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Occurrence of *Escherichia coli* and *Pasteurella multocida* in Layer Chickens in Bangladesh with Special Reference to Colistin and Quinolone Resistance

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Abstract

Pasteurella multocida and *Escherichia coli* are two important gram-negative bacterial pathogens causing septicemia and are responsible for high morbidity and mortality in poultry with economic losses. To investigate the occurrence of these two pathogens in layer chickens, a cross-sectional study was conducted with liver samples of affected layers (n=100) from July 2018 to November 2018. *P. multocida* and *E. coli* were isolated using conventional bacteriological methods and the isolates were confirmed by polymerase chain reaction using species-specific primers. The *E. coli* isolates obtained were subsequently investigated to determine their susceptibility to different antimicrobials by disc diffusion method. The minimum inhibitory concentration (MIC) of colistin in *E. coli* isolates showing phenotypical resistance to colistin was detected using the broth microdilution method. PCR further characterized isolates displaying resistance to colistin and ciprofloxacin. The occurrence of *E. coli* in layers was 35% (95% Confidence Interval 26.4 – 44.8%). No *P. multocida* was confirmed in the samples collected. All *E. coli* isolates showed phenotypical resistance to ampicillin, ciprofloxacin, sulfamethoxazole-trimethoprim, and tetracycline while 22.9% and 62.9% isolates displayed resistance to colistin and ciprofloxacin, respectively. All isolates were found to be multi-drug resistant. The minimum inhibitory concentration of colistin in *E. coli* isolates ranged from 8 to 128µg/mL, and 3 out of 8 colistin-resistant *E. coli* isolates harbored the *mcr-1* gene. All ciprofloxacin-resistant isolates contained the *gyr*A gene. About 94% and 14% of isolates harbored *gyr*B and *par*C genes, respectively. The study highlights the circulation of colistin and ciprofloxacin-resistant *E. coli* in layers in Chattogram, Bangladesh.

Introduction

Poultry farming is one of the vibrant subsectors of agriculture that contributes significantly to the development of the agro-based economy of Bangladesh (Kumar, 2019). The poultry farming system in Bangladesh is characterized by the presence of all the five types of production systems outlined by the United Nations Food and Agriculture Organization (FAO) (Epizootics, 2007). The semi-commercial (sector 3) poultry production system with low to medium biosecurity predominates in the country where the flock size varies from a few hundred to a few thousand. Modern commercial poultry production system involves various stressors,

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such as feed, high stocking density and hatchery processing, which diminish bird immunity and increase bacterial pathogen colonization. The higher bacterial load and lower immunity not only impacts bird health and growth but also jeopardizes food safety (Elghandour *et al.*, 2020). Among the reported bacterial diseases in birds, fowl cholera and avian colibacillosis are two major infections associated with high morbidity and mortality in chickens with significant economic losses (Singh *et al.*, 2014).

Fowl cholera, a highly virulent disease affecting both domesticated and wild birds, is caused by *Pasteurella multocida*. The disease poses a significant threat to the poultry industry, severely impacting profitable production (Hunter, 2001). The acute form of fowl cholera is marked by symptoms such as nasal discharge, facial swelling, comb and wattle discoloration, ataxia, head retraction, fever, reduced egg production, and depression, often leading to high morbidity and mortality rates (Mehmood *et al.*, 2016). Birds that survive the acute stage or are infected with less virulent strains may develop chronic fowl cholera, which leads to localized infections in areas such as the joints, foot pads, tendon sheaths, sternal bursa, conjunctivae, wattles, pharynx, lungs, air sacs, middle ears, bone marrow, and meninges (WOAH, 2021) (Office International des Epizooties (OIE), 2008). The causative agent, *P. multocida*, commonly resides as a commensal in the respiratory tract of birds and typically causes disease when the host is under stress (Harper *et al.*, 2006). It is particularly prevalent in the respiratory syndromes of commercial layers worldwide, especially in hot, humid climates like Bangladesh (Hossain *et al.*, 2017).

Escherichia coli is a member of the bacterial family *Enterobacteriaceae*, which typically colonizes the gastrointestinal tract of warm-blooded animals. Some strains of *E. coli* may emerge as pathogens due to the presence of virulence genes that distinguish it from the commensal strains (Rosengren *et al.*, 2009). Certain *E. coli* pathotype, designated as 'avian pathogenic *E. coli*' (APEC) causes avian colibacillosis, one of the principal causes of morbidity and mortality in poultry worldwide (Lutful Kabir, 2010). Air sacculitis and related pericarditis, perihepatitis, and peritonitis are the most common syndromes that APEC causes in poultry (Ewers *et al.*, 2003). Bangladesh has an endemic case of colibacillosis in chickens as a result of increased poultry production and, greater exposure of birds to pathogens and numerous stressors (Biswas *et al.*, 2006; Khaton *et al.*, 2008).

The emergence of antimicrobial resistance in bacterial pathogens is a global health threat (Hou *et al.*, 2023). The usage of antimicrobials in poultry production is one of the contributing factors to the development of antimicrobial resistance in bacterial commensals or pathogens circulating in the poultry population. In the absence of an effective monitoring system, the farmers in Bangladesh often use antimicrobials in their birds for therapy, prophylaxis and growth promotion (Haque *et al.*, 2020; Hosain *et al.*, 2021). The presence of antimicrobial residues in poultry meat and eggs associated with the overuse of antibiotics may also affect public health security negatively (Goetting *et al.*, 2011; Reig & Toldrá, 2008). In addition, the occurrence of antimicrobial-resistant pathogens in commercial birds may lead to heavy economic losses due to treatment failures (Olarinmoye *et al.*, 2013). *E. coli* has been used as a marker organism to monitor antimicrobial resistance in humans, livestock and poultry and some *E. coli* strains possessed by poultry are potential sources of antimicrobial resistance genes to human commensals or pathogens (Kheiri & Akhtari, 2016; Overdevest *et al.*, 2011). Colistin, once banned due to toxicity is currently considered as the 'last-resort' antibiotic for the treatment of human infections caused by the multi-drug resistant (MDR) pathogens. The detection of a transferable plasmid-borne gene (*mcr*) associated with the spread of colistin resistance across the globe raised a serious concern (Liu *et al.*, 2016). Information on antimicrobial resistance of the major poultry pathogens such as *P. multocida* and *E. coli* in Bangladesh is limited. The present study aimed to isolate and identify *Pasteurella multocida* and *Escherichia coli* from post-mortem samples of layer chickens and determine the antimicrobial susceptibility pattern of the isolates. The study also focused on detecting the MIC of colistin against the isolates and colistin/ciprofloxacin resistance genes in the isolates.

Materials and Methods

Collection of samples

Following post-mortem examination of layer chickens (n = 100), the liver samples were collected aseptically and were kept in a sterile polythene bag with an identification number. The samples were transported immediately to the ADDL-PRTC maintain the cold chain. A separate questionnaire was used for collecting bird-related information, including age, flock size, rearing system, vaccination, and use of antibiotics.

Isolation and identification of *Pasteurella multocida* and *Escherichia coli*

For microscopic observation of *Pasteurella multocida*, the liver impression smear was made and fixed by using heat. Then, the smear was stained with Giemsa stain and kept overnight. The next day stained smears were rinsed in running water to remove excess stain and dried using blotting paper. After that, the bacterium was diagnosed by having a characteristic bipolar appearance illustrated under a

microscope. Attempts were also made to isolate *P. multocida* using blood agar (Oxoid Ltd., England) as a primary isolation media. For this, the surface of the liver sample was seared with a hot spatula and the sterilized surface was cut with sterile scissors. Then, a specimen was obtained by inserting a sterile inoculating loop into the cut without touching the outer surface. The specimen was inoculated directly into blood agar and incubated at 37°C for 24 hours. Finally, the organism was preliminarily identified based on colony morphology and the standard biochemical tests (Merchand & Packer, 1967). The suspected isolates were stored at -80°C using 50% glycerol until further investigation.

For the isolation of *E. coli*, the inoculum was taken from the liver using a sterile inoculating loop and streaked onto MacConkey agar (Oxoid Ltd., England) and then incubated at 37°C for 24 hours. *E. coli* growth was detected when bright pink-colored large colonies yielded on a MacConkey agar media. Then, suspected colonies were streaked onto EMB agar (Merck, Germany, Hi-media, India) and incubated at 37°C for 24 hours. Those colonies having a characteristic appearance of metallic sheen were primarily considered as *E. coli*, later confirmed by standard biochemical tests (Merchand & Packer, 1967). All biochemically confirmed *E. coli* cultures were stored in brain heart infusion broth (BHI; Oxoid Ltd., England) at -80°C using 50% glycerol.

Confirmation of *P. multocida* and *E. coli* by polymerase chain reaction (PCR)

A species-specific PCR was used for the confirmation of *P. multocida* (Townsend *et al.*, 1998). The confirmation of *E. coli* was carried out by PCR targeting the universal marker gene such as *uspA* (the universal stress protein) (Chen & Griffiths, 1998) and *uidA* (β-glucuronidase) (Heijnen & Medema, 2006). The primer details used for the detection and confirmation of *P. multocida* and *E. coli* are given in Table 1. Amplification products were separated by agarose gel electrophoresis (2% agarose in 1X TAE) and the PCR products were visualized and documented by UV illumination (BDA digital, biometra GmbH, Germany).

Table 1. Primers used for the detection and confirmation of *Pasteurella multocida* and *Escherichia coli*

Name of Bacteria	Primer	Primer sequence (5'-3')	Fragment size (bp)	Reference
Pasteurella multocida	KMT1T7 (F)	ATCCGCTATTTACCCAGTGG	460	(Townsend *et al.*, 1998)
	KMT1SP6 (R)	GCTGTAAACGAACTCGCCAC		
Escherichia coli	uspA Up	CCGATACGCTGCCAATCAGT	884	(Chen & Griffiths, 1998)
	uspA Down	ACGCAGACCGTAGGCCAGAT		
	uidA Up	TATGGAATTTCGCCGATTTT	166	(Heijnen & Medema, 2006)
	uidA Down	TGTTTGCCTCCCTGCTGCGG		

Antimicrobial susceptibility testing

Susceptibility of *E. coli* isolates [0.5 McFarland turbidity standards (equivalent to growth of 1.5×10^8 CFU/mL)] against different antimicrobials was performed using the Kirby-Bauer disk diffusion method (Bauer *et al.*, 1966). The following antimicrobials (with respective disc potencies) were used-Ampicillin (10 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Ciprofloxacin (5 µg), Colistin (10 µg), Gentamicin (10 µg), Sulfamethoxazole-trimethoprim (25 µg) and Tetracycline (30 µg). The selection of the antibiotics was based on the commonly used antibiotics in the sector 3 poultry production system in Bangladesh. The susceptibility testing of different antimicrobials was interpreted according to the CLSI guidelines (CLSI, 2018). Isolates displayed resistance to ≥3 different classes of antimicrobials, which was defined as 'multi-drug resistant' (Darabpour *et al.*, 2011). To avoid overestimation of resistance, those isolates demonstrating intermediate were considered sensitive.

Determination of minimum inhibitory concentration (MIC) of colistin

E. coli isolates displayed phenotypical resistance to colistin was investigated further to determine the MIC of colistin in cation-adjusted Mueller-Hinton broth using the broth microdilution technique (Humphries *et al.*, 2018). In short, each well contained an inoculum of about 5x105 CFU/mL in cation-adjusted Mueller-Hinton broth. Colistin was examined at dilutions ranging from 0.125 µg/mL to128 µg/mL. *Escherichia coli* ATCC 25922 was used as a control in the test. Isolates with a colistin MIC ≤2µg/mL were categorized as susceptible and those with a colistin MIC >2 µg/mL were categorized as resistant (Humphries *et al.*, 2018).

Detection of the *mcr* genes

Screening for the presence of *mcr* genes (*mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5) was performed in all colistin-resistant *E. coli* isolates by multiplex PCR using primers and cycle conditions described earlier (Borowiak *et al.*, 2017; Rebelo *et al.*, 2018) (Table 2). The amplified products were visualized by electrophoresis using 1.5% agarose gel.

Detection of quinolone resistance genes by PCR

Using the primers listed in Table 3, PCR was used to determine if the *gyrA, gyrB,* and *parC* genes were present in *E. coli* isolates that showed resistance to ciprofloxacin. A previously isolated strain was used as the positive control, while a master mixture without any DNA template served as the negative control.

Table 2. Primers used for multiplex PCR for detection of *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5 genes

Target gene	Primer name	Primer sequence (5´--3´)	Size(bp)	Reference
mcr-1	mcr1_320bp_F mcr1_320bp_R	AGTCCGTTTGTTCTTGTGGC AGATCCTTGGTCTCGGCTTG	320	(Rebelo *et al.*, 2018)
mcr-2	mcr2_700bp_F mcr2_700bp_R	CAAGTGTGTTGGTCGCAGTT TCTAGCCCGACAAGCATACC	715	(Rebelo *et al.*, 2018)
mcr-3	mcr3_900bp_F mcr3_900bp_R	AAATAAAAATTGTTCCGCTTATG AATGGAGATCCCCGTTTTT	929	(Rebelo *et al.*, 2018)
mcr-4	mcr4_1100bp_F mcr4_1100bp_R	TCACTTTCATCACTGCGTTG TTGGTCCATGACTACCAATG	1116	(Rebelo *et al.*, 2018)
mcr-5	MCR5_F MCR5_R	ATGCGGTTGTCTGCATTTATC TCATTGTGGTTGTCCTTTTCTG	1644	(Borowiak *et al.*, 2017)

Table 3. Primer sequences used to detect ciprofloxacin-resistant *E. coli* isolates

Gene	Name of primer	Primer sequence (5´--3´)	Fragment Size (bp)	Reference
gyrA	*GyrA-184-F* *GyrA-185-R*	TACCGTCATAGTTATCCACGA TTCCTGTTTTTGCTCACCCA	312	(Wiuff *et al.*, 2000)
gyrB	*GyrB-303-F* *GyrB-304-R*	GTCCGAACTGTACCTGGTGG AACAGCAGCGTACGAATGTG	281	
parC	*ParC-305-F* *ParC-306-R*	CTATGCGATGTCAGAGCTGG TAACAGCAGCTCGGCGTATT	261	

Statistical analysis

All data were entered into a spreadsheet of Microsoft Excel 2007 and transferred to EpiTools epidemiological calculators (http://epitools .ausvet.com.au.) for data summary and descriptive statistics. The 95% confidence interval of the prevalence values was calculated by the modified Wald method using the GraphPad software QuickCalcs (https://www.graphpad.com/quickcalcs/). Also, Chi-square test was used to assess the differences among percentages.

Results

Identification of *Pasteurella multocida*

Based on the questionnaire, the dead layer birds had a clinical history of low feed intake, poor growth, respiratory distress, and diarrhea. All the flocks were treated with antibiotics like Oxytetracycline and a vaccine was given during rearing as per layer vaccination schedule. Out of the 100 liver samples investigated, seven were suspected to be positive for *P. multocida* based on Giemsa staining, colony characteristics on blood agar and biochemical tests. However, all of the seven isolates suspected as *Pasteurella multocida* were found to be negative by PCR (Figure 1).

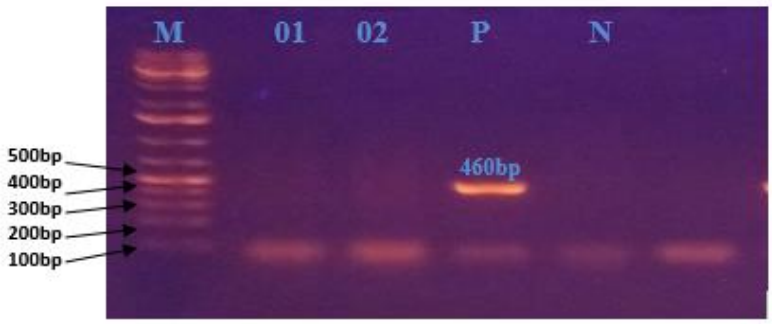

Figure 1. *P. multocida* specific PCR assay. This Figure illustrates fragments specifically amplified by PCR by means of the primers KMT1SP6 and KMT1T7. Lane 1: 1 kb plus DNA marker, Lane 4: positive control, Lane 5: negative control. Lanes 1 and 2: samples giving negative reactions in PCR.

Identification of *E. coli*

Out of the 100 liver samples examined, a total of 35 were found positive with *E. coli* based on colony characteristics on MacConkey and EMB agar plates and biochemical tests. Typical colonies were confirmed by PCR using two *E. coli*-specific primers for the marker gene (*uspA* and *uidA*). All isolates that were phenotypically positive for *E. coli* were also positive for the presence of the marker genes (Figure 2 and Figure 3).

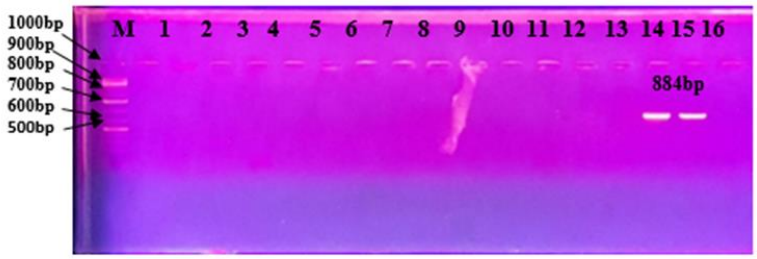

Figure 2. PCR products showing the uspA gene-sized amplicon (884bp) of a representative number of isolates

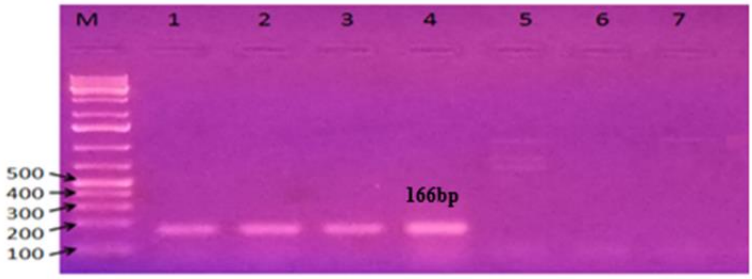

Figure 3. PCR products showing the uidA gene-sized amplicon (166bp) of a representative number of isolates

Occurrence of *E. coli*

An overall occurrence of *E. coli* along with the prevalence seen with the variables: age, feeding, litter, and use of antibiotics, are shown in Table 4. Of the 100 samples investigated 35 were found positive for *E. coli* giving a prevalence of 35% (95% CI 26.4 – 44.8%). The prevalence varied among the age groups. The highest prevalence was recorded in the finisher layer and the lowest was in starter chickens ($P < 0.05$). The chickens with commercial feed history had a proportionately higher prevalence, although statistically not significant ($P > 0.05$). Moreover, isolates of *E. coli* in liver samples were significantly ($P < 0.05$) higher in chickens reared in cages.

Table 4. Prevalence of *E. coli* isolated from liver samples of layer chickens

Variables	No. birds investigated	No. positive with *E. coli*	Prevalence (%)	95% CI (%)	P value
Age					
Starter (0-8wks)	14	1	7	0.01-33.5	0.002
Grower (9-18 wks)	29	6	21	9.5-38.8	
Layer layer (>18 wks)	57	28	49	36.6-61.7	
Feeding					
Homemade	23	6	26	12.3-46.8	0.30
Commercial	77	29	38	27.7-48.8	
Litter					
Sawdust	28	3	11	2.9-28.0	
Rice husk	15	4	27	10.5-52.3	0.002
Cage rearing	57	28	49	36.6-61.7	
Use of antibiotics					
Yes	90	35	39	29.5-49.2	0.01
No	10	00	0	-	
Total	100	35	35	26.4-44.8	

Antimicrobial susceptibility test of *E. coli* isolates

The results of the disc diffusion test by Bauer – Kirby method are shown in Figure 4. All *E. coli* isolates displayed resistance to ampicillin (AMP), tetracycline (TE), sulphamethoxazole-trimethoprim (SXT) and ciprofloxacin (CIP). More than 60% of isolates were found to be resistant to gentamicin (CN). In addition, 20% of isolates showed resistance to ceftriaxone (CRO) and cefotaxime (CTX), and 22.9% of isolates to colistin (CT).

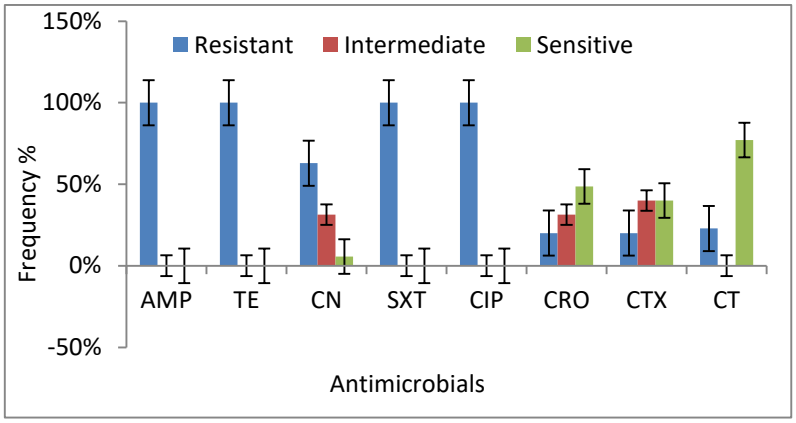

Figure 4. Results of antimicrobial susceptibility testing; proportional representations of resistant, intermediately-resistant and sensitive isolates against the tested antimicrobials with standard error bar; AMP = Ampicillin; TE = Tetracycline; CN = Gentamicin; SXT = Sulphamethoxazole-Trimethoprim; CIP = Ciprofloxacin; CRO = Ceftriaxone; CTX = Cefotaxime; CT = Colistin.

Colistin susceptibility

The broth microdilution test results for the 8 colistin-resistant *E. coli* isolates are shown in Figure 5. The minimum inhibitory concentration (MIC) of colistin in *E. coli* isolates ranged from 8 to ≥128 µg/mL.

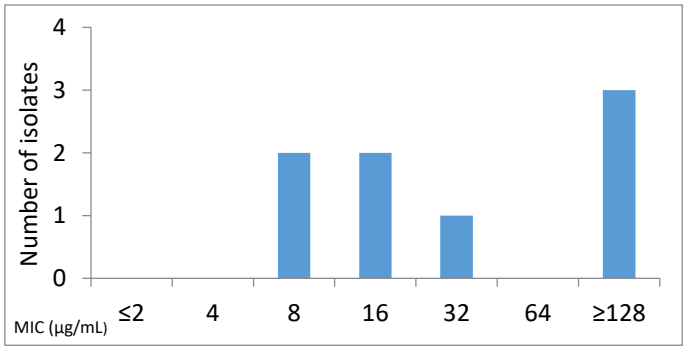

Figure 5. Minimum inhibitory concentrations of colistin in E. coli isolates (n = 8)

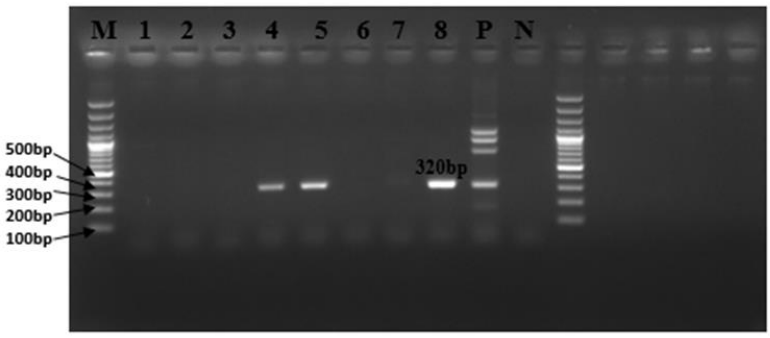

Figure 6. Visualization of the bands on an agarose (1.5%) gel for the eight E. coli and five positive control strains. Gene Ruler 1kb bp Plus DNA Ladder was used as molecular size marker and the size of each amplicon is indicated at the side

Detection of *mcr* genes

A total of 8 colistin-resistant *E. coli* isolates were selected for PCR assays to identify plasmid-mediated *mcr* (*mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5) genes. Of them, 3 isolates harbored *mcr*-1 gene (Table 5). The amplicon size of the *mcr*-1 gene is 320 bp (Figure 6). No other *mcr* genes were detected in colistin-resistant *E. coli* isolates tested.

Table 5. *E. coli* isolates exhibiting resistance to colistin sulfate with their MIC value and *mcr* gene

Sample ID	MIC value	*mcr* gene
L-8	8	*mcr*-1
L-41	8	Not detected
L-31	16	*mcr*-1
L-14	16	*mcr*-1
L-40	32	Not detected
L-22	≥128	Not detected
L-57	≥128	Not detected
L-60	≥128	Not detected

Detection of resistance genes for Ciprofloxacin

All the 35 *E. coli* isolates were tested for the presence of the *gyrA*, *gyrB* and *parC* genes whose amplicon sizes were 312 bp, 281bp and 261bp, respectively. According to the results obtained, 35 (100%) isolates harbored the *gyrA* gene, while 33 (94.3%) had the *gyrB* gene and 5 (14.3%) harbored the *parC* gene. Amplicons showing the presence of *gyrA*, *gyrB* and *parC* of some of the isolates tested are illustrated in Figures 7, 8, and 9, respectively.

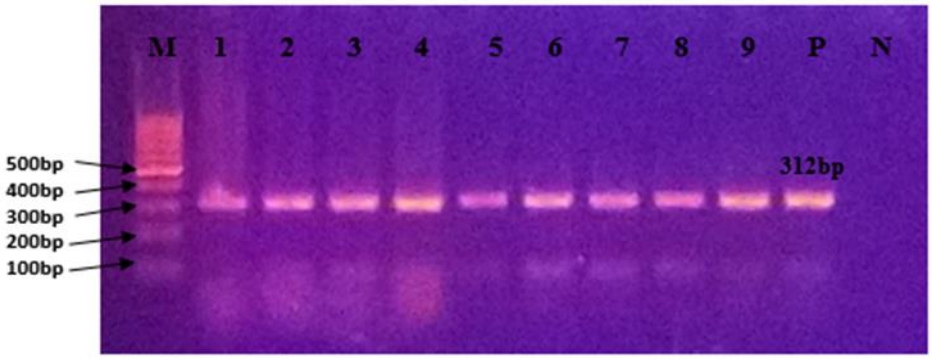

Figure 7. PCR products showing the gyrA gene-sized amplicon (312 bp) of a representative number of isolates resistant to ciprofloxacin

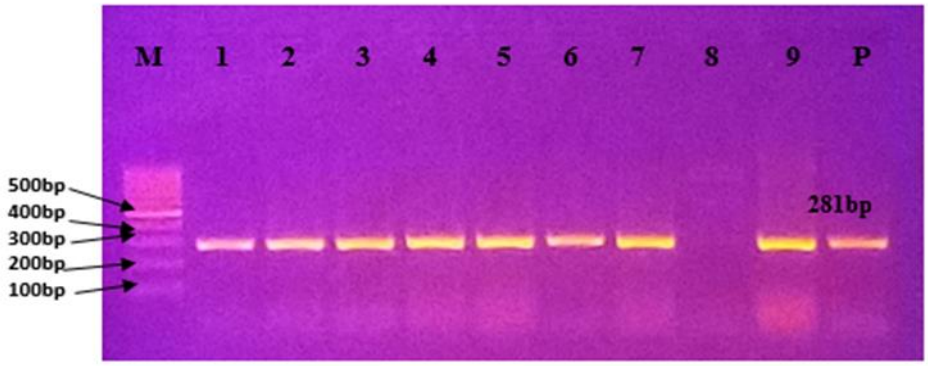

Figure 8. PCR products showing the gyrB gene-sized amplicon (281 bp) of a representative number of isolates resistant to ciprofloxacin

Multi-drug resistance pattern

The diversity of resistant phenotypes among the 35 *E. coli* isolates is presented in Table 6. A total of 9 different resistance patterns were observed. The highest number of isolates displayed resistance to the profile AMP, CIP, SXT, and TET (n=10). All 35 *E. coli* isolates showed multi-drug resistance (i.e., resistance to ≥3 antimicrobial types) with a range from 4 to 8 different antimicrobials (Table 7). Approximately 32% of isolates were resistant to six antimicrobial classes. Six percent of isolates displayed resistance to eight antimicrobial classes.

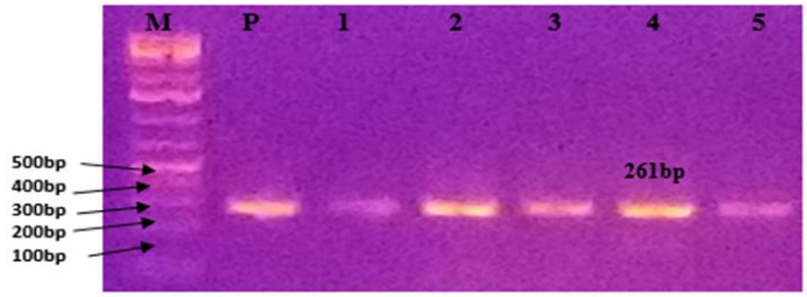

Figure 9. PCR products showing the parC gene-sized amplicon (261 bp) of a representative number of isolates resistant to ciprofloxacin

Table 6. Antimicrobial resistance pattern of *E. coli* isolated from liver samples of dead layer chickens

Sl. no	Resistance phenotype	No. of isolates displaying resistance
1	AMP, CIP, SXT, TET	10
2	AMP, CIP,CRO, SXT, TET	02
3	AMP, CIP, CTX, SXT, TET	01
4	AMP, CIP,GEN, SXT, TET	08
5	AMP, CIP,COL,GEN, SXT, TET	05
6	AMP, CIP,CRO,GEN, SXT, TET	03
7	AMP, CIP,CTX, GEN, SXT, TET	03
8	AMP, CIP,COL,CRO,GEN, SXT, TET	01
9	AMP, CIP,COL,CRO, CTX,GEN, SXT, TET	02

Table 7. Numbers and percentages of *E. coli* isolates exhibiting resistance to various antimicrobial classes

Number of antimicrobial classes to which isolates were resistant	Number (%) of resistant isolates
4	10 (28.57)
5	11 (31.42)
6	11 (31.4)
7	1 (2.85)
8	2 (5.71)

Discussion

In the present study, liver samples from dead-layer chickens are tested to determine the prevalence of *P. multocida* and *E. coli* with their antimicrobial susceptibility profiles. The presence of possible corresponding genes associated with resistance of *E. coli* isolates to colistin and ciprofloxacin was also investigated. The results of the study highlighted the circulation of colistin and fluoroquinolone-resistant *E. coli* in layer chickens in the Chattogram region of Bangladesh. Fluoroquinolones are considered a critically important antimicrobial by WHO, and finding resistance against them is a major public health concern.

P. multocida associated fowl cholera is one of the common bacterial infections of layer birds that have been reported as outbreak every year in Bangladesh (Hossain *et al.*, 2017). The present study observed localized infections with swollen wattles, enlargement of wattles and swollen footpads but failed to confirm *P. multocida* using PCR. *P. multocida* are nutritionally fastidious and grow best on media supplemented with chicken serum (Casino *et al.*, 2023). The chickens being sampled should not have been treated with antimicrobials previously for the optimal recovery of the bacteria. The chicken serum was not used in the media during the isolation process in this study and having chickens without antimicrobial treatment is probably a rarity at present, which might explain for not being able to recover the bacteria from the samples collected from the dead birds suspected to have died with fowl cholera.

In the poultry business, avian colibacillosis, caused by *E. coli*, is a serious health concern. The prevalence of *E. coli* in layer hens was found to be 35% in this study, which is nearly identical to the results (31.3%) of the earlier study (Kwon *et al.*, 2008). Another study (Rahman *et al.*, 2004a) reported a 36.73% prevalence of colibacillosis in clinically affected layers. On the other hand, a study conducted in South Korea recorded a 27.2% prevalence in layer flocks (Tonu *et al.*, 2011), which is slightly lower than the present study.

The prevalence of *E. coli* varied according to different age groups. The highest prevalence of *E. coli* was found in the age of more than 18 weeks (Rahman *et al.*, 2004a) reported Avian colibacillosis is quite common in adult layer birds and is seen in all

age groups of hens (9.52 to 36.73 %) with high prevalence rate in adult layer birds (36.73%) which supports our present study. Rahman *et al.* (2004a) also found colibacillosis in the 0-8 weeks of the age group of chickens at the rate of 13.36%, which is higher than the present study. However, several studies (Roshdy *et al.*, 2012) reported a 75% prevalence in layer chickens of more than 1 week of age, not consistent with the findings of the present study. These results were greater than those of the current study, presumably because the birds were older and in a different stage of production and because commercial hens have superior feeding, management, and immunization records. In chicken, *E. coli* is typically found in the digestive system. Stress may increase *E. coli*'s pathogenicity, which might result in the development of illness (Khaton *et al.*, 2008; Tonu *et al.*, 2011).

The prevalence of *E. coli* in chickens under the study varied among different litter management systems and the highest (38%) was found in cage-rearing systems. Due to a lack of information related to litter management system we failed to compare our findings with others. However, one study showed that the presence of bacterial, parasitic infections and cannibalism appeared highly in litter-based and free-range rearing systems than cage cage-rearing systems in layer birds (Fossum *et al.*, 2009).

The results of antimicrobial susceptibility testing revealed that all *E. coli* isolates recovered from the liver samples of layer chickens were resistant to ampicillin, ciprofloxacin, sulfamethoxazole-trimethoprim and tetracycline. Earlier studies (Rahman *et al.*, 2004b) (Biswas *et al.*, 2006) reported *E. coli* to be highly resistant to ciprofloxacin and tetracycline which is corroborative with the present study. Probable therapeutic use of tetracycline due to easy availability and cheapness was correlated with the resistance pattern of the E. coli isolates in the present study.

Colistin resistance has emerged in Gram-negative organisms due to increased usage of the antibiotic, and the rates of resistance are constantly rising (Gales *et al.*, 2011). Recently in an Indian study colistin resistant isolates were detected among human patients (Ghafur *et al.*, 2019). Colistin is considered a last-resort antibiotic for infections with multi-drug-resistant pathogens belonging to *Eneterobacteriaceae*. About 23% *E. coli* isolates exhibited resistance to this critically important antimicrobial which is worrisome. Indiscriminate use of colistin in poultry in Bangladesh could have a contributory role in the development of the resistance (Ievy *et al.*, 2020) (Masud *et al.*, 2020) (Sarker *et al.*, 2019). Following the initial screening of the *E. coli* isolates for susceptibility to colistin by disc diffusion method, the broth microdilution method was used to determine the MIC of colistin as the disc diffusion method was not found to be reliable for detecting the colistin resistance (Boyen *et al.*, 2010). There is no breakpoint available for colistin in CLSI guidelines.

The acquisition of *gyrA* in all of the *E. coli* isolates exhibiting resistance to ciprofloxacin suggests the association of *gyrA* with phenotypical resistance against ciprofloxacin. An earlier study (Kmeť & Kmeťová, 2010) also detected that *E. coli* isolates showing high resistance to ciprofloxacin harbored the *gyrA* gene. Mutations in the quinolone-resistant determining region (QRDR) of the *gyrA* and *gyrB* genes are often linked to the development of quinolone resistance, and less commonly, mutations in the topoisomerase IV genes, which encode the *parC* and *parE* genes, are linked to this phenomenon.

From Enterobacteriaceae, a plasmid-mediated colistin resistance gene (*mcr*-1) that confers colistin resistance was discovered in China (Liu *et al.*, 2016). Later colistin-resistant *E. coli* and *Klebsiella pneumoniae*, carrying the *mcr-1* gene, were reported worldwide from different sources (Poirel *et al.*, 2017). In the present study *mcr-1* gene was detected in three *E. coli* isolates. However, a high MIC value of colistin was determined in the rest of the *E. coli* isolates, suggesting the chromosome mediated colistin resistance (Poirel *et al.*, 2017).

Conclusion

This study described a 35% occurrence of *E. coli* in the liver of dead-layer chickens in Bangladesh. All the *E. coli* isolates were multi-drug resistant (MDR) and showed resistance to ampicillin, ciprofloxacin, sulfamethoxazole-trimethoprim and tetracycline. The minimum inhibitory concentration (MIC) of colistin ranged from 8 to ≥128 µg/mL in colistin-resistant *E. coli* isolates. The *mcr*-1 gene was detected in 3 of 8 colistin-resistant *E. coli* isolates. *E. coli* harboring the *mcr*-1 gene had a colistin MIC range of 8–16 µg/mL. The resistance to ciprofloxacin in the circulating strains has evolved because of the acquisition of the *gyrA*.

Ethical Statement

This research work was carried out with dead-layer birds. So, there was no mandatory to take ethical approval. However, it complies with all the rules and regulations.

Conflict of interest

The authors declare that they have no conflict of interest.

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