Detection of Antibiotic Resistance Genes in *Escherichia coli* Isolated From Healthy Livestock in Umuahia

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Abstract. The use of antimicrobials in animal farming is considered the most important factor promoting the emergence, selection, and dissemination of antibiotic-resistant microorganisms. This study aimed to evaluate the antibiotic resistance genes in *Escherichia coli* isolated from healthy livestock. A total of 240 samples were collected from four different livestock: sheep (60), cattle (60), goats (60), and poultry (60). The faecal sample was aseptically inoculated onto a MacConkey agar plate and incubated overnight at 37 °C. The isolate was identified as E. coli based on its characteristic colony morphology on MacConkey agar, with lactose fermentation and pink-coloured colonies. The Presumptive *E. coli* colonies were again cultured on Eosin-methylene-blue (EMB) agar. The disc diffusion method was used to determine the antibiotic susceptibility testing of the isolates using ten (10) antimicrobial agents. Polymerase chain reaction was used to detect the presence of resistance genes of interest, particularly blaTEM, blaSHV and blaCTX. Seven (7) isolates were selected, including three isolates from cows (C1, C2, C3), two isolates from goats (G4 and G5) and two (2) isolates from poultry (P1 and P3). blaCTX was detected in C5 but absent in C1 and C2. blaTEM and blaSHV were detected in C1, C2 and C5. blaTEM and blaSHV were detected in G4, while blaTEM was detected in G5 only. Moreso, blaTEM was detected in P1, while both blaTEM and blaSHV were present in P3. The presence of these resistance genes in the isolates would promote the proliferation of antimicrobial resistance, threatening public health.

Keywords: Antibiotics; antibiotic resistance; poultry; cattle; goat.

INTRODUCTION

Escherichia coli is a facultatively anaerobic, gram-negative, rod-shaped bacterium. They constituted part of the normal commensal bacterial flora of animals and humans. *Escherichia coli* is a common member of the intestinal microbiota of livestock. It is considered a major cause of mortality and morbidity in livestock, but regular exposure to antibiotics puts them under selection pressure to acquire antibiotic resistance. Antibiotic resistance has emerged as a major threat to human and animal health, and animal production has been identified as a hotspot for its emergence and spread [1]. Because of the rapid expansion of intensive animal production systems to meet global demand for animal protein, concerns have arisen about the potential emergence of antibiotic resistance. This concern stems from the frequent use of antibiotics in these systems to maintain animal health and enhance productivity [2].

Antibiotics are becoming increasingly ineffective and pose one of the greatest threats to humans, animals, and the environment from a one health perspective [3]. One of the major drivers and contributing factors for the emergence and spread of antibiotic resistance in livestock production settings is the frequent use of these agents, either for prophylactic (as feed additives to promote growth performance) or therapeutic purposes [4]. Similarly, antibiotic resistance has negatively impacted livestock farmers through treatment failures, production losses, and economic losses, thereby posing potential risks to the overall viability of the animal sub-sector [5]. Moreover, the incidence of antibiotic resistance in animal health is increasing due to its involvement across diverse animal species and mi-

crobes, varying livestock environments, and intricate resistance mechanisms [6, 7].

Previous studies have identified livestock, such as ruminants, as plausible reservoirs of antibiotic-resistant bacteria and resistance genes, including *Escherichia coli* [8, 9, 10]. Over the past decades, there has been an increasing trend of antimicrobial-resistant E. coli, with a higher frequency of resistance genes, many of which were acquired through horizontal gene transfer, posing a significant threat to livestock, humans, and the environment [11]. This trait may accelerate the emergence of AMR in *E. coli* from livestock and the environment, including in animal production settings.

Antibiotic-resistant (AR) *Escherichia coli* that carries antibiotic resistance genes (ARGs) is also present in multiple hosts and environmental compartments as a normal inhabitant and can be globally transmitted between animals and humans. AR occurs when bacteria develop resistance to antibiotics, allowing them to multiply and grow even in the presence of antibiotics. Animals and their products serve as potential reservoirs and vectors for the spread of antibiotic-resistant *Escherichia coli*. Therefore, this study aimed to investigate antimicrobial susceptibility and the presence of resistance genes in *Escherichia coli* from healthy livestock in Umuahia, Abia State.

MATERIALS AND METHODS

Study Area. This study was carried out in Umuahia, and samples were collected from 10 commercial livestock farms. MOUAU farm, Umudike, Arochukwu by Owerri road farm, Ahiaeke farm, Aba road farm, Auwal Sarki Audu farm, Olokoro farm, Amuzu Ubakala farm, Afaraukwu Ibeku farm, College of Veterinary Medicine, Umudike, Bende road farm.

Collection of Samples. A total of 240 samples from four different livestock, comprising sheep (60), cattle (60), goats (60) and poultry (60), were collected aseptically into sterile containers.

Isolation and Identification of Escherichia coli. The sterile swab stick used to collect a livestock faecal sample was aseptically inoculated onto a Blood Agar and MacConkey agar plate and incubated overnight at 37°C for 24 hours [12]. Presumptive *E. coli* colonies were again cultured on eosin-methylene blue (EMB) agar and then purified on

Nutrient agar. The isolation and identification were done at Ovent Research Laboratory, Uzuakoli Road, Umuahia.

Gram Staining and Microscopy. The dried smear of the isolate obtained was fixed and stained with a crystal violet solution (primary dye) for 60 seconds, rinsed with tap water, and drained to avoid dilution by the mordant. It was flooded with iodine solution (mordant for 6 seconds and rinsed. Acetone was also applied dropwise on the tilted slide and subsequently rinsed with tap water. The slides were further flooded with safranin for 30 seconds, rinsed, and air-dried. Finally, all slides were examined under a microscope at a 100x objective lens [13]. The following biochemical tests were also carried out as recommended for the biochemical screening of *Escherichia coli: triple sugar iron agar, urease production, indole production, methyl red, Voges-Proskauer, and citrate test,* using standard methods as described by [14]. *E. coli* isolates can be confirmed biochemically by the use of a traditional method called IMViC tests. This is a set of four tests that are used to differentiate members of the family Enterobacteriaceae. IMViC is an abbreviation that stands for the indole, methyl red, Voges-Proskauer, and citrate utilisation tests.

Antibiotic Susceptibility Testing (AST) of Escherichia coli Isolates. Antimicrobial susceptibility testing of the *Escherichia coli* isolates was done on Mueller-Hinton agar using the Kirby-Bauer disk diffusion method, following the guidelines provided by the Clinical and Laboratory Standards Institute CLSI [15]. The *Escherichia coli* isolates was subjected to AST using 10 different antibiotics from various antibiotic classes including: Gentamicin (GEN,10 mcg); Ofloxacin (OFX,10 mcg); Augmentin (AU, 30 mcg); Peflacine (PEP, 10 mcg); Ceftazidime (CTZ, 30 mcg); Ciprofloxacin (CPX, 10 mcg); Ceporex (CEP, 10 mcg); Ceftriaxone (TRX, 30 mcg); streptomycin (S, 30 mcg) and cefuroxime (CEF, 30 mcg) in the disc diffusion assay. The diameters of the zones of inhibition were measured and compared with the zone size, as per CLSI guidelines [15].

Double Disk Synergy Test (DDST). Resistant isolates were further screened for the detection of Extended-Spectrum Beta-Lactamases (ESBL) using the double-disk synergy test as described by [16]. The double-disc synergy test with cefotaxime, ceftazidime, and amoxicillin-clavulanic acid

discs was used to screen for the ESBL phenotype [17].

Plasmid Analysis. Plasmid analysis was performed on the identified highly resistant strains of Escherichia coli. Plasmid DNA extraction was done using FavorPrepTMPlasmid Mini Kit according to the manufacturer's instructions.

Molecular Identification of the Isolates

Extraction of DNA Using NIMR Biotech Genomic DNA Extraction Kit. Genomic DNA extraction was carried out for all isolates using the NIMR Biotech genomic DNA extraction kit in accordance with the manufacturer's instructions. In detail, a loopful of culture of each isolate was harvested into 1 ml of sterile water in separate 1.5 ml microcentrifuge tubes. The samples were pelleted by centrifugation at 10,000 revolutions per minute (rpm) for 1 minute, and the supernatants were discarded. 200 µl of lysis buffer was added to each tube containing the pelleted samples, followed by thorough mixing for a few seconds using a vortex mixer. The samples were then incubated at 55 °C for 10 min in a heating block (Biobase). After incubation, the samples were allowed to cool, then centrifuged at 10,000rpm for 30 seconds. After this step, 200 µl of absolute ethanol was added to each sample, and the supernatants were carefully transferred into well-labelled spin columns fitted into collection tubes without dislodging the pellets. The spin columns were centrifuged at 10,000 rpm for 1 min, and the flow-through was discarded. The DNA, now trapped on the silica membrane in the spin columns, was washed with 500 µl of wash buffer 1 and 2, respectively. At each wash stage, the spin columns were centrifuged at 10,000 rpm for 30 s to allow the wash buffer to pass into the collection tubes. After this, the spin columns were spin dried at 14,000 rpm to remove any traces of ethanol. DNA samples were eluted into well-labelled, nuclease-free 1.5 ml microcentrifuge tubes with 50 µl of elution buffer. After elution, the DNA samples were stored at -20 °C until further analysis.

16S rRNA Gene Amplification and Sequencing. 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) using 16S rRNA primer pairs (27F:5'-AGAGTTTGATCCTGGCTCAG-3', 1492R: 5'-TACGGYTACCTTGTTACGACTT-3'). A 50 µl total reaction volume was prepared for each isolate's DNA samples with 5X HOT FIREPol Blend Master mix with 7.5 mM MgCl$_2$ (Solis Biodyne) which was brought down to 1X concentration containing 1X Blend Master mix buffer (Solis Biodyne), 1.5 mM MgCl$_2$, 200µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 25pMol of each forward and reverse primer (BIOMERS, Germany), 2 unit of Hot FIREPol *DNA* polymerase (Solis Biodyne), Proofreading Enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in a PTC-200 gradient thermal cycler (Eppendorf) with an initial denaturation at 95°C for 5 minutes, followed by 35 amplification cycles of 30 seconds at 95°C, 1 minute at 55°C, and 2 minutes at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The resulting amplicons were separated on a 1.5% agarose gel, and electrophoresis was carried out at 80V for 1 hour and 30 minutes. After electrophoresis, DNA bands were visualised by ethidium bromide staining and a UV- Trans-illuminator. A 100 bp DNA ladder was used as a molecular weight standard. After this process, the amplicons were sent for sequencing using the SeqStudio genetic analyser (Sanger sequencing). The resulting sequences were used for BLAST analysis to identify the isolates.

Detection of Extended-Spectrum Beta-Lactamase Genes. For the ESBL screening, Multiplex Polymerase Chain Reaction was carried out to detect the presence of the three prominent ESBL genes (TEM, SHV and CTX-M) in the bacterial DNA samples using the following specific set of primers; TEM (Forward: 5-TTT CGT GTC GCC CTT ATT CC-3; Reverse: 5- ATC GTT GTC AGA AGT AAG TTG G-3), SHV (Forward: 5-CGC CTG TGT ATT ATC TCC CT-3; Reverse: 5- CGA GTA CTC CAC GAG ATC CT), CTX-M (Forward: 5- CGC TGT TGT TAG GAA GTG TG-3; Reverse: 5- GGC TGG GTG AAG TAA GTG AC -3). The PCR reaction was carried out using the Solis BioDyne 5X HOT FIREPol Blend Master Mix. PCR was performed in a 20 µl reaction mixture. The reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM MgCl$_2$, 200µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25 pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol *DNA* polymerase (Solis Biodyne), Proofreading Enzyme, 2 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in a PTC-200 gradient thermal cycler (Eppendorf) with an initial denaturation at 95°C for 5

minutes, followed by 35 amplification cycles of 1 minute at 95°C, 1 minute at 58°C, and 1 minute at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The resulting amplicons were separated on a 1.5% agarose gel, and electrophoresis was carried out at 80V for 1 hour and 30 minutes. After electrophoresis, DNA bands were visualised by ethidium bromide staining using a UV- Trans-illuminator. A 100 bp DNA ladder was used as a molecular weight standard.

Statistical Analysis. Comparative resistance rates for *E. coli* strains from different livestock were statistically analysed using paired comparisons. ANOVA was used to analyse comparative rates among the four animals. Mean analyses were carried out using ANOVA (Analysis of Variance) at the 5% significance level.

RESULTS AND DISCUSSION

A total of 240 samples were collected from four different livestock, comprising sheep (60), cattle (60), goats (60), and poultry (60), for fresh faecal droppings.

Table 1 – Distribution of organisms isolated from samples

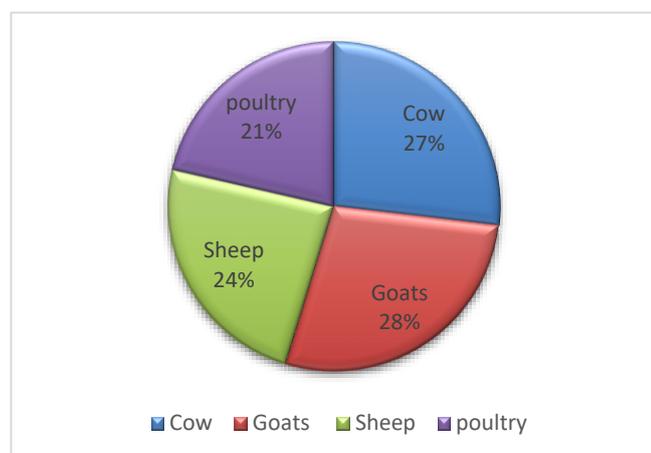| No of samples	No growth	*S.A*	*E. coli*
Cow	1	5	54
Goats	0	4	56
Sheep	2	10	48
Poultry	2	15	43
Total	5	34	201

Figure 1 – Frequency of isolation of *Escherichia coli* from the respective livestocks

Two hundred and one isolates were selected based on their Gram reaction, cell morphology, and biochemical tests. The presumptive *E. coli* colonies were cultured on Eosin-methylene blue (EMB) agar and then purified on a Nutrient agar plate. Eosin Methylene Blue (EMB) agar is a selective and differential medium for isolating and identifying Gram-negative bacteria, particularly Enterobacteriaceae like *Escherichia coli*. Lactose fermentation is indicated by a colour change (dark purple or black colonies with a metallic green sheen). In the Gram reaction, E. coli appears Gram-negative, with rod-shaped bacilli appearing as single cells or in pairs. Results of antimicrobial susceptibility testing of *E. coli* isolates (n = 201) from samples of livestock. The *E. coli* isolates were categorised as resistant and susceptible according to the CLSI [15] guideline. The results in Table 2 show that the isolates that were sensitive to the antimicrobials are Ofloxacin, Pefloxacin, Ceforex, and Ceftriaxone. The isolates that were resistant to the antimicrobials are Augmentin, Ceftazidime, Gentamycin, Ciprofloxacin, Streptomycin and Cefuroxime.

Table 2 – Antibiotic Resistance Profile of *E. coli* isolates

Antibiotic	The antimicrobial susceptibility test			
	Sensitive		Resistant	
	N	(%)	N	(%)
Ofloxacin (OFX)	162	(80.6)	39	(19.4)
Augmentin (AU)	66	(32. 8)	135	(67. 2)
Peflacine (PEF)	148	(73.6)	53	(26.3)
Ceftazidime (CTZ)	68	(33.8)	133	(66.2)
Gentamycin (CN)	39	(19.4)	162	(80.6)
Ciprofloxacin(CPX)	45	(22.4)	156	(77.6)
Ceporex (CEP)	156	(77.6)	45	(22.4)
Ceftriaxone (TRX)	184	(91.5)	17	(8.5)
Streptomycin (S)	71	(35.3)	130	(64.7)
Cefuroxime (CEF)	80	(39.8)	121	(60.2)

Notes: Low: ≤10%, moderate: >10% to 20%; high: >20% to 50%; very high: >50% to 70%, extremely high: >70%.

Most *Escherichia coli* isolates exhibited multidrug resistance, meaning resistance to at least one agent in three or more antibiotic classes.

Table 3 – Multidrug Resistance and ESBL Profile of the Isolates

	No. (%) MDR	No. (%) ESBL positive
Cow (n=54)	14 (9.8)	9 (6.3)
Goats (n=56)	10 (2.3)	7 (4.9)
Sheep (n=48)	21 (14.7)	12 (8.4)
Poultry (n=43)	31 (21.7)	20 (14.0)
Total (n=201)	76 (53.1)	48 (48.6)

Notes: MDR = Multidrug Resistance (The number of antibacterial agents varied from 3 to 8 antimicrobial agents); ESBL= extended spectrum beta-lactamases; ESBL positive was determined when each of the third-generation cephalosporin had a >5mm zone of inhibition alone than when used together with Amoxicillin-Clavulanate.
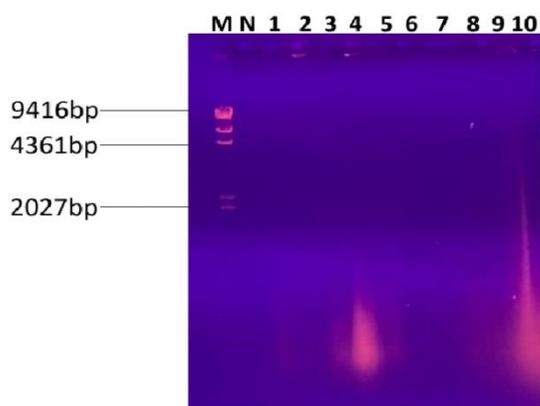

Figure 3 – Agarose gel electrophoresis of plasmid

Notes: Lane M= DNA marker (Lambda HIND 111). Lane 1=R1, Lane 2=R2, Lane 3=R3, Lane 4=R4, Lane 5=R5, Lane 6=R6, Lane 7=R7, Lane 8=R8, Lane 9=R9, Lane 10=R10; A plasmid was found in lane 7 at 3.5 kb.

Table 4 – ESBL Results of the Isolates

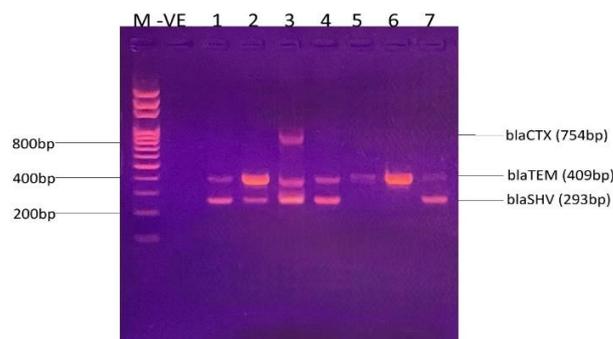| PCR code	Sample ID	ESBL genes		
		BlaCTX	blaTEM	BlaSHV
1	C1	NEG	POS	POS
2	C2	NEG	POS	POS
3	C5	POS	POS	POS
4	G4	NEG	POS	POS
5	G5	NEG	POS	NEG
6	P1	NEG	POS	NEG
7	P3	NEG	POS	POS

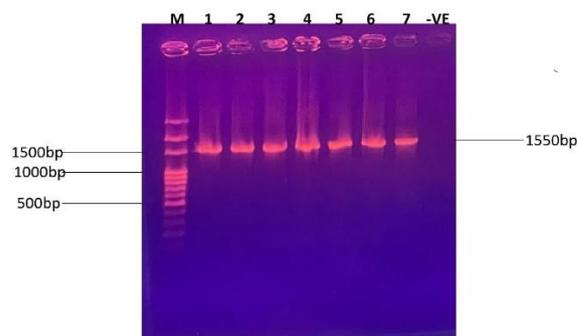Figure 4 – ESBL Gel Image of the Isolates

Figure 5 – ESBL Gel Image of the Isolates

The genotypic identification of the most promising *Escherichia coli* isolates (C5 and P3), based on their high prevalence of ESBL genes in the bacterial DNA samples, was performed using 16S rRNA gene sequencing. The C5 and P3 isolates were identified as *Escherichia coli* 3-907R-A10-01 and *Escherichia coli* 4-907R-B10-04, respectively, as shown in Table 5.

Table 5 – Sequence Id and Identified Organism

No	A	B	C
1	SEQUENCE ID	IDENTIFIED ORGANISM	% IDENTITY
2	3-907R-A10-01	*Escherichia coli*	89.5
3	4-907R-B10-04	*Escherichia coli*	98.3

The inappropriate use of antibiotics in livestock farming also contributes to the threat of antibiotic resistance. According to the Organisation for Economic Cooperation and Development (OECD), there will be a 67% increase in the amount of antimicrobials used in food animals worldwide from 63,151 tons in 2010 to 105,596 tons in 2030 [18]. The emergence of antibiotic resistance in livestock is attracting attention due to its significant consequences for animal and human health [19]. This study revealed that the

Escherichia coli isolates that were resistant to the antimicrobials were Augmentin (AU) 67.2%, Ceftazidime (CTZ) 66.2%, Gentamycin (CN) 80.6%, Ciprofloxacin (CPX) 77.6%, Streptomycin (S) 64.7%, and Cefuroxime (CEF) 6.2%. This high resistance level, even though the farms under study do not have a history of supplementing animal feed with antibiotics as growth promoters, nor are antibiotics used prophylactically extensively. It is therefore probable that other factors and pressures have brought about this high incidence of resistance in the absence of antibiotic exposure.

The high figures presented here may not be out of place but rather reflect resistance events in other hosts (humans) sharing the same environment with these animals, as observed in this study. It is common for people to defecate in and around surrounding compounds, bushes or to urinate just at the corner of the house. Such poor, unhygienic disposal of human excreta definitely exposes these animals to normal human enteric flora that may harbour novel resistant traits. Furthermore, an increase in the risk of acquisition of resistant organisms by animal hosts, due to the selective use of antibiotics in human hosts in the same environment, has been described [20], with humans in that environment previously exposed to various antibiotics. The high level of drug resistance observed in E. coli isolated from apparently healthy cattle in this study is comparable to findings from other workers on *E. coli* isolates from healthy livestock from various parts of Nigeria. For instance, using a disk diffusion method, Authors [21] reported 67.6% (710/1051) resistance for streptomycin among *E. coli* isolated from apparently healthy cattle ready for slaughter in Ekiti State, Nigeria, and 62.5% (n = 80) from livestock in the South Eastern Nigeria [22, 23] reported 56.3% (n = 240) resistance for streptomycin among *E. coli* isolated from healthy cattle at a major cattle market in Ibadan, Oyo State, South Western, Nigeria. In this study, using the antibiotic breakpoint method, 64.7% (130/201) of isolates showed resistance to streptomycin.

The highest level of resistance in the commensal *E. coli* studied [21] in cattle at Ekiti State was 88.1% (892/1051) in gentamicin. However, gentamicin was not among the antibiotics used for breakpoint determination but was among those studied. At the same time, a considerably low resistance of 17.2% to Gentamycin was observed among the isolates [22]. In this study, the highest level of resistance to gentamicin was 80.6% (162/201). Authors [23] reported 51.3% (n = 240) resistance for Ciprofloxacin among *E. coli* isolated from healthy cattle at a major cattle market in Ibadan, Oyo State, South Western, Nigeria. In this current work, the second-highest level of resistance was observed with Ciprofloxacin at 77.6% (156/201). As a result, it was discovered that they routinely used certain antibiotics. For example, streptomycin was often used without prescription whenever they found any of the animals coughing, oxytetracycline during cold seasons, and penicillin-streptomycin for all manner of infections, all these without careful attention to the correct dose/dosage or veterinary prescriptions. These unwholesome practices must have contributed to the high level of drug-resistant E. coli isolated from healthy livestock, driven by selective pressure.

The large proportions of the bacteria studied are not susceptible to the respective breakpoints of the antibiotics, indicating their tolerance to these antibiotics and the possibility of difficulty treating infections caused by these organisms with the concerned antibiotics [24]. Since there is the possibility of acquiring a drug resistance mechanism through interbacterial transfer [25], the current work underscores the public health risk of the possible transfer of this high drug resistance trait from this apparently nonpathogenic E. coli to pathogenic animal pathogens and humans. A significant public health concern is that multidrug-resistant commensal *E. coli* strains may serve as a reservoir of resistance genes that could be transferred to pathogenic bacteria. The high prevalence of multidrug-resistant E. coli observed in this study suggests a need for improved education and communication about antibiotic use in human and veterinary medicine.

CONCLUSIONS

Because of the rapid expansion of intensive animal production systems to meet global demand for animal protein, concerns have arisen about the potential emergence of antibiotic resistance. In conclusion, this study revealed that the C5 and P3 isolates identified as *Escherichia coli* 3–907R–A10-01 and *Escherichia coli* 4–907R–B10-04 were isolated from cow and poultry, respectively. The use of antibiotics as growth promoters or supplements in livestock is also a factor contributing to the spread of antibiotic resistance genes. One of the many reasons

behind the irrational use of antibiotics is a lack of awareness and knowledge of antibiotic use and antibiotic resistance. Similarly, antibiotic resistance has negatively impacted livestock farmers through treatment failures, production losses, and economic losses, thereby posing potential risks to the overall viability of the animal subsector. The detection of antibiotic resistance genes (ARGs) in livestock highlights the critical need for responsible antibiotic use and robust surveillance in agricultural settings. The presence of ARGs in livestock poses significant risks to animal and human health, as well as the environment. To mitigate these risks, it is essential to promote the judicious use of antibiotics in livestock production; implement robust monitoring and surveillance systems for ARGs; develop and adopt alternative strategies for disease prevention and treatment; and foster collaboration between the human and animal health sectors. By addressing antibiotic resistance in livestock, we can help protect public health, ensure food safety, and preserve the effectiveness of antibiotics for future generations.

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