pro (Origin: Data Analysis and Graphing Software) to illustrate the antimicrobial properties of the isolates. These tools provided an in-depth view of the data by making it easier to create intricate Venn diagrams and Heat Maps. ArcMap 10.7 was utilized to create the research area map, with a shape file obtained from (www.diva-gis.org). The choropleth was produced using this mapping data effectively showed the sample sizes and prevalence of different explanatory variables.

RESULTS

Cultural and Biochemical Properties of *E. coli*: Based on microbiological growth on EMB agar, the overall prevalence of *E. coli* was 80.97% (200/247). Gram-negative (pink) rod-shaped *E. coli* bacterium observed under the microscope. Results of several biochemical tests (Sugar fermentation, MRVP, citrate utilization, motility, indole, and urease tests) revealed the similar findings.

Prevalence of *E. coli* isolates by molecular assay: It was concluded from the PCR results (Fig. 2) of this study that *E. coli* was detected in 80.97% (95% CI: 75.51-85.67) of captive wild and migratory birds. Specifically, 77.31% (92 out of 119) of migratory birds and 84.38% (108 out of 128) of wild captive birds tested positive for *E. coli*. According to the location, Tilagor Eco- Park found statistically (p<0.001) the highest prevalence (89.47%; 51/57) and Haripur found the lowest prevalence (32.00%; 8/25). Most of the captive birds like Dove (*Columba livia domestica*), Golden Pigeon (*Columba aurea*), Grey Parrot (*Psittacus erithacus*), Myna (*Acridotheres tristis*), Parrot (*Psittaciformes*), Peacock (*Pavo cristatus*), Red jungle fowl (*Gallus gallus*), Silver Pigeon (*Columba argentina*) and Vulture (*Cathartes aura*) showed 100% positivity for *E. coli* (Table 2).

Identification of Genes Associated with Antimicrobial Resistance: Diverse categories of resistance-associated genes, including STEC, virulence genes, ESBL-resistant genes, and antibiotic-resistant genes, were identified and documented in Table 3. The identified resistant genes were shown in Fig. 2E-2J. Antibiotic resistance profiling showed that three isolates exhibited the presence of all (*tetA*, *mefA*, *qnrS*, *sul1* and *strA*) the tested antibiotics resistance genes (Fig. 2C). In comparison to bla_{OXA}, the genes conferring antimicrobial resistance, namely bla_{TEM} and bla_{SHV}, were identified in 91.50% (183/200) and 9% (18/200) of the *E. coli* isolates, respectively (Table 3). The prevalence of the *qnrS* resistant gene was significantly greater across the total isolates, with 87.50% (175 out of 200) demonstrating resistance; this was a statistically significant finding (p<0.0001). The Spearman correlation coefficient (ρ) was calculated to evaluate the relationships between *E. coli* and various genes. The results revealed a weak positive correlation (ρ = 0.1–0.3) between *E. coli* positive isolates and the genes *fimC*, bla_{TEM}, *strA*, and *tetA*. Additionally, a moderate positive correlation (ρ = 0.4–0.6) was observed between *strA* and *fimC* (ρ = 0.43), *tetA* and *fimC* (ρ = 0.44), as well as *qnrS* and bla_{TEM} (ρ = 0.47). Notably, a strong positive correlation was found between *strA* and *tetA* (ρ = 0.92), indicating a significant association between these two genes (Fig. 3A).

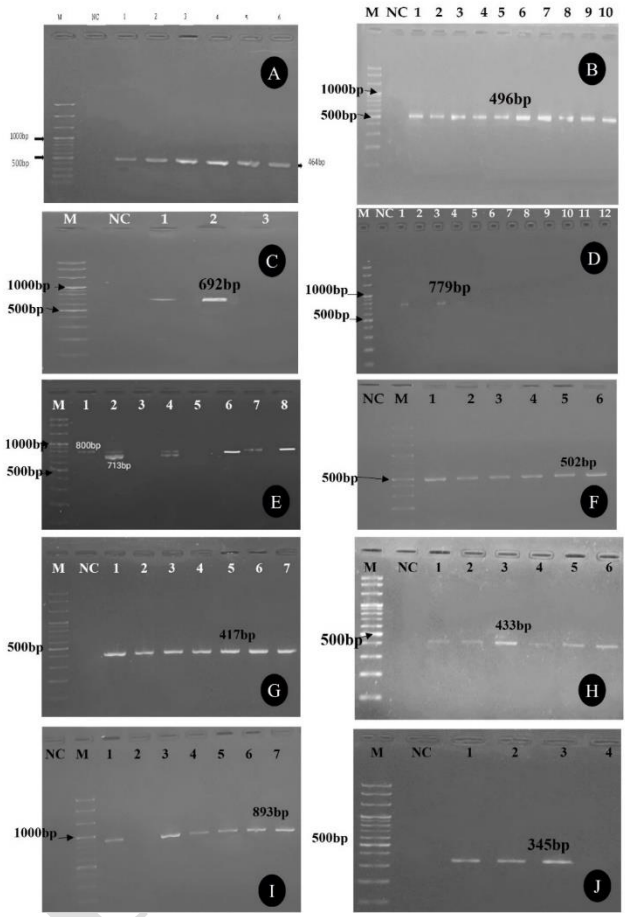

Fig. 2 (A-J): Electrophoresis on 1.5% agarose gel showing specific amplified band of different gene of *E. coli* amplification by PCR; Lane M: 100 bp Marker DNA; Lane NC: Control (– ve); A: Lane (1-6) reaction specific (+ ve) for *PhoA* gene (464 bp); B: Lane (1-10) reaction specific (+ve) for *fimC* virulent gene (496 bp); C: Lane 1-2 (+ve) for *iucD* virulent gene (692 bp); D: Lane 1, 3 (+ve) for *stx2* STEC gene (779 bp); E: Multiplex PCR for ESBL producing bla_{TEM} (800 bp) and bla_{SHV} (713 bp); F-J: Showing specific band 502 bp, 417 bp, 433 bp, 893 bp and 345 bp for *tetA, qnrS, Sul1, strA and mefA*, respectively.

Virulence Gene Identification and Co-Existence: Out of 200 *E. coli* isolates, 167 (83.5%) tested positive for the virulence gene *fimC*, while 17 (8.5%) were positive for the virulence gene *iucD* (3). Notably, only three isolates tested positive for both virulence genes. The difference in percentage between *fimC* and *iucD* was highly significant (p < 0.001), as illustrated in Fig. 2B. In the uniplex PCR testing, both *fimC* and *iucD* genes displayed distinct bands of 496bp (Fig. 2B) and 692bp (Fig. 2C), respectively. The Venn diagram shows the coexistence of virulence genes (*fimC*, *iucD*) and beta-lactam resistance genes (bla_{TEM}, bla_{SHV}). Only two isolates had both virulence and resistance genes. Six isolates were positive for *fimC*, bla_{TEM}, and bla_{SHV}, while five were positive for *iucD*, bla_{TEM}, and bla_{SHV}. Additionally, two isolates had both beta-lactam resistance genes, and three were positive for five antibiotic resistance genes (*tetA*, *qnrS*, *sul1*, *strA*, *mefA*), as shown in Fig. 3B and 3C.

Detection of STEC: The *stx2* gene was found in 24 isolates (12%; 24/200), while none of the isolates tested positive for

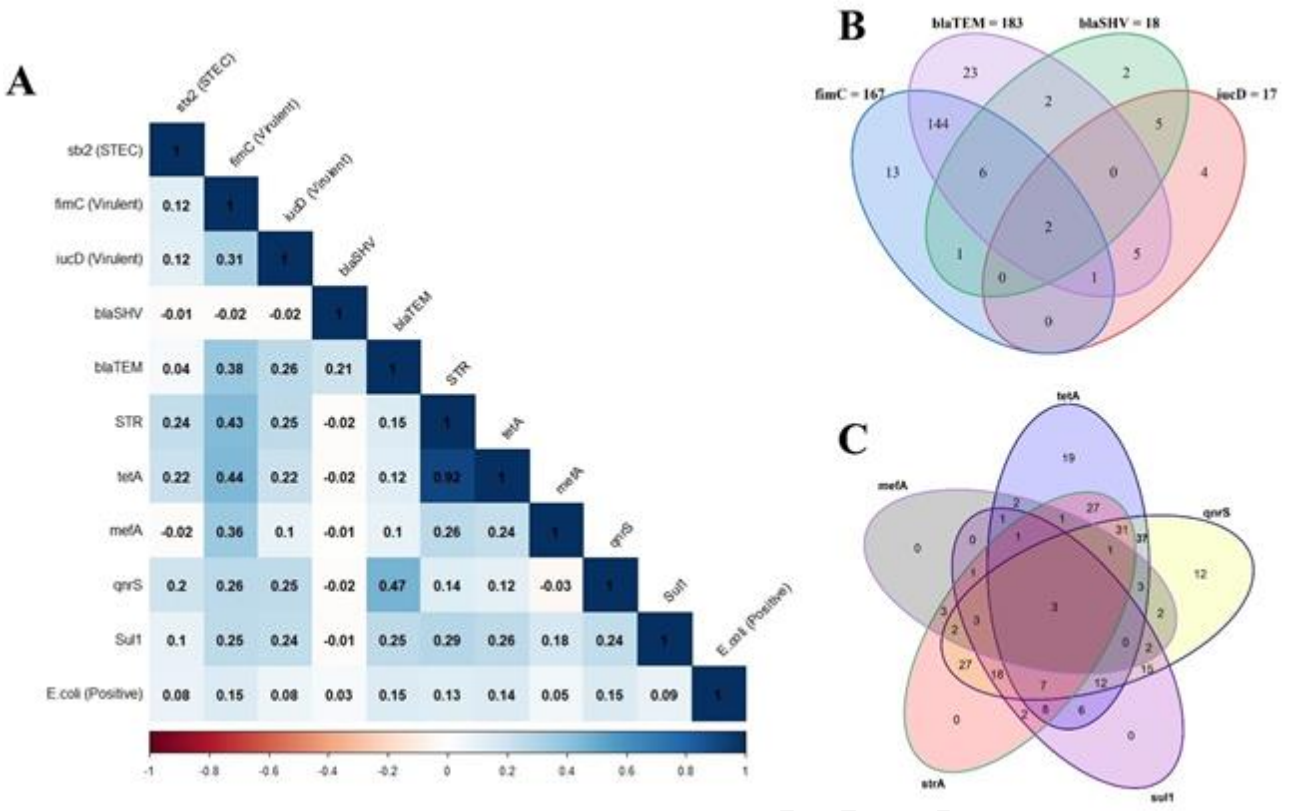

Fig. 3 (A-C): (A) Spearman correlation among the different category of identified genes; (B) Venn diagram showing the interaction of the isolates between Virulent and ESBL resistant genes; (C) Venn diagram showing interaction among different antimicrobial resistant genes.

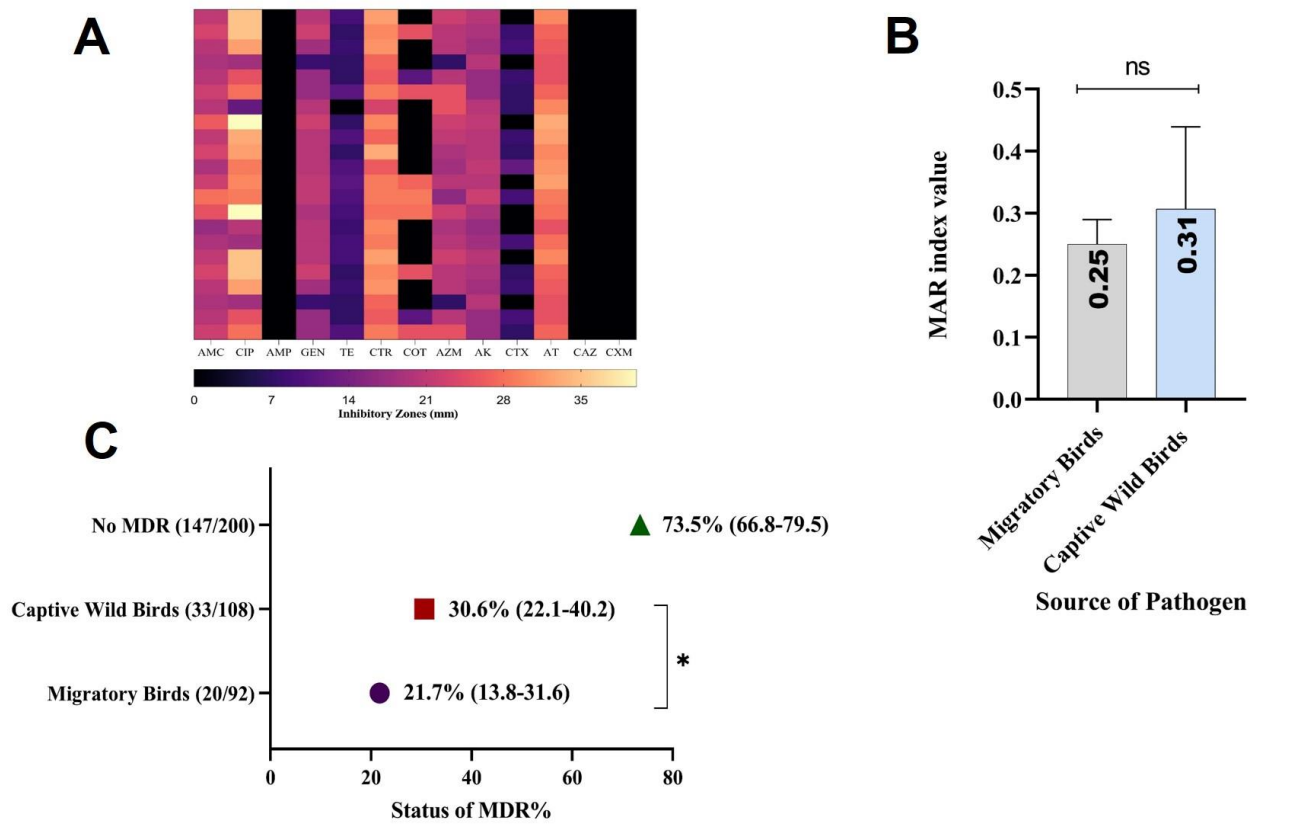

Fig. 4: 4A: Heat Map showing the resistance pattern with their average zone of inhibition (mm) indicated as color scale; CTX = cefotaxime; TE = tetracycline; CIP = ciprofloxacin; AZM = azithromycin; COT = trimethoprim-sulfamethoxazole; GEN = gentamicin; CXM = cefuroxime; CAZ = ceftazidime; CTR = ceftriaxone; AMP = ampicillin; AMC = amoxicillin+clavulanate; AK = Amikacin; AT = aztreonam. Figure- 4B: MAR index value; Figure- 4C: Status of MDR %; MDR: Multi-drug resistant, MAR: Multiple antibiotic resistant; NS: Non-significant; *indicates *p* value less than 0.05.

Table 2: Univariate analysis showing the prevalence of *E. coli* in migratory and wild captive birds

Category	Explanatory Variable	n/N	Prevalence% (95% CI)	Fisher's Exact Test/ χ^2	*p*-value
Type of Birds				1.997	0.16
	Migratory	92/119	77.31% (68.73-84.48)		
	Wild captive birds	108/128	84.38% (76.90-90.19)		
	Total	200/247	80.97% (75.51-85.67)		
Location				45.90	<0.001
	Eco- Park (Tilagore)	51/57	89.47% (78.48-96.04)		
	Haripur (Sylhet)	8/25	32.00% (14.95-53.50)		
	Zoo (Moulavibazar)	84/94	89.36% (81.30-94.78)		
	Tanguar Haor (Sunamganj)	57/71	80.28% (69.14-88.78)		
Birds' species				29.72*	0.014
	Black Crowned Heron	3/6	50.00% (11.81-88.19)		
	Dove	4/4	100.00% (39.76-100.00)[a]		
	Eagle	3/6	50.00% (11.81-88.19)		
	Golden Pigeon	2/2	100.00% (15.81-100.00)[a]		
	Grey Parrot	8/8	100.00% (63.06-100.00)[a]		
	Grey Heron	6/8	75.00% (34.91-96.81)		
	Horn bill	4/5	80.00% (28.36-99.49)		
	Kalim	9/11	81.82% (48.22-97.72)		
	Kite	2/3	66.67% (9.43-99.16)		
	Macaw	2/3	66.67% (9.43-99.16)		
	Mathura	1/3	33.33% (0.84-90.57)		
	Myna	8/8	100.00% (63.06-100.00)[a]		
	Parrot	7/7	100.00% (59.04-100.00)[a]		
	Peacock	10/10	100.00% (69.15-100.00)[a]		
	Purple Heron	1/3	33.33% (0.84-90.57)		
	Red Jungle Fowl	4/4	100.00% (39.76-100.00)[a]		
	Silver Pigeon	2/2	100.00% (15.81-100.00)[a]		
	Sun Conure	4/5	80.00% (28.36-99.49)		
	Vulture	3/3	100.00% (29.24-100.00)[a]		
	Wild Bird (Non-specific)	24/26	92.31% (74.87-99.05)		

[a]Superscript Means One-sided 97.5% Confidence Interval; N= Total tested, n= positive case, CI= Confidence interval; *Superscript means Fisher's Exact Test where minimum 20% cell have expected count less than 5.

Table 3: Detection of genes (STEC, Virulent, ESBL resistant, Resistant gene) from isolated (n = 200) *E. coli* in migratory and wild captive birds.

Category of Gene	Identified Gene	PCR positive	Prevalence% (95% CI)	χ^2	*p*-value
STEC				25.53	<0.001
	stx1	0	0.00% (0.00-1.83) *		
	stx2	24	12.00% (7.84-17.33)		
Virulent Gene				226.45	<0.0001
	fimC	167	83.50% (77.62-88.36)		
	iucD	17	8.50% (5.03-13.26)		
ESBL Resistant Gene				465.65	<0.0001
	bla_{TEM}	183	91.50% (86.74-94.97)		
	bla_{SHV}	18	9.00% (5.42-13.85)		
	bla_{OXA}	0	0.00% (0.00-1.83) *		
Antibiotic Resistant Gene				525.09	<0.0001
	tetA	159	79.50% (73.23-84.87)		
	qnrS	175	87.50% (82.10-91.74)		
	sul1	79	39.50% (32.68-46.64)		
	strA	134	67.00% (60.02-73.47)		
	MefA	25	12.50% (8.26-17.90)		

*Superscript Means One-sided 97.5% Confidence Interval; CI: Confidence interval; STEC: Shiga-toxin producing *Escherichia coli.*

the *stx1* gene. In the multiplex PCR assays, a distinct band of 779bp size was observed for the *stx2* gene, while no corresponding band was detected for the *stx1* gene (Fig. 2D).

Antimicrobial Susceptibility Patterns: The heat map (Fig. 4A), using a color scale to represent zones of inhibition from 0 to 40 mm, graphically illustrates the antibiotic sensitivity patterns of the *E. coli* isolates. Notably, all 200 isolates showed complete (100%) resistance to multiple antibiotics, including ampicillin, ceftazidime, cefuroxime, cefotaxime, and tetracycline. In contrast, the isolates demonstrated complete (100%) sensitivity to ceftriaxone, amikacin, and aztreonam.

Comparison of Antimicrobial Susceptibility Test Results: All isolates exhibited complete resistance to tetracycline and cephalosporins, including cefuroxime, ceftazidime, and cefotaxime. However, *E. coli* isolates from captive wild birds showed lower sensitivity to amoxicillin + clavulanate (87.5%) and ciprofloxacin (62.5%) compared to migratory bird isolates (100% and 85.71%, respectively). Captive birds exhibited higher sensitivity to trimethoprim-sulfamethoxazole (87.5%) compared to migratory birds (57.14%). Additionally, captive birds were 100% sensitive to azithromycin, while migratory birds showed 85.71% sensitivity.

Status of MDR and MAR Index: Among the 200 *E. coli* isolates, 53 (26.5%; 95% CI: 20.5-33.2) were identified as multidrug-resistant (MDR), with 21.7% (20/92) of MDR isolates originating from migratory birds and 30.6% (33/108) from captive wild birds (Fig. 4C). The MAR index

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Table 4: Comparison between migratory bird and captive wild bird of antimicrobial susceptibility test of *E. coli* isolates by disk diffusion method.

Antibiotics	Migratory Birds (n=92)		Captive wild Birds (n=108)	
	Sensitive(S) %	Resistant(R) %	Sensitive(S) %	Resistant(R) %
Penicillins				
amoxicillin+clavulanate (AMC, 20/10 µg)	100	0	87.5	12.5
ampicillin (AMP, 10 µg)	0	100	0	100
Cephalosporins				
ceftriaxone (CTR, 30 µg)	100	0	100	0
ceftazidime (CAZ, 30 µg)	0	100	0	100
cefuroxime (CXM, 30 µg)	0	100	0	100
cefotaxime (CTX, 30 µg)	0	100	0	100
Tetracyclines				
tetracycline (TE, 30 µg)	0	100	0	100
Fluoroquinolones				
ciprofloxacin (CIP, 5 µg)	85.71	14.29	62.5	37.5
Macrolide				
azithromycin (AZM 15 µg)	85.71	14.29	100	0
Sulfonamides				
trimethoprim-sulfamethoxazole (COT, 1.25/23.75 µg)	57.14	42.46	87.5	12.5
Aminoglycosides				
gentamicin (GEN, 10 µg)	85.71	14.29	100	0
amikacin (AK 30 µg)	100	0	100	0
Monobactam				
aztreonam (AT, 30 µg)	100	0	100	0

ranged from 0.23 to 0.46, averaging 0.27. The highest MAR index value of 0.46 was observed in 16 isolates from captive wild birds, indicating significant drug resistance in captive conditions. In comparison, 16 isolates from migratory birds had a MAR index of 0.31 (Fig. 4B).

DISCUSSION

Millions of migratory birds travel globally each year, playing a key role in the spread of antibiotic-resistant bacteria. Their interactions with wildlife, other species, and human-altered ecosystems facilitate this bacterial transmission across vast distances (Yuan *et al*., 2021; Elsohaby *et al.,* 2021). In light of the constrained knowledge regarding MDR bacteria in migratory and captive wild birds, our study focused on detecting MDR-STEC *E. coli*.

This phenomenon is underscored by the findings of our study, revealing a staggering 77.31% prevalence rate of *E. coli* among migratory birds. This prevalence rate notably exceeded the figures documented in previous studies conducted in Bangladesh, Northern Italy, and Egypt (Rashid *et al*., 2015; Dotto *et al*., 2016; Fahim *et al*., 2019). The findings closely aligned with those reported in a study from Bangladesh who found the prevalence of *E. coli* from migratory bird was 83.33% (Islam *et al*., 2021). However, our findings indicated a lower prevalence rate when compared to a prior investigation conducted by Shobrak and Abo-Amer, (2015). Several factors may contribute to these observed variations, including disparities in detection methodologies, regional and seasonal dispersion patterns, sample sizes, and the diverse array of migratory bird species studied. Furthermore, it is crucial to acknowledge the potential influence of stressful circumstances on the shedding of pathogens by migrating birds during their arduous journeys (Rashid *et al*., 2015). These multifaceted elements collectively shape the intricate dynamics of antibiotic-resistant pathogen prevalence among avian populations, highlighting the complexity of this ecological phenomenon.

Moreover, the findings from this study highlighted a relatively higher incidence of *E. coli* among captive wild birds, standing at 84.38%. This prevalence rate exceeded that reported in a study by Zurfluh *et al*., (2019), which documented the prevalence of 53.7%. However, it fell short of the remarkably high prevalence rate of 93.3% observed in a study (Shobrak and Abo-Amer, 2015). It is essential to emphasize the differences in prevalence rates among captive wild birds may stem from management risk factors in captivity as well as differences in sampling methods and laboratory techniques employed across various research endeavors.

In case of virulent gene detection, our study revealed varying prevalence rates, with the *fimC* gene exhibiting a significantly higher occurrence at 83.5%, in contrast to the lower prevalence of the *iucD* gene at 8.5%. Notably, previous research in Bangladesh identified the *fimC* and *iucD* virulence genes at rates of 67.27% and 29.09% respectively, indicating a shift in these prevalence rates (Islam *et al*., 2021). Additionally, migratory birds in Italy were found to harbor APEC-associated virulence genes (Bertelloni *et al*., 2019). The *iucD* gene's involvement in APEC pathogenesis, mediating aerobactin synthesis and iron acquisition pathways, underscores its significance (Subedi *et al*., 2018). When APEC colonizes avian hosts, it poses a risk through environmental dissemination, potentially endangering humans, animals, and other avian species through the fecal route (Hu *et al*., 2022). Furthermore, the threat of APEC to both cattle and human populations might be exacerbated by biological and environmental stressors. Crucially, our study identified APEC-associated virulence genes in migrating birds, raising concerns as these genes have also been found in humans and wild mammals, as reported by Fr̈mmel *et al*., (2013). This underscores the potential transmission risks between avian species, humans, and other wildlife, highlighting the complex interplay of these virulence genes within various ecosystems.

This study showed that Shiga toxin-producing *E. coli* (STEC) was comparatively rare among wild and migratory birds, affecting just 12% of the isolates. Interestingly, out of 200 isolates, only 24 had the *stx2* gene found. On the other hand, according to a previous study conducted in Bangladesh, 35% (8 out of 23) of the isolates had STEC and showed either the *stx1* or *stx2* gene (Johura *et al*., 2017). Researchers have looked into the frequency of Shiga toxin-producing *E. coli* (STEC) in different bird species from different parts of the world also noted that 4.5% of isolates from Iranian broilers had the *stx2* gene identified (Ghanbarpour *et al*., 2011). Intriguingly, similar to our current study, as well as findings by Kobayashi *et al*., (2002). *E. coli* derived from wild birds in their research did not exhibit the presence of either *stx1* or *stx2* genes. These observations highlight the variable prevalence of STEC and gene distribution among avian populations across different regions and underscore the need for continued research to elucidate these patterns.

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Wild birds, owing to their frequent movement and exposure to various ecological factors, may have an elevated risk of disseminating antimicrobial resistance (AMR) determinants to humans and other wildlife species (Arnold *et al.*, 2016). Our study showed that *E. coli* exhibited high resistance to various antibiotics such as ampicillin, ceftazidime, cefuroxime, cefotaxime, and tetracycline. Likewise, a separate study conducted in Bangladesh found comparable resistance patterns in *E. coli* strains isolated from migratory birds (Islam *et al*., 2021). This research emphasizes how widely antibiotics are used in Bangladeshi veterinary and human healthcare settings. A Multiple Antibiotic Resistance (MAR) index above 0.20 suggests that the isolates predominantly originate from a resistant source Emon *et al*., (2024). In our study, 53 isolates had MAR index values exceeding 0.20, with an average of 0.27, indicating moderate levels of bacterial contamination in the samples.

The development of antibiotic resistance in these bacteria is probably caused by a number of causes, the most important of which is the strong selective pressure brought on by the indiscriminate and widespread use of antibiotics (Perry and Wright, 2013). This highlights the imperative requirement for initiatives to control antibiotic misuse and to vigilantly monitor and address antimicrobial resistance (AMR) in both human and veterinary medicine.

In our current investigation, we identified specific antibiotic resistance genes, including bla_{TEM} (91.5%), bla_{SHV} (9%), *tetA* (79.5%), *qnrS* (87.5%), *sul1* (39.5%), *strA* (67%), and *mefA* (12.5%), responsible for conferring resistance to particular antibiotics. In the same geographic region of Bangladesh, studies have frequently detected resistant genes such as bla_{TEM}, bla_{SHV}, *tetA*, *qnrS*, *sul1*, *strA* in various organisms (Emon *et al.*, 2024; Liza *et al.*, 2024; Naser *et al.*, 2024). This suggests a potential for horizontal gene transfer in the captive wild bird populations, contributing to the spread of antibiotic resistance.

It is plausible that these resistance isolates in migratory and wild birds may have been acquired from environments contaminated by human waste or secretions (Islam *et al*., 2021). The coexistence of multiple resistance genes underscores the genotypic complexity underlying antibiotic resistance, which may potentially foster the development of multidrug-resistant (MDR) pathogens. Unfortunately, resistance patterns indicate a prevalent misuse and overuse of various antibiotic classes, often without due consideration of the detrimental consequences of antimicrobial resistance (AMR) (Byrne *et al*., 2019). A study by Mukerji *et al*., (2019) reported the elevated resistance levels to crucial antimicrobials like extended-spectrum cephalosporins and fluoroquinolones were documented among wild birds. These outcomes point to a concerning pattern of antibiotic overuse and misuse, as well as the possibility of cross-contamination in different environmental contexts, which can promote the emergence of antibiotic resistance. Multidrug-resistant bacteria are becoming more common, which is especially alarming as they pose a serious threat to public health by making it more difficult to administer effective therapy.

This study is an initial attempt to identify the virulence and resistance genes of *E. coli* from wild birds that migrate through the Sylhet region of Bangladesh that are kept in captivity. Whole genome sequencing would offer a more thorough identification of virulence and resistance genes in the isolated organisms, despite the fact that this is only a starting investigation. Parallel to this, including *E. coli* isolates from a wider variety of avian species-both migratory and domestic would enhance the study's scope and reliability. This study has some limitations. The molecular identification of virulence and resistance genes was performed but whole genome sequencing could provide more comprehensive insights into the genetic makeup of the isolates. Additionally, environmental factors, such as the birds' diets or habitats, which may influence the spread of antimicrobial resistance, were not thoroughly investigated. Finally, the study did not assess the potential for horizontal gene transfer between bacterial species, which could further complicate the resistance scenario.

Conclusions: This study highlights the alarming prevalence of antimicrobial-resistant *E. coli* in both migratory and captive wild birds, particularly the identification of multidrug-resistant (MDR) Shiga toxin-producing *E. coli* (STEC). The significant occurrence of virulence genes, such as *fimC* and *iucD*, and resistance genes like bla_{TEM} and *qnrS* in *E. coli* of migratory and captive wild birds indicates potential public health threats as they act as reservoirs and disseminators of these pathogens. Notably, the detection of STEC strains and their resistance to critical antibiotics, including tetracycline and cephalosporins, emphasizes the pressing need for a One Health approach. Importantly, the emergence of co-existing resistance genes within the *E. coli* isolates suggests a complex interplay between different resistance mechanisms, which may enhance the survival and adaptability of these pathogens in both avian and human hosts. Collaborative monitoring and containment strategies are crucial to mitigate the risk of zoonotic transmission and the spread of antibiotic resistance from wildlife to human populations.

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