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Research article

Multidrug resistant *Escherichia coli* in backyard poultry and captive pigeons in Chattogram, Bangladesh

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A B S T R A C T

The emergence of antimicrobial resistance, particularly multidrug resistance (MDR) in *Escherichia coli* (*E. coli*), is becoming a major concern in developing countries like Bangladesh. In this study, we aimed to identify the MDR *E. coli* in backyard poultry and pet pigeons in the Chattogram district of Bangladesh. For this, *E. coli* isolates were identified from 61 cloacal swabs (36 from backyard poultry and 25 from pet pigeons) and revealed 82% (50/61) prevalence in backyard poultry and pet pigeons. *E. coli* isolates were further investigated for the variations in the antimicrobial resistance profiles of 12 selected antimicrobial belonging to 7 classes of antibiotics by the disk diffusion method. The antimicrobial susceptibility assay showed high resistance against erythromycin (88%), amoxicillin (82%) and tetracycline (64%). In contrast, isolates were found to be sensitive against ceftriaxone (98%), gentamicin (92%), and chloramphenicol (80%). In addition, antibiotic resistance genes (*sul1* or *sul2*) against sulfonamides were identified in 22% of total isolates through polymerase chain reaction (PCR). In total, 44 (88%) isolates found resistance to ≥3 antimicrobials; among them, 4 (8%) isolates showed resistance to ≥7 antimicrobials tested. The result clearly shows that, MDR *E. coli* isolates are commonly present in backyard poultry and pigeons in this area.

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1. INTRODUCTION

Conventional backyard farming of poultry, such as, chickens, ducks, geese, guinea fowl, and turkeys can contribute up to 70% of the total poultry production in low-income countries like Bangladesh (Alders et al., 2018). However, Enterobacteriaceae, particularly *E. coli*, is of great concern of this farming. The proximity between backyard poultry and humans may accelerate the transmission of this pathogen between the 2 host species (Vanessa, 2011). It is one of the most common avian bacterial pathogens causing diseases, leading up to 30% of poultry mortality (Georgnaras et al., 2001). In addition to backyard poultry, pigeons reared as pet birds are considered as potential reservoir for pathogenic *E. coli* strains with zoonotic potential too (Sonntag et al., 2005; Silva et al., 2009; Mia et al., 2022).

Antibiotic resistance in zoonotic pathogens has been considered as the greatest threat to public health and an emerging challenge for today's

world (Dadgostar, 2019). Antibiotics are often used in poultry farming systems as therapeutics as well as preventive interventions. However, using of suboptimal doses has often been associated with developing antimicrobial resistance (AMR) in poultry. Moreover, over the time, *E. coli* also has developed a remarkable capacity to acquire antibiotic resistance genes through horizontal gene transfer (Capita and Alonso-Calleja, 2013). Therefore, many *E. coli* strains in poultry are the reservoir of antibiotic resistance genes that may transmit to humans (Kheiri and Akhtari, 2016; Nhung et al., 2016). The possession of antibiotic-resistant bacteria by backyard poultry and other captive pet birds could increase this problem if they spread these bacteria to the close contact humans. There is also a chance of potential infection to the consumers by antibiotic-resistant bacteria through cross-contamination or insufficiently cooked poultry meat. Therefore, more emphasis should be given to identify the presence of antibiotic-resistant *E. coli* in backyard poultry and other pet birds to reduce the risk of transmission of AMR to people engaged in backyard poultry farming.

Several studies have demonstrated the prevalence and higher rate of AMR of *E. coli* in chicken (broiler and layer) in Bangladesh (Akond et al., 2009; Jakaria et al., 2012; Ievy et al., 2020; Mandal et al., 2022). Studies in different countries have also reported the emergence of AMR and multidrug-resistant (MDR) in backyard poultry (Borzi et al., 2018; Hedman et al., 2019; Sarba et al., 2019). However, very little or no study has been conducted yet to identify the occurrence of MDR in *E. coli* in backyard poultry and captive pigeon in Chattogram, Bangladesh. Notably, in the current world, the emergence and global spread of MDR are considered as significant threat to the public health. Therefore, this study investigated the MDR pattern of *E. coli* isolated from backyard poultry and captive pigeons reared close to the humans living in Chattogram, Bangladesh.

2. MATERIALS AND METHODS

Ethical considerations

All the techniques employed on the experimental animals were approved by the Animal Experimentation Ethics Committee of Chattogram Veterinary and Animal Sciences University under the memo no. CVASU/Dir(R&E)EC/2022/349/6.

Specimen collection

In this study, we collected a total of 36 cloacal samples from backyard poultry (including chicken- 20, duck- ten, and guineafowl- six), and 25 from captive pigeons from August 2021 to May 2022 in Chattogram, Bangladesh. Descriptive statistics based on sex, health condition and sources of birds were provided in table 1. Sterile cotton buds were used for the collection of cloacal swab samples. The birds were selected randomly from different households in the Chattogram district.

Identification of *Escherichia coli*

Each cloacal swab sample was immediately placed in a sterile collection tube containing 10 ml sterilized Buffer Peptone Water (BPW), and the tube was kept in an ice box. After collection, the samples were taken to the laboratory for microbiological analysis following standard protocol for isolation and identification within 24 to 72 hours (Das Gupta et al., 2017). Briefly, swab samples in Buffer Peptone Water (BPW) were streaked onto MacConkey agar and incubated aerobically for 24 hours at 37°C. Lactose-fermenting colonies (pink or red coloured colonies) on MacConkey agar were selected and sub-cultured on Eosin methylene blue (EMB) agar. Green metallic sheen colonies were identified as *E. coli*. All strains were further tested using standard biochemical techniques described by Garcia and Isenberg (2007). *E. coli* positive isolates were then preserved at -80°C with 15% sterile glycerol until use. Finally, *E. coli* isolates were confirmed by PCR using primers for a housekeeping gene *adk* (Zhang et al., 2021). The sampled birds were diverse based on their sex, health status and origin. All bacterial isolates were preserved at -20^{o}C in Brain Heart Infusion broth (BHI) with 50% glycerol.

Antimicrobial susceptibility testing

The disk diffusion method was used to test the antimicrobial susceptibility of 50 isolates against 12 selected antimicrobials (Bauer et al.,

1966). In this disk diffusion method, bacteria were grown for 24 h on Mueller-Hinton agar. Then, five to 10 bacterial colonies were selected and suspended in 0.85% sterile physiological saline solution and adjusted to a 0.5 McFarland turbidity standard, equivalent to 108 CFU/mL. Using a sterile cotton swab, the bacterial inoculum was spread onto the Mueller-Hinton agar plate and placed the antibiotics discs on top of the agar plates. All *E. coli* isolates were tested against the selected antibiotics (Oxoid Ltd., Cambridge, UK): amoxicillin (AMX)- 25 µg, ampicillin (AMP)- 10 µg, cefepime (FEP)- 30 µg, chloramphenicol (C)- 30 µg, ceftriaxone (CRO)- 30 µg, ciprofloxacin (CIP)- 5 µg, cefalexin (CL)- 30 µg, erythromycin (ERY)- 15 µg, gentamycin (GEN)- 10 µg,nalidixic acid (NA)- 30 µg,tetracycline (TET)- 10 µg, and trimethoprim-sulfamethoxazole (SXT)- 25 µg. The antimicrobials were selected based on availability and those commonly used for treating *E. coli* infections in animals and birds. Based on the sizes of the zone of inhibition, isolates were considered "resistant (R)", "intermediately resistant (I)", and "sensitive (S)" against the tested antimicrobials (Table 2). The standard characterization method for the antimicrobial susceptibility test recommended by the Clinical and Laboratory Standards Institute (CLSI, 2021) were followed. *E. coli* isolates exhibiting resistance to three or more antimicrobials tested were considered MDR isolates.

DNA extraction

The conventional crude boiling method was used to extract the genomic DNA of *E. coli* isolates. After thawing at room temperature, the preserved isolates were inoculated in 5% citrated bovine blood agar plates and then incubated at 37°C for 24 hours. After that, 200 µl of sterile deionized water was taken in a 1.5 ml Eppendorf tube for each isolate. Then, a loop full of 2-3 fresh well-isolated colonies were picked up and transferred to the Eppendorf tube, vortexed boiled at 99°C for 15 minutes and immediately placed on the ice. Then the Eppendorf tube containing homogeneous cell suspension was centrifuged at 13,000 rpm for 5 minutes. Finally, 100 µl of supernatant was collected and stored at -20 °C for future use as DNA template in PCR.

PCR assays for sulfonamide resistance genes

Uniplex PCR assay was used to identify two resistance genes against sulfonamides: *sul1* and *sul2*. Information about primers used in this study is described in Table 3. The reaction volume for each PCR assay was 20 µl consisting of 2 µl of the prepared DNA template; 2 µl of primers (each 1 µl) at 20 pmol, 10 µl of PCR master mix (DreamTaq Green PCR Master Mix) (Thermo Scientific, Invitrogen, USA) and 6 µl Nuclease free water. The PCR reaction was performed on a Thermo-cycler (Applied Biosystems, USA) under the following steps: 3 minutes of initial denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing temperature (specific for each primer) for 40 seconds, synthesis at 72°C for 1 minute, followed by 8 minutes for the final extension at 72°C. The PCR product for a specific gene was electrophoresed using 5 µl of the final PCR reaction mixture on a 1.5% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, USA). A 1kb plus molecular size marker (New England Biolabs, USA) was included in each gel during electrophoresis. An aliquot of the reaction mixture without template DNA was used as a negative control.

Table 1. Distribution of samples from backyard poultry and captive pigeons based on sex, health condition and sources of birds.

Variables	Chicken (20)	Duck (10)	Guineafowl (6)	Captive pigeon (25)
Sex	Male = 9 Female = 11	Male = 5 Female = 5	Male = 4 Female = 2	Male = 15 Female = 10
Health condition	Healthy = 14 Sick = 6	Healthy = 8 Sick = 2	Healthy = 6 Sick = 0	Healthy = 21 Sick = 4
Location	City = 9 Village = 11	City = 4 Village = 6	City = 0 Village = 6	City = 25 Village = 0

Table 2. Panel of antibiotics used, their concentrations and zone diameter (in mm) interpretative standards for *Escherichia coli* (CLSI, 2021)

Group of Antimicrobial agents	Antimicrobial agents	Disk contents	Zone diameter		
			R	I	S
Penicillin	Ampicillin (AMP)	10 µg	≤13	14-16	≥ 17
β–lactamase inhibitor	Amoxicillin (AML)	10 µg	≤13	14-17	≥ 18
Amino glycosides	Gentamicin (CN)	10 µg	≤12	13-14	≥ 15
Cephems	Ceftriaxone (CRO)	30 µg	≤19	20-22	≥ 23
	Cefepime (FEP)	30 µg	≤18	19-24	≥ 25
	Cefalexin (CL)	30 µg	≤14	15-17	≥ 18
Macrolids	Erythromycin (E)	15 µg	≤13	14-22	≥ 23
Tetracycline	Tetracycline (TE)	30 µg	≤11	12-14	≥ 15
Fluoroquinolones	Ciprofloxacin (CIP)	5 µg	≤21	22-25	≥ 26
Quinolones	Nalidixic acid (NA)	30 µg	≤13	14-18	≥ 19
Folate pathway inhibitor	Trimethoprim-Sulfamethoxazole (SXT)	1.25/23.7 µg	≤10	11-15	≥ 16
Phenicoles	Chloramphenicol (C)	30 µg	≤12	13-17	≥ 18

Table 3. Oligonucleotide primer sequences for *sul1*, and *sul2* genes, annealing temperature, size of the PCR product, and reference of each primer used.

Primer	Primer Sequence (5'– 3')	Target gene	Annealing temp.(°C)	Size of product (bp)	References
sul1 F	CGG CGT GGG CTA CCT GAA CG	*sul1*	51	433	Sunde, 2005
sul1 R	GCC GAT CGC GTG AAG TTC CG				
sul 2 F	CCT GTT TCG TCC GAC ACA GA	*sul2*	59	435	Chang et al., 2007
sul 2 R	GAA GCG CAG CCG CAA TTC AT				

Table 4. Frequency of *E. coli* in studied subjects.

Study subjects	Scientific name	No of samples	No of *E. coli* isolates	Frequency of *E. coli* isolation
Chicken	*Gallus gallus*	20	17	85% (95% CI: 63.12–95.61)
Duck	*Anas platyrhynchos*	10	8	80% (95% CI: 47.94–95.41)
Guineafowl	*Numida meleagris*	6	5	83% (95% CI: 41.78–98.86)
Pigeon	*Columba livia*	25	20	80% (95% CI: 60.42–91.59)
Total		61	50	82% (95% CI: 70.35–89.80)

Data management and analysis

Data management was performed using Microsoft Office Excel 2010. The Chi-square ($\chi 2$) test was carried out to estimate the difference between the study groups. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3. RESULTS

Prevalence of *E. coli*

A total of 61 cloacal swab samples were examined, of which 50 (82%) isolates were finally confirmed as *E. coli* through cultural, biochemical, and molecular characterization. The proportion of birds harbouring *E. coli* is presented in Table 4. Of the 20 tested samples from chicken, 17 (85%) were positive for *E. coli*. The detection of *E. coli* in duck, guineafowl and pigeon were 80%, 83% and 80%, respectively.

Antimicrobial sensitivity

The antibiotic susceptibility patterns of *E. coli* against 12 antimicrobials are shown in Figure 1. The proportion of isolates resistance to erythromycin, amoxicillin and tetracycline were

88%, 82%, and 64% respectively. On the other hand, 98% isolates showed sensitivity to ceftriaxone, 92% to gentamicin, and 80% to chloramphenicol. The frequencies at which different zones of inhibition to 12 antimicrobials tested are displayed in Figure 2. Of the tested isolates, 24 (48%) *E. coli* isolates have shown resistance to trimethoprim-sulfamethoxazole. However, in PCR assay, we have observed that 6 (12%) and 5 (10%) isolates were carrying *sul1* and *sul2* gene, respectively (Figure 3). A total of 11 (22%) isolates were resistant to sulfonamide based on the PCR assay. About 88% (44 out of 50) of *E. coli* isolates were resistant to 3 or more antimicrobials tested. Among them 4 isolates from pigeon and chicken were resistant to more than 7 selected antimicrobials (Figure 4). No significant difference in MDR patterns was observed in isolates from backyard poultry and pigeons (P = 1.0).

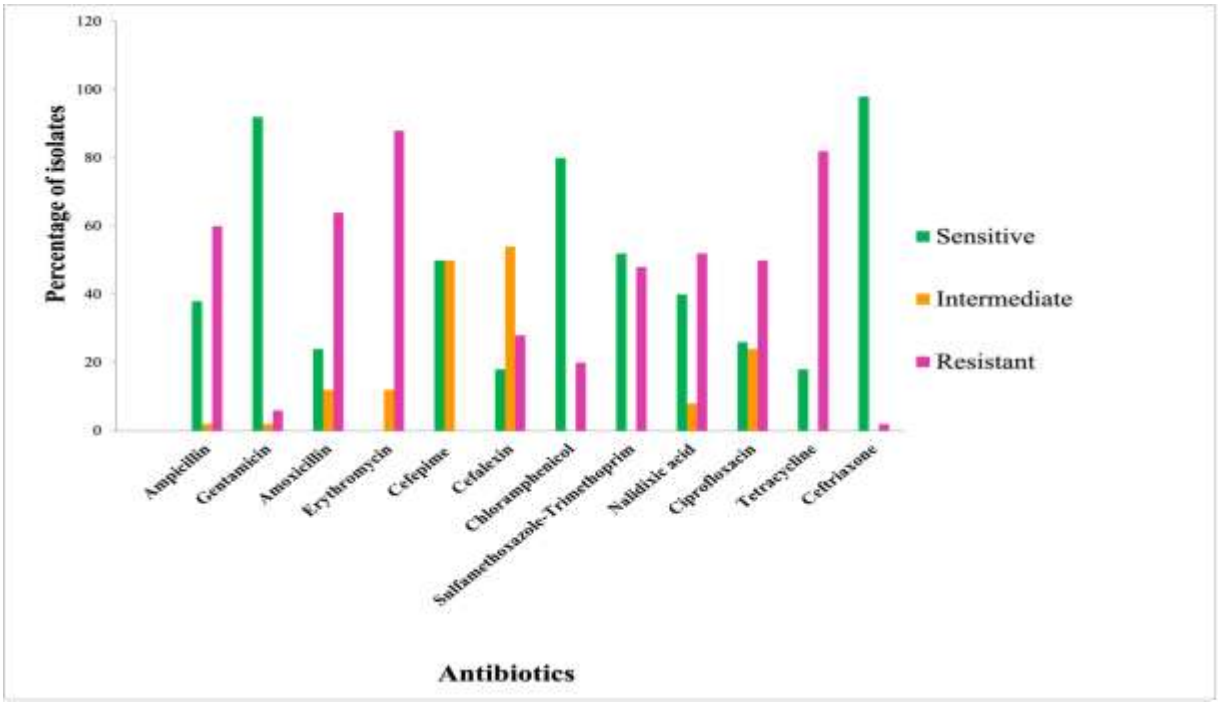

Figure 1. Results of antimicrobial susceptibility testing of *E. coli* from backyard poultry and captive pigeons. Green, orange, and pink colour of the bar diagram representing sensitive, intermediately resistant, and resistant *E. coli* isolates, respectively, against the tested antibiotics.

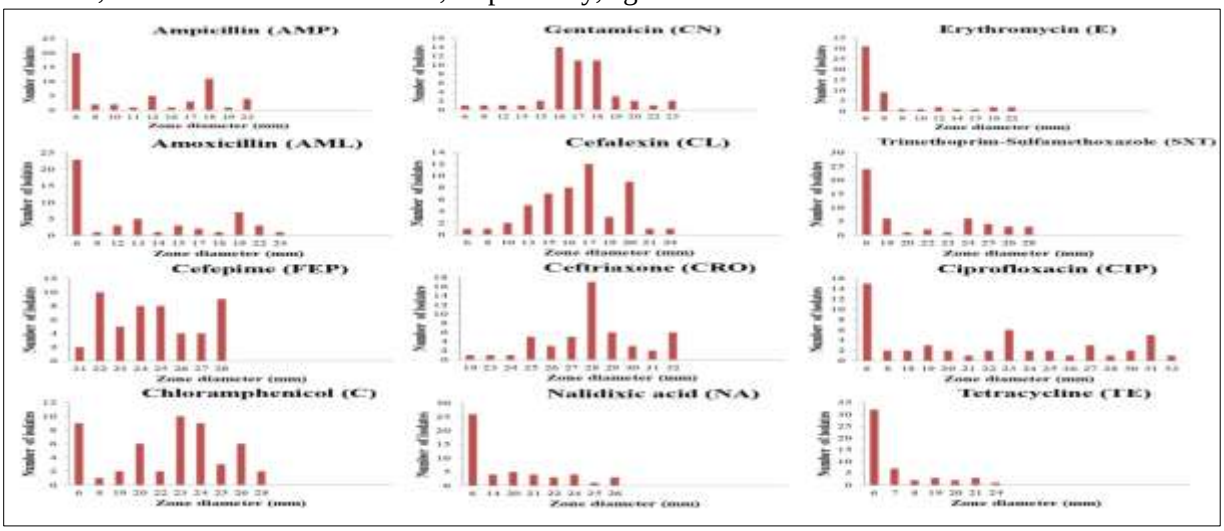

Figure 2. Frequency distribution of the isolates based on the different zone of inhibition to 12 antimicrobials tested (zone diameter was measured in millimetres).

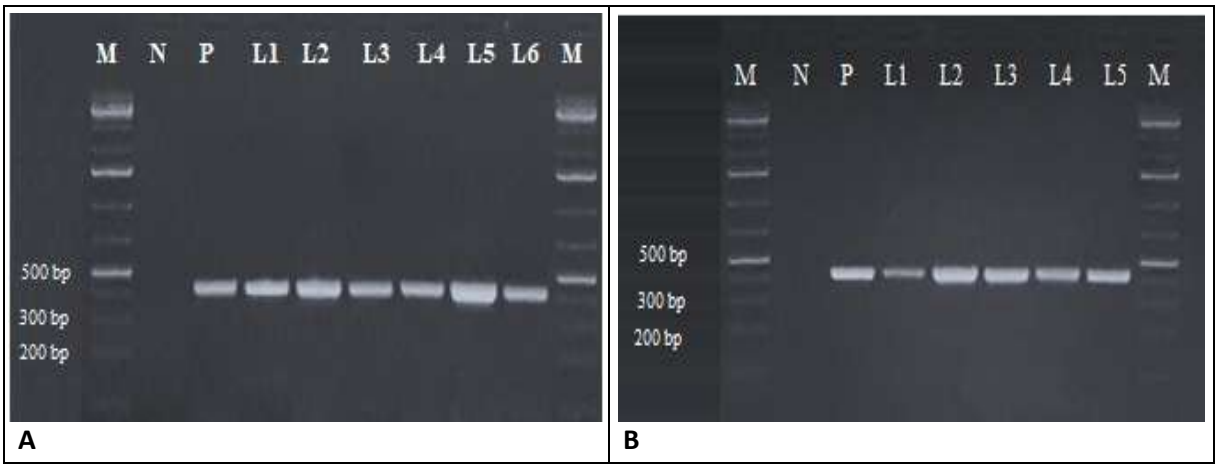

Figure 3. Results of PCR assay for 2 antimicrobial resistance genes: *sul1* and *sul2*. A. *sul1* gene (433 bp) amplicon: lane M- 1 kb plus DNA ladder; N- Negative control, lane P- Positive control, lanes L1–L6-*sul1*-positive isolates; B. *sul2* gene (435 bp) amplicon, lane M- 1 kb plus DNA ladder; N- Negative control, lane P- Positive control, lanes L1–L5-*sul2*-positive isolates.

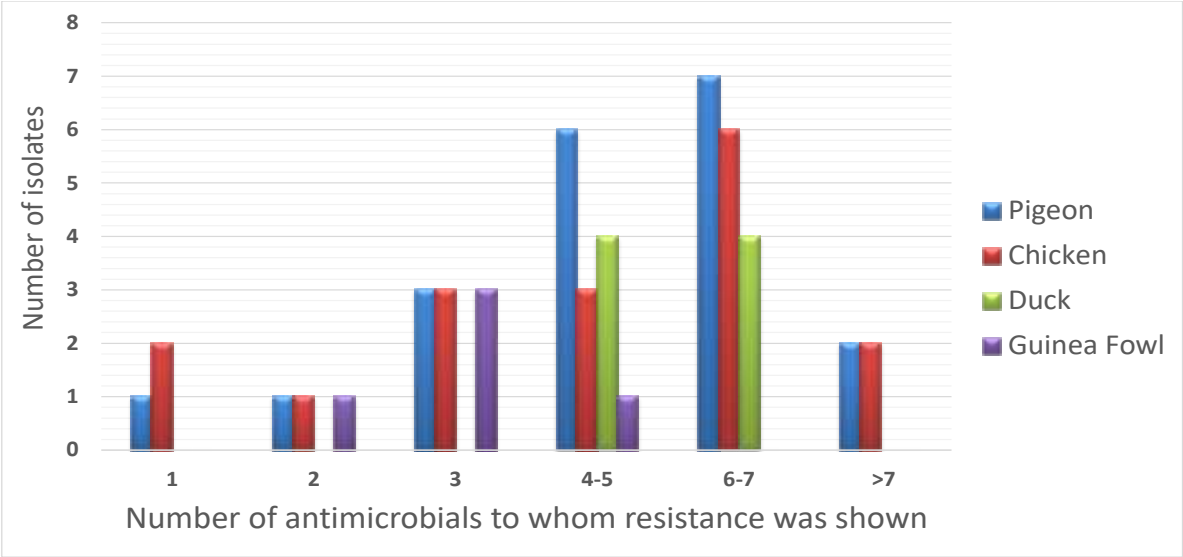

Figure 4. Multi-drug resistance profiles of *E. coli* from backyard poultry and pigeon.

4. DISCUSSION

The main aims of this study were to investigate the occurrence of AMR among *E. coli* isolated from backyard chickens and captive pigeons and further characterize the isolates based on their MDR pattern. In this study, *E. coli* was isolated from 83% and 80% of cloacal swabs samples in backyard poultry and pigeons, respectively. The frequency of isolation of *E. coli* from backyard poultry in this study was comparatively higher than the previously reported studies in Malaysia (52%) (Ibrahim et al., 2021), Egypt (34%) (Ibrahim et al., 2019), Ethiopia (32.5%) (Sarba et al., 2019) and Saudi Arabia (31-40%) (Al-Marri et al., 2021). In addition, the frequency of *E. coli* isolation in pigeons in this study was higher than previously reported in pigeons in Dhaka, Bangladesh (52.5%) by Karim et al. (2020). In their study, they collected oral and cloacal swab samples (20 swab samples from each site) for isolation of *E. coli* from pigeons, whereas we only used cloacal samples (25 swab samples) for our study. The differences in the frequency of *E. coli* isolation may be due to the sample type, sampling size, geographical location, and rearing system.

The emergence of antibiotic resistance and its persistence in host and transmission remain as a major concern, especially in developing countries, where small holding farming system

is more common (Bui et al., 2018; Hedman et al., 2019). In recent years, few studies have been performed for antibiotic resistance of *E. coli* from cattle on smallholdings and cattle reared on bathan (semi-intensive rearing system), buffalo and goats in Bangladesh (Das Gupta et al., 2017; Das Gupta et al., 2013; Gupta et al., 2018; Islam et al., 2013). However, studies on MDR *E. coli* from backyard poultry and captive pigeons in Bangladesh are scanty if present. Therefore, this study investigated the MDR pattern of *E. coli* in backyard poultry and pet pigeons. We observed that *E. coli* isolates exhibited variable resistance to the 12 antimicrobials tested. High resistance was observed against erythromycin (88%), tetracycline (82%), and amoxicillin (64%). Higher proportions of *E. coli* isolated from pigeon were resistant to amoxicillin (63%) and erythromycin (62%), which corroborate the result of a recent study in Bangladesh (Karim et al., 2020). Sulfonamides are the most used antibiotics for treating birds and animals in Bangladesh. Therefore, in addition to the disk diffusion method, *E. coli* isolates were also screened for the presence of two antimicrobial resistance genes for sulfonamide: *sul1* and *sul2*. The results revealed that 22% of the isolates showed resistance to sulfonamides based on PCR assay. In this study, the proportion of sulfonamides-resistant *E. coli* isolates was lower than erythromycin, tetracycline and amoxicillin-resistant isolates. However, sulfonamides have been used for a long time in poultry and pet birds.

We also found that 6 (12%) isolates were resistant only to ≤ 2 antimicrobials. In contrast, the remaining 44 (88%) isolates showed resistance to 3 or more antimicrobials and were therefore regarded as MDR isolates. The higher proportion of MDR might be attributed to the extensive, haphazard, and continuing usage of similar antimicrobials in backyard poultry and pet birds like pigeons. The emergence of MDR *E. coli* isolates in backyard poultry, and pet birds is a public health concern (Brower et al., 2017; Shrestha et al., 2017; Rahman et al., 2020). Moreover, the backyard poultry and pet bird environment may enhance the persistence of MDR pathogens and transmission to individuals in contact. Therefore, monitoring MDR occurrence regularly among *E. coli* in backyard poultry and pet birds is recommended, as this organism can transmit to humans of close contact through food, contaminated drinking water or environments.

5. CONCLUSION

Our study aimed to investigate MDR *E. coli* isolates from backyard poultry and pet pigeons in the Chattogram district of Bangladesh. Overall, the prevalence of *E. coli* was more than 80% (50/61) in the studied subjects, and about 88% (44/50) of the isolates were found as MDR. The isolates were highly resistant to erythromycin, tetracycline, and ampicillin. This may be associated an extensive use of these antimicrobials for the treatment of backyard chicken and pigeons in Bangladesh. In addition, around 22% (11/50) of the isolates contained resistant genes against sulfonamides. This result indicates that antimicrobial resistance in backyard poultry is challenging for Bangladesh, and immediate action should be taken to overcome this situation. Therefore, rational use of antibiotics, continuous surveillance of antimicrobial uses, and detection of MDR regularly are necessary to reduce the risk of MDR *E. coli* in backyard poultry and pet birds.

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