ISSN: 2536-7153 SJMLS

Sokoto Journal of Medical Laboratory Science 2023; 8(4): 18 - 27

SJMLS - 8(4) - 003

Multidrug resistant strains of Escherichia coli from Faeces of Layer birds in Jamalpur, Bangladesh

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Abstract

Escherichia coli (*E. coli*) is a facultative anaerobic bacterium commonly found in the intestines and is mostly harmless. However, *E. coli* has the ability to cause a wide range of diseases in the gastrointestinal, urinary, and nervous systems of man and animals. Indiscriminate use of antibiotics in animal production, hospitals, and agriculture are among the major sources for the development of antimicrobial resistance. Through horizontal gene transfer, *E. coli* possesses a number of resistance genes that have made it resistant to many antibiotics. The aim of this study was to detect *E. coli and determine the* antimicrobial resistant pattern of *E. coli* isolated from poultry faecal samples from Jamalpur, Bangladesh. The antibiotic susceptibility pattern was assessed using disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute and the detection was achieved using the polymerase chain reaction (PCR). From the forty (40) faecal samples, fifteen (15) were positive based on the culture and biochemical identification method. PCR analysis confirmed nine (9) isolates to be PCR positive. The results showed that all 15 isolates were resistant to at least eight (8) antibiotics from at least four (4) different classes of antibiotics indicating multidrug resistance of the isolates. The results show a very high risk of antibiotic resistance with an MAR index greater than 0.2 for all the *E. coli* isolates. Interventions using a one-health approach to tackle the menace of antimicrobial resistance (AMR) are required.

Keywords: antimicrobial resistance feces, multidrug resistant (MDR) *E. coli,* poultry

Introduction

Escherichia coli (*E. coli*), is a member of the Enterobacteriaceae family and a major cause of foodborne infections, although a common inhabitant of gastrointestinal tract of poultry, animals, and man (Levine, 1987). However, uncontrolled use of antimicrobial agents in veterinary medicine and humans leads to the discharge of antimicrobial resistant (AMR) bacteria from untreated sewage, livestock farms, wastewater treatment facilities, aquaculture ponds, and landfills (Van Boeckel, *et al*., 2015; Lin *et al*., 2020). In addition, the excessive use of these antibiotics in poultry production and animal husbandry is considered a main cause of antibiotic resistance (Ventolla, 2015). It has been reported that the *E. coli* strains isolated from contaminated faeces, eggs, and poultry products are resistant to commonly used antibiotics (Barlli *et al*., 2020). This resistance can be acquired through horizontal gene transfer or gene mutations while multidrug-resistant bacteria (MDR) usually harbor several drug resistant genes (Sun *et al*., 2019). These genes are transferred to other bacteria and eventually found in the food chain. The rapid emergence of multidrug-resistant *E. coli* strains has also resulted to significant morbidity and mortality in humans (Gandra, 2019).

Fecal *E. coli* from laying hens can contaminate the eggs in many instances and the bacteria is transferred to farm workers and the consumer

market through handling and eventual consumption. This eventually finds its way into the food chain and continues to threaten the treatment of initially susceptible disease conditions. The efficacy of currently available antibiotics is decreasing because of the emergence of resistant strains of *E. coli* and other bacteria in the environment, food, and hospitals (Ventola, 2015). *E. coli* in the avian intestinal tract that has been exposed to antibiotics may persist for a long time even in the absence of antibiotics and later be transferred to eggs or the food chain for human consumption (Chaslus *et al*., 1987).

In developing countries, antibiotic resistance is so complex and difficult, due to irrational use of antibiotics both in the clinical and agriculture settings, low socioeconomic status, poor sanitation, and resistance to commonly used antibiotics are scarcely investigated (poor surveillance systems) (Manyi-Loh *et al*., 2018). Hence, it is crucial to continuously study the antimicrobial resistant pattern of *E. coli* isolated from poultry fecal samples that are used on agricultural land.

Methodology

Study Area and design

The study is a descriptive cross-sectional study. The study was conducted in Jamalpur Bangladesh for a period of 3 months, with samples taken weekly from randomly selected layer farms within the district. Forty (40) cloacal swabs were aseptically collected from different farms, labelled and transported to the veterinary Pharmacology Lab of the Bangladesh Agricultural University, for bacteriological analysis.

Isolation of *E. coli*

E. coli was isolated and identified based on standard bacteriological procedure (ISO, 2001). First, cloacal swab samples were pre enriched with 1 ml sterile Buffered peptone water (BPW), then transferred into nutrient broth (NB), and incubated at 37˚C for 24 h. The culture was then streaked onto MacConkey agar and incubated at 37˚C for 24 h. Three presumptive *E. coli* colonies (pink to dark pink, dry and donut-shaped, surrounded by a dark pink area) were then sub cultured on nutrient agar to obtain pure culture, and identification was performed using Gram staining and biochemical tests (catalase, oxidase, indole, methyl red, Voges Proskauer test, and sugar fermentation test using triple sugar iron agar). Biochemical identification was according to developed standard operating procedures (SOPs) (Jackson *et al*., 2001).

Identification of *E. coli* by Polymerase Chain Reaction (PCR)

Bacterial DNA was extracted by the boiling method (Dashti *et al*., 2009). Extracted DNA was quantified using nanodrop spectrometer (NanoDrop 1; Thermo Fisher Scientific, USA) and more than 100 ng/µl concentration of DNA was maintained to use for PCR assay. The PCR targeted the *fli*C gene with a 401 bp, with the following primer sequence

Forward: 5'- ATAATCTACGCCGCCAACT-3'
Reverse: 5'- GACTCCATCCAGGACGAAA-3'

A 100 µl reaction mixture containing 2 µl of template DNA (approximately 50 ng), 1 µM (each) primer, 200 µM (each) deoxyribonucleotide triphosphate, 10 µl of 10 X PCR Buffer. (Perkin-Elmer), and 2.5 U of Taq polymerase and 2.5 U of TaqStart antibody. The cyclic conditions used for amplification of *fli*C gene of *E. coli* include initial denaturation at 95˚C for 5 min for 1 cycle, denaturation at 94˚C for 30 secs annealing at 62˚C for 30 secs, extension at 72˚C for 30 secs and final extension 72˚C for 2 min at 35 cycles (Wang *et al*., 2002). About 5 µL of the PCR products was electrophoresed in 1.0% Agarose gel stained with Ethidium bromide, at 100V for 45min and visualized under UV illuminator (SYNGENE, Biosystems 2000). A 100bp DNA molecular ladder (Promega, Corporation, USA) was included to determine the size of the amplified products.

Antimicrobial Resistance Profiling (ARP) of the Isolates

AMR profiling of the *E. coli* was performed by disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018). Eighteen antimicrobials from eight different classes were used in the AMR profile test. These included (1)

Quinolones (levofloxacin (5µg), ciprofloxacin (5µg)), (2) Macrolides (azithromycin (15µg, erythromycin (15µg), (3) Beta-Lactams (amoxycilin (25µg), cephalexin (15µg), cephalsporins , cefixime (15µg), ceftriazone (15µg), cefuroxime (30µg), cefotaxime (25µg).), (4) Tetracyclines (doxycycline (25 µg), tetracycline (30µg), (5) Aminoglycoside (gentamicin (30µg), neomycin (15 µg,) Amikacin (), (6) Para amino benzoic acid (PABA) derivatives (sulfur (25µg), (7) Amphenicols (chloramphenicol (30µg), and (8) Polymixin E (colistin (10µg)). *E. coli* (ATCC 25922) strain was used as a reference strain. The isolates were categorized as resistant, intermediate, or sensitive based on the diameter of the zone of inhibition according to CLSI guidelines. As there is no standard zone of inhibition mentioned for erythromycin with Enterobacteriaceae, the interpretation was performed based on the zone of inhibition for *Staphylococcus* spp. *E. coli* showing resistance to at least one antibiotic in three or more different classes of antibiotics was defined as multidrug-resistance (MDR) (Magiorakos *et al*., 2012).

Results

The results of the study revealed that among the 40 fecal samples collected for analysis 15 were found to be culture positive as *E. coli* on EMB agar media and 9 were PCR positive (Table 1).

Table 1: Results of culture and PCR of the cloacal swab samples

S/N	ID/N	Culture and identification	PCR
1	NS1	positive	positive
2	NS2	Positive	Positive
3	NS3	Positive	Positive
4	JS1	Positive	Positive
5	JS2	Positive	Positive
6	JS4	Positive	Positive
7	JS5	Positive	-
8	JS6	Positive	-
9	JS7	Positive	-
10	SS4	Positive	Positive
11	SS9	Positive	-
12	SS5	Positive	Positive
13	SS7	Positive	Positive
14	SS1	Positive	-
15	SS4	Positive	-

Detection of *E. coli* by PCR

The PCR result for the detection of *Escherichia coli* isolates using the *fli*C gene primers, showed that only nine (9) (NS1, NS2, NS3, JS1, JS2, JS4, SS4, SS5, SS7**)** out of the 40 *Escherichia coli* isolates were positive for the *fli*C gene (Figure 1).

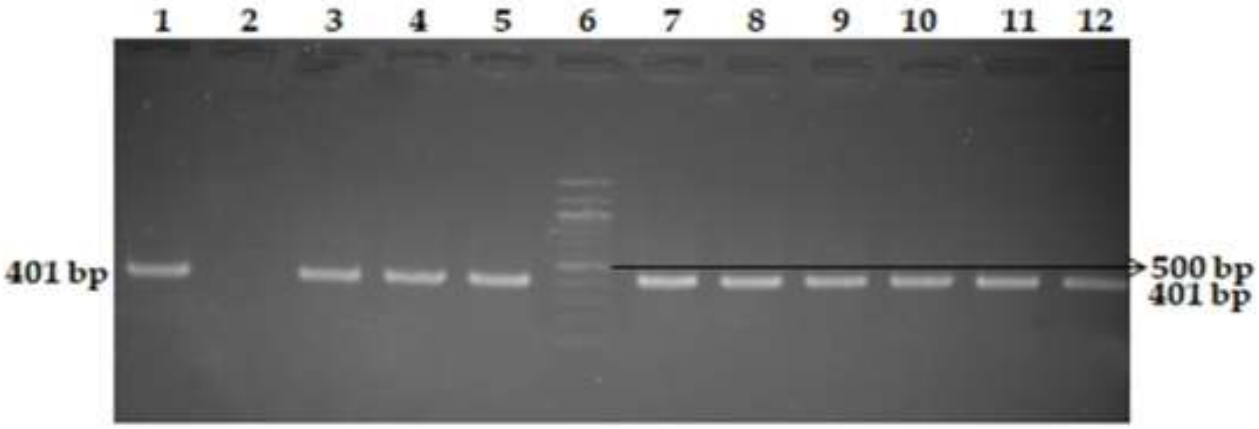

Figure 1. Amplification of *fli*C gene for the detection of *Escherichia coli*. Lane 1: positive control (401 bp), lane 2: negative control, lanes (3, 4, 5, 7, 8, 9, 10, 11 & 12) positive for *fli*C gene, lane 6: 100 bp DNA ladder;

Percentage resistance of isolates to 18 antibiotics

All the isolates tested were resistant (100%) to amoxycillin, ciprofloxacin, cefixime, chloramphenicol, cephalexin, cefotaxime, cefuroxime, erythromycin and tetracycline. They also resisted azithromycin and doxycycline (87%), followed by sulfur (86%), levofloxacin (73%) and resistance to amikacin was the least (26.6%) (Figure 2).

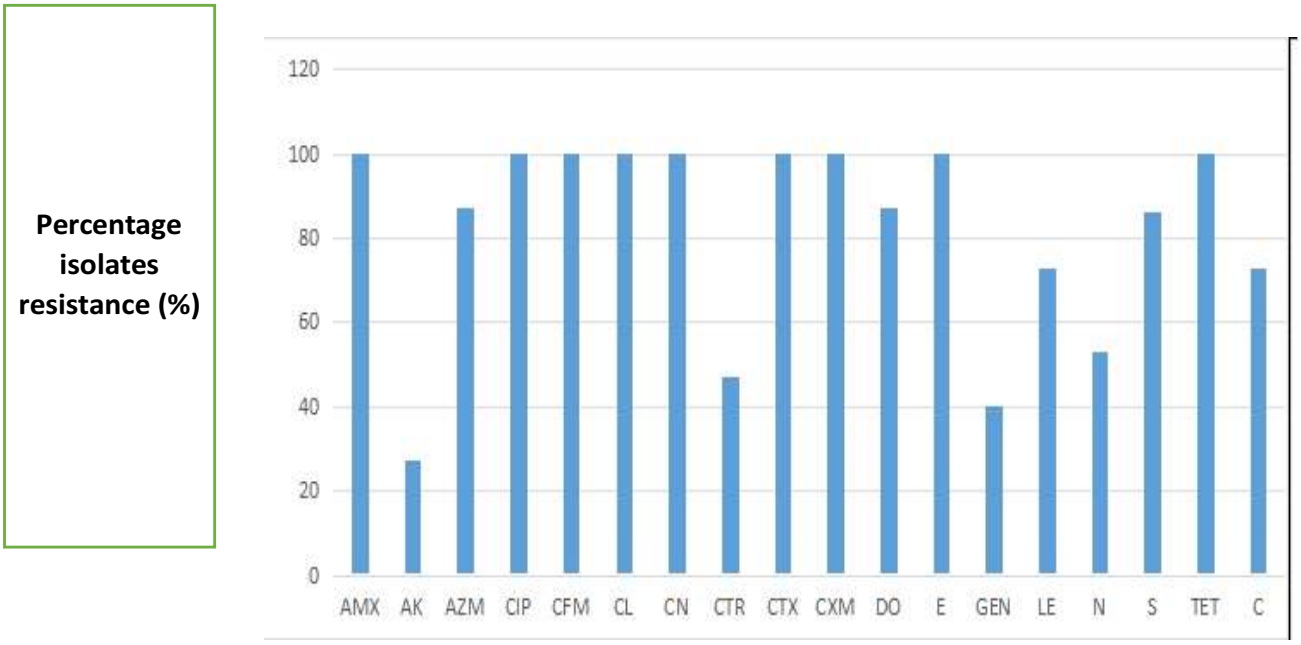

Figure 2: Percentage resistance of *Escherichia coli* to a panel of 18 antibiotics

Keys: amoxicillin (AMX), amikacin (AK), azithromycin (AZM), ciprofloxacin (CIP), cefixime (CFM), chloramphenicol (CL), cephalexin (CN), ceftriazone, (CTR), cefotaxime (CTX), cefuroxime (CXM), doxycycline (DO), erythromycin (E) gentamicin (GEN), levofloxacin (LE), neomycin (N), sulfur (S), tetracycline (TET), colistin (C).

Antibiogram of *E. coli* isolates

The susceptibility testing of the 15 *Escherichia coli* isolates gave 13 different antimicrobial resistance patterns (Antibiogram) with all of the isolates showing multiple resistance (Table 2).

Table 2: Antibiogram of *E. coli* isolates

S/NO.	ISOLATES	ANTIBIOGRAM RESISTANCE PATTERN
1.	NS1	AMX, AK, AZM, CIP, CFM, CL, CN, CTR, CTX, DO, E, S, TET, C
2.	NS2	AMX, AK, AZM, CIP, CFM, CL, CN, CTR, CTX, CXM, DO, E, GEN, LE, N, S, TET, C
3.	NS3	AMX, CIP, CFM, CN, CTR, CTX, DO, E, LE, S, TET, C
4.	JS1	AMX, AK, AZM, CIP, CFM, CL, CN, CTR, CTX, DO, E, LE, N, S, TET, C
5.	JS2	AMX, AK, AZM, CIP, CFM, CL, CN, CTR, CTX, DO, E, N, S, TET, C
6.	JS4	AMX, AZM, CIP, CEF, CL, , CN, CTR, CTX, DO, E, LE, TET, C
7.	JS5	AMX, AZM, CIP, CFM, CL, CN, CTX, CXM, E, GEN, N, TET, C
8.	JS6	AMX, AZM, CIP, CFM, CL, CN, CTX, CXM, DO, N, S, TET, C
9.	JS7	AMX, AZM, CIP, CFM, CL, CN, CTX, CXM, DO,E, GEN,LE, N, S, TET, C
10	SS4	AMX, AZM, CIP, CFM, CL, CN, CTX, CXM, DO,E, GEN,LE, N, S, TET, C
11.	SS9	AMX, AZM, CIP, CFM, CL, CN, CTX, CXM, DO,E, LE, S, TET, C
12.	SS5	AMX, AZM, CIP, CFM, CL, CN, CTX, CXM, DO, E, GEN, LE, N, S, TET, C
13.	SS7	AMX, AZM, CIP, CFM, CL, CN, CTX, CXM, DO, E, LE, S, TET
14.	SS1	AMX, AZM, CIP, CFM, CL, CN, CTX, CXM, E, LE, S, TET,
15.	SS4	AMX, CIP, CFM, CL, CN, CTR, CTX, CXM, DO, E, GEN, LE, N, S, TET

Multiple drug resistant and Antibiotic Resistance Index

The multiple antibiotic resistances of the *Escherichia coli* isolates are shown in Table 3. The results revealed that all the isolates were resistant to at least eight (8) antibiotics from at least four (4) classes of antibiotics (Table 3). The results of this study also revealed that all the isolates 15(100%) had MAR index greater than 0.2. (Table 4).

Table 3: Multiple drug resistance pattern of *Escherichia coli* isolates

Resistance pattern	Frequency	Percentage (%)
Resistant to ≤3 antibiotics	0	0
Resistant to ≥	15	100
Total	15	100

Table 4: Multiple antibiotics resistance (MAR) index of *Escherichia coli* isolates

MAR index	Frequency	Percentage (%)
≤	0	0
≥	15	100
Total	15	100

Discussion

Antimicrobial drugs are supplemented in poultry feeds at sub-therapeutic levels for growth promotion, prevention or reduction of disease outbreaks, improvement of digestion, and increase in feed conversion ratio (Adebayo and Adeola, 2005). However, increased use of these drugs has been shown to contribute to the increasing prevalence of bacterial antibiotic resistance in humans and animals (Apata, 2009). *Reports have revealed widespread* presence of antibiotic-resistant pathogens in poultry farms and products in many areas around the world (Adebayo and Adeola, 2005).

Antibiotic resistance is of great public health concern because the antibiotic-resistant bacteria associated with the animals may be pathogenic to humans, easily transmitted to humans via food chains, and widely disseminated in the environment via animal wastes. These may cause complicated, untreatable, and prolonged infections in humans, leading to higher healthcare cost and sometimes death**.** *Escherichia coli*, followed by *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* are six pathogens that have been linked to high fatality of patients once infected. In 2019 these bacteria have been responsible for 929,000 (660 000–1 270 000) fatalities associated with AMR (Murray *et al*., 2022).

Van Epps and Blanley (2016) reported high amounts of fluoroquinolones, sulfonamides, and tetracyclines antibiotics in animal manure all of which the World Health Organization has listed as critically important drugs for human health. It was reported that even proper management of compost piles did not demonstrate a significant increase in antibiotic degradation. Anaerobic digestion was also not effective for some key antibiotics, including lincosamides and select sulfonamides and fluoroquinolones (Van Epps and Blanley, 2016). Thus, the need to ensure that antibiotics are only used rationally based on professional recommendations.

Thirteen (13) antibiogram patterns were observed in this study, which suggest a high diversity of antimicrobial resistance of the *Escherichia coli* isolates. Antibiograms are a guide to clinicians and pharmacist in selecting the best empirical antimicrobial treatment in the event of pending microbiology culture and susceptibility results. They are also useful tools for detecting and monitoring trends in antimicrobial resistance.

All the isolates tested were resistant (100%) to amoxycillin, ciprofloxacin, cefixime, chloramphenicol, cephalexin, cefotaxime, cefuroxime, erythromycin and tetracycline. They also resisted azithromycin and doxycycline (87%), followed by sulfur (86%), levofloxacin (73%) and resistance to amikacin was the least (26.6%) (Figure 2). Similarly, Kolář *et al*. (2012) also reported their isolates having increased frequencies of resistance to ciprofloxacin in 10% of the strains.

Tetracycline, cefotaxime, amoxycillin high resistance as seen in our study was also reported by Sonola *et al*. (2021). In a similar study carried out in Nigeria by Awogbemi *et al*. (2018) *E. coli* showed 100% resistance to amoxicillin but lower resistance to gentamicin. *In addition, E. coli* isolated from Portuguese poultry was reported to have high resistance to tetracycline (70%) (Mendonca *et al.,* 2016). Kolář *et al*. (2012) also reported 97% of *E. coli* strains to be resistant to tetracycline, 51% were resistant to ampicillin. Adesiyun *et al*. (1993) reported the

high prevalence of resistance to tetracycline, rifampicin, streptomycin, erythromycin, amoxicillin, vancomycin and doxycycline) can be attributed to the uncontrolled/widespread use of these antimicrobials as growth promoters since the farmers have unlimited access to these agents and their use. These findings along with our results disagree with the reports of Tafida *et al*. (2013) who reported susceptibility to tetracycline and sulfamethoxazole.

Many of these antibiotics are accessible as over the counter medications (Rugumisa *et al*., 2021). *E. coli* isolates in this study showed increased susceptibility to amikacin, ceftriazone, gentamicin, and colistin with only three isolates were resistant to amikacin and 6 for gentamicin. This is similar to the work of Mendonca *et al*. (2016) in Portugal who reported only 17% resistance in *E. coli* isolates. Interestingly *E. coli* obtained in another research was reported to be 100% susceptible to gentamicin, neomycin, and ciprofloxacin (Okorie *et al*., 2016). Sonola *et al*. (2021) also reported *E. coli* isolates were more susceptible to gentamicin. About seven isolates in this research were resistant to ceftriazone and only two for colistin. However, there is high incidence of intermediate susceptibility to colistin in 12 isolates.

However, a study from Jamaica showed *E. coli* isolated from broiler chickens was resistant to kanamycin (91.2%) which is also an aminoglycoside belonging to the same class as amikacin that was found mostly effective on *E. coli* in this study (Miles *et al*., 2006). All isolates were resistant to amoxycillin, cefoxime, cephalexin, cefuroxime, and erythromycin. Similar report by Mendonca *et al. (*2016) *indicated that E. coli* isolated from Portuguese poultry was reported to have high resistance to ampicillin (63%) which is also a beta lactam antibiotic like amoxycillin (Mendonca, 2016). However only 20% of the *E. coli* isolates were resistant to ampicillin from a study carried out in Jamaica by Miles *et al*. (2006).

Contrary to our findings which showed that all 15(100%) of the *E. coli* isolates were multidrug resistant, in the Netherlands a study by Van Den (2001) reported only 23% of multidrug resistant *E. coli* from poultry source and also 34.7% prevalence was found in the study of Aworh *et al*. (2021). However, a related study conducted in Bangladesh among poultry and poultry environment reported a much higher prevalence of *E. coli* (82%) from chicken faecal samples when compared with the findings from this study also show a high prevalence of multidrug resistance.

The high concentrations of resistant isolates can be attributed to the indiscriminate use of these antibiotics in animal production chain and by humans (Kimera *et al*., 2021; Mgaya *et al*., 2021) . Also improper waste management from households, hospital settings, and the environment can contaminate the soil on which food crops for animal and human consumption are grown with such residues of antimicrobial agents (Badowski *et al*., 2011; Pickering *et al*., 2012). This makes the cycle favorable for breeding the resistant strains of bacteria we find all around us. In Bangladesh, there was a 10% isolation rate for multidrug resistant *E. coli* just from household soils where many of the poultry are dwelling with the farmers (Montealegre *et al*., 2018).

The spread of bacterial resistance in the population can be revealed by measurement of multiple antibiotic resistances (MAR) index (Kruperman, 1985). The MAR index was determined for the 15 *Escherichia coli* isolates. The results revealed that all 15(100%) of the *Escherichia coli* isolates had MAR index greater than 0.2. MAR index higher than 0.2, an indication that these isolates originated from a high-risk source of contamination e.g., farms that are exposed to antibiotics (Wang *et al*., 2002). Any bacterial strain exhibiting MAR index values lower than 0.2 is thought to originate from sources, in which antibiotics are seldom or never used (lower risk). Infections caused by resistant microorganisms may result in failure to respond to treatment, result in prolonged illness, higher health care expenditure and greater risk of death (Adzitey *et al*., 2013). Our findings indicate high-risk source of contamination where several antibiotics are used. Multidrug resistant *E. coli* might be a threat not only for the poultry sector but also for public health.

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Citation: Aminatu Abubakar Sani, Usman Bashir Usman, Hauwa'u Umar Mungadi. Multidrug resistant strains of Escherichia coli from Faeces of Layer birds in Jamalpur, Bangladesh. ***Sokoto Journal of Medical Laboratory Science*; 8(4): 18-27.** https://dx.doi.org/10.4314/sokjmls.v8i4.3