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High incidence of *Escherichia coli* resistance to colistin from chicken farms in Kathmandu Valley

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Abstract

Overusing antimicrobial growth promoters in poultry farming plays a significant role in spreading drug-resistant *Escherichia coli*. The widespread practice of colistin for any purpose in chickens can potentially disseminate resistant genes from the environment to humans, posing an undeniable threat to livestock and public health. In Nepal, few reports describe the incidence of *E. coli* bacteria, which are resistant to colistin. Henceforth, this study's purpose was evaluation of colistin-resistant *E. coli* from ten chicken farms (commercial and backyard) in Kathmandu Valley. In total 43 *E. coli* isolates were obtained from fifty chickens. All isolates were resistant to colistin, as detected by their growth on MacConkey Agar containing 4 µg/mL colistin. Antibiotic susceptibility tests were used to define the isolates further. 70.0% of isolates were categorized as multidrug-resistant, while the colistin-resistant isolates showed low resistance to Imipenem. The questionnaire data showed the rampant colistin administration in chicken feed, which may have contributed to the proliferation of *E. coli* isolates resistant to antibiotics in the valley. These findings highlight the importance of advanced investigation into commercial and backyard livestock to facilitate safe poultry practices.

Keywords: Chicken, *Escherichia coli*, Colistin, Resistance

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Introduction

There is widespread practice of antibiotics in poultry production [1]. Farmers often use antimicrobials to ease the efficient digestion of food and eliminate infectious bacteria, resulting in healthier food animals favoring economic uplift [2]. However, this reliance on antibiotics presents significant concerns. While the minimal quantity of antibacterial agents reduces the number of susceptible bacterial species, they can also foster resistance in the normal microbiome of chickens [3]. Extensive use of antibiotics encourages bacteria to develop chromosome mutations and acquire multidrug-resistant plasmids or transposon, aiding them to thrive in a harsh environment [4]. Various bacterial mechanisms, including drug efflux, enzymatic inactivation, and target protection, confer concurrent resistance to a broad spectrum of antimicrobial drugs, developing multidrug resistance (MDR) [5]. The World Health Organization is particularly concerned about the use of antibiotics in poultry, as these compounds have a potential adverse effect on human medicine. The presence of antimicrobial residues in meat and eggs has a direct impact on human health [6,7]. Furthermore, there is a possibility of diffusing antibiotic-resistant determinants into the human gut microbiome, increasing the risk of drug-resistant bacteria in humans and posing a severe threat to public health [8].

Escherichia coli is a predominantly gram-negative gut bacterium of humans, animals, and birds [9]. Most *E. coli* are commensal, while some isolates cause intestinal and extra-intestinal diseases, collectively termed 'Colibacillosis' [10]. Moreover, *E. coli* constitutes numerous resistance genes, leading to treatment failure in human and veterinary infections [11,12]. Horizontal gene transfer is one way for *E. coli* to accumulate resistance genes for enterobacterial gene pool or other bacterial species [13]. The virulent and resistant *E. coli* is equipped with machinery and channels assisting the diffusion of bacteria between animals and humans. Hence, *E. coli* is considered a model bacteria to specify the level of antimicrobial resistance in the bacterial population [14].

Colistin is one of the extensively used antibiotics incorporated routinely as a growth promotor to prevent and cure infections in food animals like pigs and poultry [15]. Polymyxin E, better known as colistin, is a cationic polypeptide that can destroy Gram-negative bacteria, including Enterobacteriaceae [16]. This protein disrupts and permeabilizes gram-negative bacterial cytoplasmic membranes, leaking the intracellular bacterial components and eventually causing cell death [17].

According to a surveillance report by WHO (2018), the antibiotic - colistin is a last resort for treating MDR bacterial infections in both human and veterinary medicine [18]. Given that, there is limited study on the prevalence of colistin-resistant E. coli in food-producing animals. In 2016, Liu et al. first identified a plasmid-mediated mcr-1 gene conferring resistance to colistin in E. coli isolates from animals and humans in China [16]. Later, the *mcr* gene was also reported in *Klebsiella pneumoniae*, *Enterobacter cloaca*, and *Salmonella spp.* These bacteria were recovered from animals, the environment, and humans in South America, Europe, Africa, and other parts of Asia [19–23]. This progressive development of resistance to colistin has increased complications and mortality in Poultry [24].

In Nepal, people have been persuaded to apply antimicrobials like colistin in poultry without proper certification. There aren't many publications about the resistant microbial species in Nepali chicken farms despite an increasing number of colistin-resistant strains in food animals like chickens being found worldwide [25–27]. The main aim of our study was to monitor the *E. coli* strains from chickens resistant to colistin. Cloacal swabs of chickens were collected from commercial and backyard farms in Kathmandu Valley to generate an antibiotic susceptibility pattern. The results would be beneficial in illustrating the microbiological profile of poultry in Nepal. By identifying the specific resistance mechanisms in these strains, we can better tailor interventions to mitigate the impact of antimicrobial resistance in poultry production.

Materials and Method

Collection of samples

Fifty cloacal swab samples were collected, 25 from commercial farms and the remaining from backyard farms. A sterile cotton swab was inserted approximately 1 inch into the cloaca and rotated slowly for about 10 seconds [28]. Then, the swab was placed into the screw-capped tube with sterile peptone broth. It was then transported to the Microbiology Laboratory at St. Xavier's College, Kathmandu, in an icebox. The sample was then incubated at 37°C for 24 hours.

Ethical approval was obtained from the Nepal Health Research Council (NHRC), Reg. 664/2018, before sample collection from the chickens.

Isolation of *E. coli*:

The cloacal swab was streaked on Eosin Methylene Blue Agar. The colonies with green metallic sheen were sub-cultured on Nutrient Agar. Gram's Staining and Biochemical Tests, which include the Catalase test, Oxidase test, Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Triple sugar iron test, Oxidation Fermentation test, and Urease test, were performed for identification of *E. coli*.

Detection of colistin-resistant *E. coli*:

E. coli isolates were inoculated on MacConkey agar containing 4µg/mL colistin. Those isolates showing pink-colored colonies were considered colistin-resistant *E. coli* per the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2016.

MIC of colistin to *E. coli*:

The agar dilution method was used to determine colistin's minimal inhibitory concentration (MIC) to *E. coli*. Mueller Hinton agar (MHA) plates with colistin, concentration starting from 4µg/mL to 32µg/mL, were prepared. The isolates were inoculated on the plates then incubated at 37°C for 24 hours. The lowest concentration of antibiotics that inhibit the visible growth of *E. coli* isolate was considered MIC for the isolate [29].

Antibiotics Susceptibility Testing of colistin-resistant *E. coli* isolates:

The antibiotics susceptibility test for the isolates was carried out using the Kirby Bauer disk diffusion method on MHA according to the CLSI guidelines, 2016. The isolates were evaluated against antibiotics, namely, Ampicillin (10 µg), Cefotaxime (30µ g), Amoxyclav (30 µg), Imipenem (10 µg), Amikacin (30 µg), Nalidixic Acid (30 µg), Ciprofloxacin (5 µg), Tetracycline (30 µg), Cotrimoxazole (25 µg) and Erythromycin (15 µg). These drugs belong to different antibiotics class with varied mode of action against bacteria. The zone of inhibition was measured and interpreted in terms of sensitivity, intermediate, and resistance to each antibiotic as per CLSI guidelines (**Supplementary Table 1**). The isolates with resistance to antimicrobial agents of three or more classes were defined as MDR *E. coli* [30].

The overall outline of the procedure followed for this study is provided in Supplementary **Figure 1**.

Results

Commercial farms have elevated usage of Antibiotics:

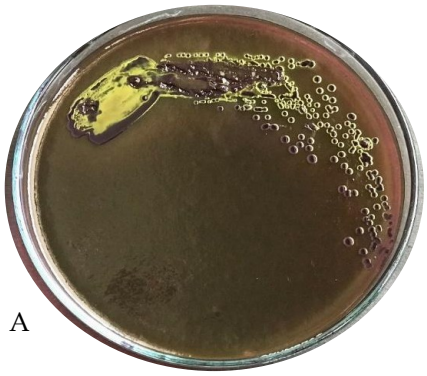
The epidemiological data of 10 sampling farms are presented in **Table 1**. All the farms owned solely chickens. Eight out of 10 farms were established in an alienated area, away from public residence. All commercial and backyard farms have mentioned using Maize as a chicken supplement. Chemical solutions containing algicide, bactericide, fungicide, and virucide

Table 1: Epidemiological data of the Chicken farms

Farms	Epidemiological data				
	Isolated from public residence	Other livestock animal existence	Antibiotics used	Feed supplement / additive	Vaccination
Commercial					
Satdobato	+	-	+	+	-
Kirtipur	-	-	+	+	+
Nagarkot	+	-	-	+	+
Suryabinayak	+	-	+	+	+
Nagdhunga	+	-	+	+	+
Backyard					
Nakhipot	-	-	+	+	-
Imadole	+	-	-	+	-
Nagarkot	+	-	-	+	-
Bhindabasini	+	-	-	+	-
Kaushaltar	+	-	+	+	-

+ Yes - No

were added to all commercial farms' drinking water. The farm in Kirtipur reported elevated antibiotic usage for the broilers, believing they show more antibiotic resistance than layers. The backyard farm from Imadol fed mustard oil to the sick chickens as a traditional treatment measure. Aside from Satdobato, commercial farms maintained a vaccination regimen regularly, whereas backyard farm owners did not. have any such practices.



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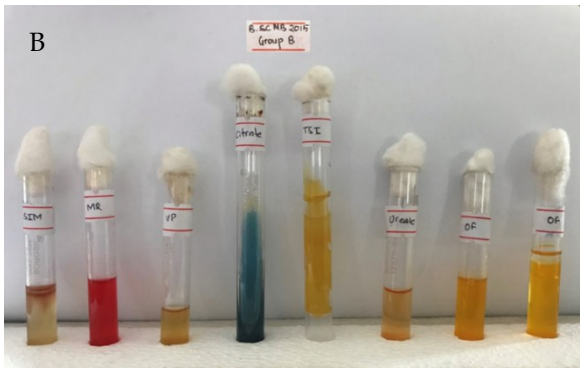


B

Figure 1: (A) Green metallic sheen colonies of *E. coli* on the EMB agar; (B) Biochemical test results of *E. coli* SIM (+) MR (+) VP (-) Citrate (-) TSI (A/A Gas+ H_2S-) Urease (-) OF(Fermentative)

E. coli was successfully isolated from cloacal swabs

In total, 43 *E. coli* were isolated from 50 cloacal swabs of chickens, accounting for 86.0% of the total sample. Commercial farms had a greater incidence of *E. coli* than backyard poultry. The green metallic sheen on the EMB agar **(Figure 1A)** and the results of biochemical tests **(Figure 1B)** confirmed the isolated bacterium as *E. coli*. The isolation of *E. coli* from the cloacal swab of chickens was predicted since it is a persistent commensal in the avian intestinal tract and rectum [31]. Other isolates included *Bacillus spp., Acinetobacter spp., Salmonella spp.,* and *Proteus spp.*, which are normal flora of chickens. Likewise, Hakkani et al. (2016) observed *E. coli* as the most abundant coliform in the colon and cecal chicken pouches, along with *Salmonella spp.* and *Staphylococcus spp.* [32]. In addition, Yulistiani et al. (2017) isolated the following Enterobacteriaceae: *Salmonella spp., Shigella spp., Citrobacter spp., Klebsiella spp., Proteus spp., Yersinia spp., Enterobacter spp., Serratia spp.,* and *Edwardsiella spp.* at traditional markets in Surabaya, Indonesia, from chicken meat and further showed their antibiogram pattern [33].

High prevalence of colistin-resistant *E. coli*:

All *E. coli* isolates grew on the MacConkey agar incorporated with 4µg/ml colistin, indicating colistin resistance **(Figure 2);** in contrast, Wang et al. (2018) found no substantial evidence of colistin resistance in bacteria from chicken populations [34]. Additionally, Joshi et al. (2019) stated that 27 *E. coli* isolates and 18 isolates had MIC of colistin 8 µg/mL in chicken farms of Kathmandu Valley [25]. Besides, the isolates were subjected to MHA incorporated with different colistin concentrations. MIC of colistin-resistant *E. coli* isolates from both commercial and backyard farms are given in **Table 2**. The highest MIC was 32 µg/mL for 11 *E. coli* isolates belonging to both commercial and backyard farms.

Table 2: MICs of colistin-resistant *E. coli* by Agar dilution method

Farm	MIC of Colistin			
	4 µg/mL	8 µg/mL	16 µg/mL	32 µg/mL
Commercial	2	-	15	4
Backyard	4	-	11	7
Total	6	-	26	11

Figure 2: Growth of *E. coli* on the MacConkey agar with 4µg/mL colistin

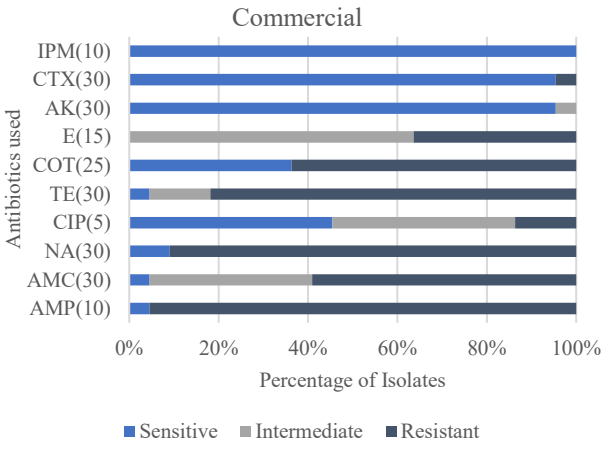
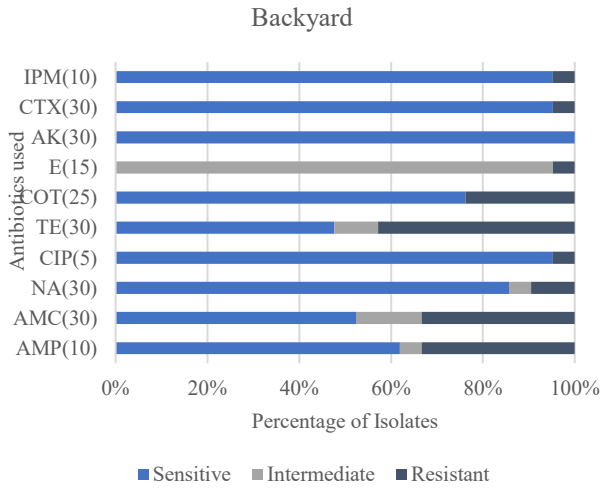


Figure 3: Antibiotic susceptibility pattern of colistin-resistant *E. coli* isolates



Colistin-resistant *E. coli* exhibited varied antibiotic susceptibility patterns:

Figure 3 illustrates the resistance profile of 43 colistin-resistant *E. coli* from commercial and backyard farms for ten antibiotics. Imipenem was effective against all colistin-resistant *E. coli* from commercial farms, followed by cefotaxime (95.5%) and Amikacin (95.5%). 90.9% were resistant to Nalidixic Acid and 81.80% to Tetracycline, while Yulistiani et al. (2017) had a higher resistance to Tetracycline than Nalidixic Acid [33]. Likewise, among 22 isolates from the backyard farm, more than 50% of the isolates were resistant to Amoxyclav. Similar to the results of Gwida & El-gohary (2015), Nguyen et al. (2016), and Yassin et al. (2017), where Ampicillin and Tetracycline-resistant *E. coli* were susceptible to Amoxyclav [24,35,36].

Similarly, all *E. coli* from backyard farms were sensitive to Amikacin, followed by Nalidixic Acid, akin to 84.00% sensitivity to Nalidixic Acid, observed by Langata et al. (2019) in Kenya [11]. The highest resistance was seen towards Tetracycline, shown by nine *E. coli* retrieved from a backyard farm [11]. Fascinatingly, twenty-one colistin-resistant *E. coli* isolates from commercial farms were resistant to ampicillin, whereas only seven colistin-resistant *E. coli* from backyard farms were resistant to ampicillin. Similarly, 90.9% of *E. coli* resistance to *E. coli* isolated from chickens on commercial farms were resistant to Nalidixic acid. In comparison, only 9.5% of isolates from backyard farms showed resistance to the same antibiotics, reflecting contrasting antibiograms in these two farms.

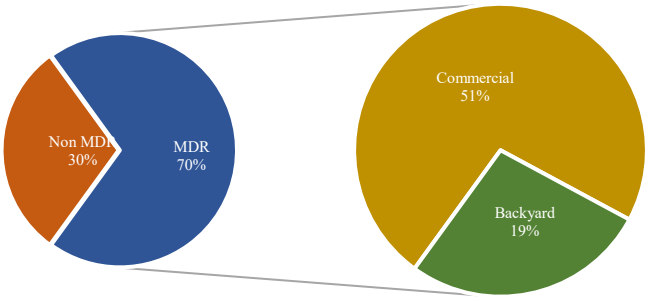


Figure 4: Multidrug-resistant profile of colistin-resistant *E. coli* isolates

Finally, Thirty *E. coli* isolates were discovered to be resistant to multiple classes of antibiotics, categorized as MDR colistin-resistant *E. coli* **(Figure 4),** like MDR *E. coli* isolated by Roslee et al. in 2016 and Yulistiani et al. in 2017 [33,37]. Interestingly, the two isolates with the

highest MDR profile were from the broiler farm in Kirtipur and they were resistant to 6 out of eight tested antibiotics: Ampicillin, Amoxyclav, Erythromycin/Ciprofloxacin, Cotrimoxazole, Nalidixic Acid, and Tetracycline. The B24M6 isolate from Bindabasini farm exhibited the most severe MDR pattern, showing resistance to six tested antibiotics, namely, Ampicillin, Amoxyclav, Cefotaxime, Cotrimoxazole, Nalidixic Acid, and Tetracycline.

Discussion

Nepal is a developing country in Southeast Asia that has primitive animal farming practices and policies compared to other developed countries worldwide. Farmers have persistently begun to employ antibiotics such as colistin to improve egg and meat output while preventing microbial diseases to sustain the market competition while making a profit. Likewise, all farms feed chickens with maize feed supplements, which do not indicate additional medications, implying that the chickens consume antimicrobials without being documented. Commercial farms have also been using 100 grams of colistin powder added to 500 liters of drinking water every 5-7 days to prevent gastrointestinal infections such as colibacillosis and salmonellosis, which may contribute to microbial resistance in poultry.

All *E. coli* isolates were resistant to colistin at 4 µg/mL. Compared to the MIC result of Joshi et al. (2019), this study found the highest MIC value of colistin (measured by the agar-dilution method) in chicken farms in Nepal [25]. Furthermore, recent studies have confirmed an incline in the frequency of colistin-resistant *E. coli* in the Nepalese poultry sector [38,39]. The highest MIC value - 32 µg/mL was observed for 33.30% of isolates from backyard chicken farms amidst denial of feeding antimicrobial agents, in any forms to chickens. Four isolates from commercial farms (C6M1, C9M9, C17M2, and C25M6) with MIC of 32µg/mL belonging to Suryavinayak and Kirtipur farms, have openly disclosed the use of colistin in drinking water for the safeguard. This extensive application of the drug may have introduced colistin resistance in the chicken with the possibility of transfer of colistin-resistant genes among chickens in the farms. Sobur et al. (2019) reported the massive usage of colistin has developed ways to prevent the inhibitory effect of the drug in the livestock and poultry industry [40]. With no discovery of new drugs, 100% resistant to colistin in vitro indicates a greater possibility that the antibiotic's treatment may be ineffective in vivo.

All colistin-resistant *E. coli* isolates from commercial farms displayed resistance to multiple classes of drugs, while 38.10% of isolates were MDR in backyard farms. Commercial farms have a higher prevalence of MDR colistin-resistant *E. coli* than backyard farms, possibly due to the overuse of antibiotics in commercial farms. Many commercial farms have revealed the use of Tylosin, a feed additive and bacteriostatic macrolide, with only a few farms administering cotrimoxazole and ampicillin to prevent possible bacterial infections. Consequently, none of the colistin-resistant *E. coli* were sensitive to Erythromycin, antibiotics belonging to macrolides suggesting resistance to the drug of one class may protect another drug of the same group. Likewise, 63.6% of colistin-resistant *E. coli* from commercial farms and 23.8% from backyard farms were resistant to cotrimoxazole, but Agyare et al. (2018) found minimal resistance to this specific sulphonamide antibiotic [3]. The affluent usage of cotrimoxazole in poultry has prompted the development of an inhibitory mechanism in E. coli to neutralize the effect of cotrimoxazole, thus expanding bacterial resistance against sulphonamide drugs [1]. Despite this, the high resistance pattern of some antibiotics, such as Nalidixic Acid and Tetracycline, may not be linked to drug usage in farms. These specific antibiotics were not commonly used in the farm samples, indicating the availability of alternative elements for antibiotic resistance in poultry.

Interestingly, only one colistin-resistant *E. coli* isolates, B12M5 from the backyard farm of Imadol, with the highest MIC of 32 µg/ mL to colistin, was found to be carbapenem-resistant (Imipenem). Given this study's limited resistance to Imipenem, it cannot be considered the medicine of choice for treating poultry infections because this is an in vitro test. An in-vivo drug testing is necessary to confirm the inhibitory action of Imipenem against colistin-resistant *E. coli*.

Finally, Colistin-resistant *E. coli* have created an alarming situation, and a multifaceted approach is essential sustain the Nepal's poultry industry. To begin with, poultry farmers should be provided with education in making them aware of the risk involving extensive use of antimicrobials for poultry production. Moreover, policy maker needs to enforce strict regulation on the use of antibiotics, particularly drugs like colistin as well as robust surveillance to ensure compliance. Various training workshops and educational programs for poultry farmers on the dangers of antibiotic overuse, resistance development, and alternative disease management practices can be conducted at the

community level. Along with appropriate management systems, it is also necessary to invest in the development of alternative medicine to strength poultry produce and health. This calls for a collaboration between researchers, the pharmaceutical industry, agricultural stakeholders, and policy makers to develop and implement innovative solutions for antimicrobial resistance.

Conclusion

Colistin, also known as polymyxin, is categorized as a last-resort antibiotic class by the World Health Organization. The current findings show a high incidence of poultry-originated *E. coli* resistant to colistin in-vitro. Identification of colistin-resistant *E. coli* with *mcr* gene screening is advised for confirmation. Furthermore, the extensive use of antimicrobials in chicken feed is linked with emergence of *E. coli* resistance to multiple antibiotics, highlighting critical need for stringent antibiotic usage policies and monitoring systems. These colistin-resistant bacterial species also have a great possibility of being disseminated through infantile poultry practices on Nepalese farms. Hence, this study emphasizes adopting a comprehensive measure in poultry community to limit the misuse of antibiotics to ensure both animal and public health.

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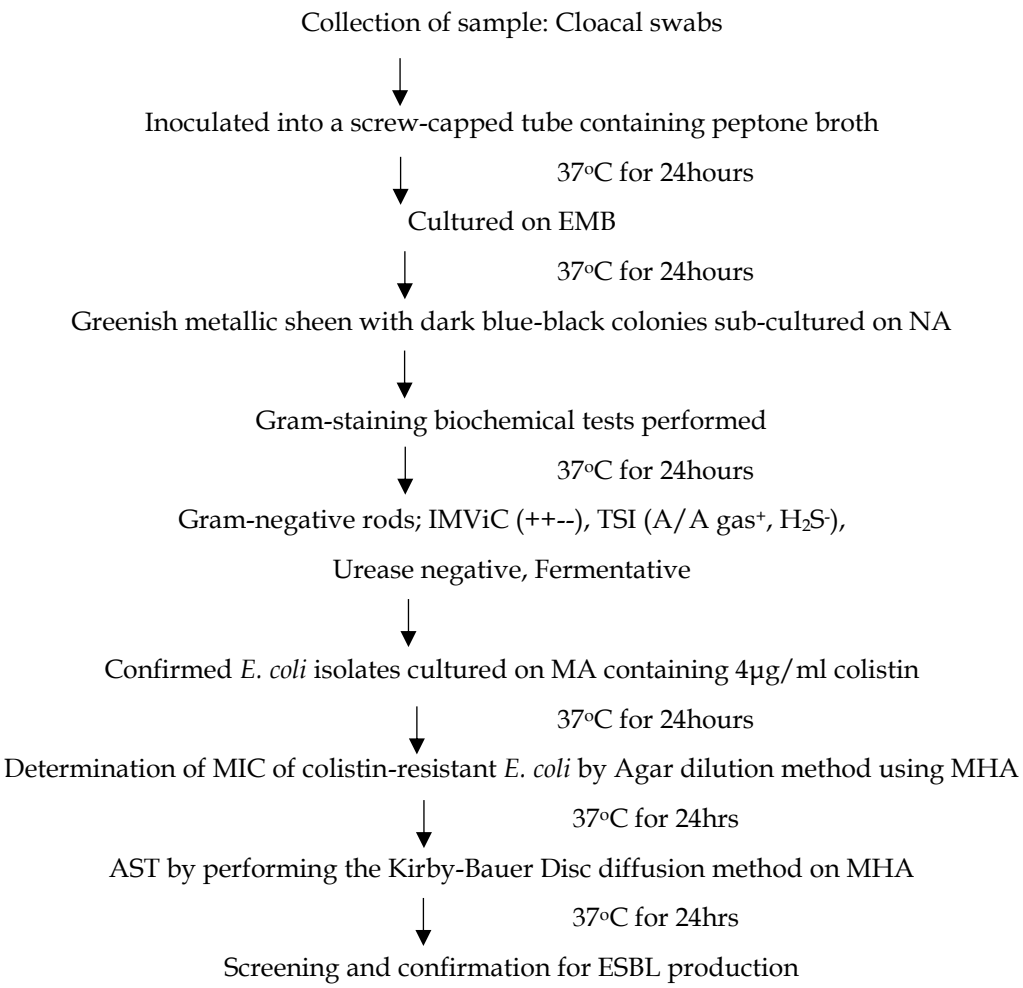
Supplementary

Supplementary Table 1. Standard zone diameter interpretation standards for *E. coli* by disk diffusion method of antimicrobial susceptibility test as per CLSI guideline, 2016.

Antimicrobial Agent	Antibiotics Class	Mode of action	Symbol	Disk Content (µg)	Zone Diameter Interpretive Criteria (nearest whole mm)		
					Sensitive	Intermediate	Resistant
Ampicillin	β lactams	Cell wall synthesis inhibition	AMP	10	≥ 17	14–16	≤ 13
Amoxyclav (Amoxicillin-clavulanate)	2nd line of antibiotics	Cell wall synthesis inhibition	AMC	30	≥ 18	14–17	≤ 13
Cefotaxime	β lactams	Cell wall synthesis inhibition	CTX	30	≥ 26	23–25	≤ 22
Imipenem	Carbapenem	Cell wall synthesis inhibition	IMP	10	≥ 23	20–22	≤ 19
Amikacin	Aminoglycoside	Inhibitors of protein biosynthesis	AK	30	≥ 17	15–16	≤ 14
Erythromycin	Macrolides	Inhibitors of protein biosynthesis	E	15	≥23	14-22	≤13
Tetracycline		Inhibitors of protein biosynthesis	TE	30	≥ 15	12–14	≤ 11
Ciprofloxacin	Quinolone	Inhibitors of DNA replication	CIP	5	≥ 21	16–20	≤ 15
Nalidixic acid	Quinolone	Inhibitors of DNA replication	NA	30	≥ 19	14–18	≤ 13
Cotrimoxazole (Trimethoprim-sulfamethoxazole)	2nd line of antibiotics	Folic acid metabolism inhibitors	COT	25	≥ 16	11–15	≤ 10



Supplementary Figure 1: Flow chart showing isolation, identification, MIC determination, and antibiotic susceptibility pattern testing of colistin-resistant *E. coli* from cloacal swabs of chickens.