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Antibiotic resistome and virulence profile of antibiotic-resistant bacteria in healthy chickens from registered broiler farms in Cagayan Province

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ABSTRACT

This study assessed the resistance profile, antibiotic resistome, and virulence characteristics of bacteria isolated from healthy broiler chickens in registered farms in Cagayan Province, Philippines. Using a descriptive cross-sectional design, 900 cloacal swab samples were collected from four broiler farms over three production cycles and processed for bacterial isolation, identification, and antimicrobial susceptibility testing. Enterobacteriaceae isolates, mainly *Escherichia coli* and *Salmonella* spp., were tested by Kirby-Bauer disc diffusion against clinically relevant antibiotics, and one representative multidrug-resistant *E. coli* isolate underwent whole-genome sequencing and annotation. Phenotypic results showed widespread multidrug resistance across farms and sampling periods, with particularly high resistance to tetracycline and trimethoprim-sulfamethoxazole, and notable resistance to ciprofloxacin and ceftazidime. *Salmonella* isolates also consistently showed tetracycline resistance. Farm interviews indicated no reported antibiotic use, yet resistance remained prevalent, suggesting that transmission may be sustained through plasmid-mediated horizontal gene transfer, sanitation gaps, transport-related contamination, litter persistence, and human or vehicle movement. Whole-genome analysis of the representative MDR isolate identified *E. coli* with a complex resistome and virulence repertoire, including the plasmid-associated virulence marker *cvaC* and multiple resistance determinants linked to efflux, target alteration, and regulatory mutations such as *marR*, *soxR*, and *soxS*. These findings establish a baseline for antimicrobial resistance in broiler farms and highlight the need for stronger surveillance, biosecurity validation, and antimicrobial stewardship in poultry production.

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INTRODUCTION

The Philippine poultry industry is a major component of the country's livestock sector and is largely centered on chicken production, particularly broiler and layer operations. As of the third quarter of 2023, the Philippine Statistics Authority reported a total chicken inventory of about 202.8 million birds, with broilers comprising 34.7% and layers 22.3%, underscoring the dominance of meat- and egg-producing chickens in national production (Philippine Statistics Authority, 2023). Broiler meat output also rose during the same period, while national chicken meat production reached approximately 1.50 million metric tons in 2023, reflecting the industry's important role in supplying affordable and accessible animal protein to Filipino consumers (USDA, 2024; Halili, 2024). Production remains concentrated in regions such as Central Luzon, CALABARZON, and Northern Mindanao, while the sector continues to support rural livelihoods, employment, and broader agricultural growth through farm *operations*, feed milling, processing, transport, and retail (FAST, 2024; Halili, 2024).

Because poultry production is highly intensive and economically important, antimicrobial use has become deeply embedded in poultry farming both globally and in the Philippines. Worldwide poultry production continues to expand as a major source of animal protein, and antibiotics have historically been used for treatment, prevention, and growth promotion in order to sustain flock productivity under intensive production systems (Hedman *et al.*, 2020; De Mesquita Souza Saraiva *et al.*, 2021). In the Philippine setting, studies have shown frequent use of antibiotics such as enrofloxacin, colistin, gentamicin, oxytetracycline, erythromycin, norfloxacin, and fosfomycin, including drugs classified as critically important for human medicine (Barroga *et al.*, 2020; Pineda-Cortel *et al.*, 2024). Although regulations require veterinary prescription for many antimicrobial products, off-label and over-the-counter access remain concerns, allowing antibiotics to be administered through water or feed for mass treatment, disease prevention, or productivity enhancement (Pineda-Cortel *et al.*, 2024). These practices raise serious food safety concerns because improper use and insufficient withdrawal periods may leave antimicrobial residues in poultry meat and eggs.

Antimicrobial residues are trace amounts of veterinary drugs or their metabolites that remain in animal-derived foods after treatment, and they are often associated with medication given too close to slaughter, excessive dosage, prolonged treatment, or failure to observe withdrawal periods. International and national frameworks such as Codex Alimentarius, Philippine National Standard PNS 48:2016, and the National Veterinary Drug Residue Monitoring Program have been established to define maximum residue limits and monitor food safety, yet studies continue to report violative residues in poultry products (Ang and Hanzel, 2023; Oyedeji *et al.*, 2021). Global evidence shows that poultry residue studies are concentrated in Asia, where concerns over antibiotic use and food safety remain high (Treiber and Beranek-Knauer, 2021). In the Philippines, limited monitoring coverage, especially among smaller farms, makes residue detection and risk assessment more difficult, strengthening the concern that poultry meat and eggs may sometimes exceed safe residue limits and pose potential hazards to consumers (Imperial *et al.*, 2022).

Beyond residues, the intensive use of antimicrobials in poultry production accelerates the emergence of antimicrobial resistance (AMR) in both commensal and pathogenic bacteria. Poultry-associated organisms such as Es*cherichia coli, Salmonella enteric*a, and *Campylobacter* can develop resistance and spread it through food, direct contact, and environmental contamination, creating an important public health threat. International reviews have shown especially high resistance in avian pathogenic *E. coli* to older drugs such as ampicillin and tetracycline, while *Salmonella* and *Campylobacter* increasingly exhibit resistance to fluoroquinolones and cephalosporins that are important in human medicine (Nhung *et al.*, 2017). Philippine studies reflect the same pattern: wet market chicken samples

in Manila have yielded highly multidrug-resistant *E. coli*, while chicken meat has also been found to carry resistant *Salmonella enterica* and *Campylobacter jejuni* and *Campylobacter coli* at concerning rates (Lim *et al.*, 2016; Dimaapi *et al.*, 2024; Nagpala *et al.*, 2025). These findings indicate that poultry products may serve as pathways for the transmission of resistant bacteria to humans, especially when meat is undercooked or handled improperly, or when poultry waste contaminates water and agricultural environments (Alam *et al.*, 2019; Nagpala *et al.*, 2025).

In response to these risks, both detection systems and government interventions have been strengthened to improve food safety and antimicrobial stewardship in poultry production. Residue detection commonly relies on screening and confirmatory methods such as four plate tests, ELISA, HPLC, and LC-MS/MS, with the latter two regarded as gold-standard approaches for accurate identification and quantification of residues (Kamouh *et al.*, 2024; Ramatla *et al.*, 2017). In the Philippines, the Bureau of Animal Industry and related agencies align testing protocols with international standards, including the 2024 Department of Agriculture circular that requires screening followed by confirmatory LC-MS/MS for positive samples. On the policy side, the government has created the Inter-Agency Committee on Antimicrobial Resistance, implemented the National Action Plan on AMR, enforced the "No Prescription, No Dispensing" rule for veterinary drugs, and supported surveillance and research initiatives across One Health sectors (Pineda-Cortel *et al.*, 2024). Together, these measures reflect the recognition that controlling AMR in poultry requires not only regulation and monitoring, but also stronger biosecurity, farmer education, and sustained collaboration among government, industry, and research institutions (BAI, 2023; DOST-PCAARRD, 2025). This study aims to assess the resistance profile, antibiotic resistome, and virulence characteristics of bacteria isolated from healthy broiler chickens in registered farms in Cagayan Province, Philippines through elucidating the whole genome of a representative multidrug-resistant isolate. This study also aims to determine the genetic determinants associated with antimicrobial resistance and pathogenicity, and to provide baseline genomic data that may contribute to the surveillance, management, and control of resistant bacterial strains in poultry production.

MATERIALS AND METHODS

Experimental design

Descriptive Cross-Sectional with observational design was applied in this study to evaluate the prevalence and patterns of antibiotic-resistant bacteria in healthy broiler chickens. This design was appropriate for capturing a picture of resistance at a specific time across the four identified farms, allowing for comparisons based on farm management practices that may influence resistance patterns. The study was conducted in three months period (August to October 2025) to consider first the approval from the poultry farm managers and the start of chicks loading to data collection which was done in three consecutive harvest period, interviews, laboratory analysis, and data interpretation.

Sample collection

The sampling sites were four (4) registered broiler poultry farms located in Barangays Gadu and Nangalisan, Solana, Cagayan, Camasi, Peñablanca, Cagayan and Centro, Pamplona, Cagayan.

These municipalities were selected based on their compliance with the Republic Act 8485 also known as the Animal Welfare Act, operational practices, and willingness to participate. The sample type was cloacal swabs collected from the above-mentioned four (4) registered broiler farms with seventy-five (75) samples from each farm. Three trials were conducted which resulted in a total of 900 samples collected over the end of three consecutive production cycles or harvest periods. Sample collection was done one week prior to the anticipated harvest date.

Moreover, an interview form containing the following information was laid out in order to correlate farm

practices with the laboratory results: antibiotic usage, biosecurity measures, vaccination programs, and usage of feed additives

Isolation and identification of bacterial pathogens

For the isolation of bacterial pathogens, gram-negative organisms were targeted for this assay prioritizing *Escherichia coli* and *Salmonella* spp.

In order to effectively isolate the target organisms, selective media were used including MacConkey Agar, Eosin Methylene Blue Agar (EMBA), Xylose Lysine Deoxycholate (XLD) Agar. Subsequent isolation and plating were done until pure isolates of putative enteric bacteria were obtained. Isolates were then identified using Analytical Profile Index (API) 20E kit. Colonies conform to the morphological and biochemical characteristics of *Escherichia coli* and *Salmonella* spp. were subsequently subjected to Antimicrobial Susceptibility Test (AST) by Kirby-Bauer Disc Diffusion assay.

Antimicrobial susceptibility test

For the Antibiotic Resistance Testing, a panel of clinically relevant antibiotics was used based on Food and Agriculture Organization (FAO) recommendation, these antibiotic discs include Amikacin, Gentamicin, Cefotaxime, Imipenem, Ceftazidime, Meropenem, Chloramphenicol, Sulfamethoxazole-Trimethoprim, Ciprofloxacin and Tetracycline.

The Kirby-Bauer disc diffusion method was employed to determine the resistance profiles of the isolated bacterial species. Plates were incubated for 18-24 hours at 37°C prior to reading of results.

Resulting Zone of Inhibition (ZOI) were interpreted according to the standard breakpoints indicated in the standard manual of the Clinical Laboratory Standards Institute (CLSI) M-100 document. Isolates showing pattern of resistance against two or more classes of antibiotics are considered multi-drug resistant.

DNA extraction of representative MDR isolate and whole genome analysis

One representative MDR Isolate showcasing resistance to more than 2 classes of antibiotics was subjected for molecular screening and whole genome analysis. The DNA of the representative isolate was extracted using boiling method. Colonies are harvested from a 24- hour culture and were suspended in a 1.5 ml microtube containing nuclease-free water. Isolate is then heated at 95°C in a heat block for 5 minutes to extract the DNA and centrifuged to isolate the DNA-containing supernatant.

Extracted DNA was then quantified using Qubit fluorometer and subjected to Whole Genome Sequencing utilizing the rapid barcoding protocols of Oxford Nanopore Technologies MinION mk1D with a 50x coverage. Sequence reads were assembled de novo using Flye (Kolmogorov, 2021) and polished using Medaka (ONT Ltd., 2020). Assembled whole genome was mapped using Proksee (Grant *et al.*, 2023) and annotated using Bakta (Schwengers *et al.*, 2021) and Prokka (Seemann *et al.*, 2014). Resistance-coding and related genes were identified through the CARD Resistance Gene Identifier database (CARD RGI) (Jia *et al.*, 2017). Species identification was done utilizing FastANI tool to determine average nucleotide identity based on closely related identity from NCBI GenBank.

RESULTS AND DISCUSSION

Throughout the collection period from August to October, a total of 900 cloacal swab samples were collected, pooled, and analyzed.

Antibiotic susceptibility assay

The putative isolates of Enterobacterales were subjected to antimicrobial susceptibility test using Kirby-Bauer disc diffusion test. Isolates identified to conform with the biochemical profile of Enterobacteriaceae family were tested for the disc diffusion test. Table 1 shows the summary of results of the AST assay showcasing identified species of Enterobacteriaceae showing resistance to antibiotics.

Table 1. Summary of isolates from four farms from August to October showcasing resistance to different classes of antibiotics

Farm code	Month of collection					
	August		September		October	
	Bacterial ID	Resistance profile	Bacterial ID	Resistance profile	Bacterial ID	Resistance profile
F1	*E. coli* F1-01	C, CIP, SXT, TE	*E. coli* F1-C2	C, CIP, SXT, TE	*E. coli* F1-C3-01	C, CIP, SXT, TE
	Salmonella F1-01	C, TE	*Salmonella* F1-C2-1	C, TE	*E. coli* F1-C3-02	C, CIP, SXT, TE
					Salmonella F1	C, TE
F2	*E. coli* F2	CTX, CTZ, C, CIP, CN, SXT, TE	*E. coli* F2-02	CTX, CTZ, C, CIP, CN, SXT, TE	*E. coli* F2-C3-1	CTX, CTZ, C, CIP, CN, SXT, TE
	Salmonella F2-1	CIP, TE	*Salmonella* F2-01	CIP, TE	*E. coli* F2-C3-2	CTX, CTZ, C, CIP, CN, SXT, TE
			Salmonella F2-02	CIP, TE	*Salmonella* F2-C3-03	CIP, TE
					E. coli F2-C3-3	CIP, CN, SXT
F3	*E. coli* F3-1	CTX, CTZ, C, CIP, CN, SXT, TE	*E. coli* F3-C2-1	CTX, CTZ, C, CIP, CN, SXT, TE	*E. coli* - F3-C3-01	CTX, CTZ, C, CIP, CN, SXT, TE
	E. coli F3-2	CTX, CTZ, C, CN, TE	*E. coli* F3-C2-2	CTX, CTZ, C, TE	E.coli F3-C3-02	CTX, CTZ, C, TE
	Salmonella F3-1	C, TE	*E. coli* F3-C2-3	CTX, CTZ, C, CIP, CN, SXT, TE	*Salmonella* F3-C3	C, TE
			Salmonella F3-C2	C, TE		
F4	*E. coli* F4-1	C, SXT, TE	*E. coli* F4-C2-1	C, SXT, TE	*E. coli* F4-1	C, SXT, TE
	Salmonella F4-1	C, TE	*Salmonella* F4-C2	C, TE	*Salmonella* F4-1	C, TE

Analysis of resistance profiles of the isolates highlights high prevalence of resistance to Tetracycline and Trimethoprim/Sulfomethoxazole. This result particularly aligns with findings from previous publications documenting very high prevalence of tetracycline and trimethoprim/sulfamethoxazole-resistant *E. coli* particularly in food-producing animals such as broiler chickens (Aberkane *et al.*, 2023, Ahmed *et al.*, 2025). Tetracycline resistance in *E. coli* is most often mediated by acquired *tet* genes (e.g., *tet* (A), *tet* (B)) that encode efflux pumps or ribosomal protection proteins; these genes are widespread in animal isolates. Trimethoprim and sulfonamide resistance is typically encoded by *dfr* (trimethoprim resistance) and *sul* (sulfonamide resistance) gene variants (Martínez-Álvarez *et al.*, 2022, Mensah *et al.*, 2022). Reviews and molecular surveys show that these genes are common in poultry *E. coli* and explain the phenotypic patterns observed in antibiotic susceptibility testing.

Furthermore, a relatively high prevalence of resistance to Ciprofloxacin and Ceftazidime can also be observed across all collection sites in different collection periods. This, too, also aligns with many major findings on the high prevalence as well of CIP and CTZ- resistant isolates of *E. coli* (Hussein *et al.*, 2025). Resistance is primarily driven by mutations in the quinolone resistance-determining regions (QRDR) of the gyrA (Ser83Leu, Asp87Asn) and parC (S80I) genes. Moreover, Ceftazidime-resistant isolates are frequently multi-drug resistant (MDR), often showing resistance to tetracycline, ampicillin, and aminoglycosides (Martínez-Álvarez *et al.*, 2022). On the other hand, results also show consistent prevalence of tetracycline-resistance in *Salmonella* isolates across different collection periods on all the farms. Resistance to tetracycline by Salmonella isolates from broiler chickens have all been previously documented (Pavelquesi *et al.*, 2021, Azevedo *et al.*, 2025). Resistance is largely conferred by acquired *tet* genes, with *tetA* the most commonly identified in *Salmonella*, and *tetB* occurring less often.

While all farms indicated no use of antibiotics in the overall management of the chickens, other sources of resistance can still be inferred. Observed lack of biosecurity measures such as absence of footbath, lack of foot traffic management, and overall inadequacy of farm barriers which should limit exposure to contaminants that may harbor AMR. Insufficient sanitation, such as failing to properly clean, disinfect, or disinfect empty poultry houses (resting period) between flocks, allows resistant *E. coli* and Salmonella to survive in litter,

dust, and equipment (feeders, drinkers) (Tilahun and Ofa, 2026a). Furthermore, horizontal gene transfer (HGT) facilitates rapid spread of antibiotic resistance (AMR) between commensal *E. coli* and pathogenic Salmonella, primarily via plasmids (conjugation) and bacteriophages.

This exchange, often driven by environmental stress, allows susceptible pathogens to acquire multidrug resistance, challenging broiler health and food safety (Oladeinde *et al.*, 2019, Oladeinde *et al.*, 2021).

Genomic analysis of representative MDR

One putative multi-drug-resistant *E. coli* isolate was subjected to Whole Genome Sequencing using Oxford Nanopore Technologies minION mk1D. The isolate had a total genome size of 3,726,439 base pairs. Fig. 1 shows the whole genome map of the representative MDR isolate.

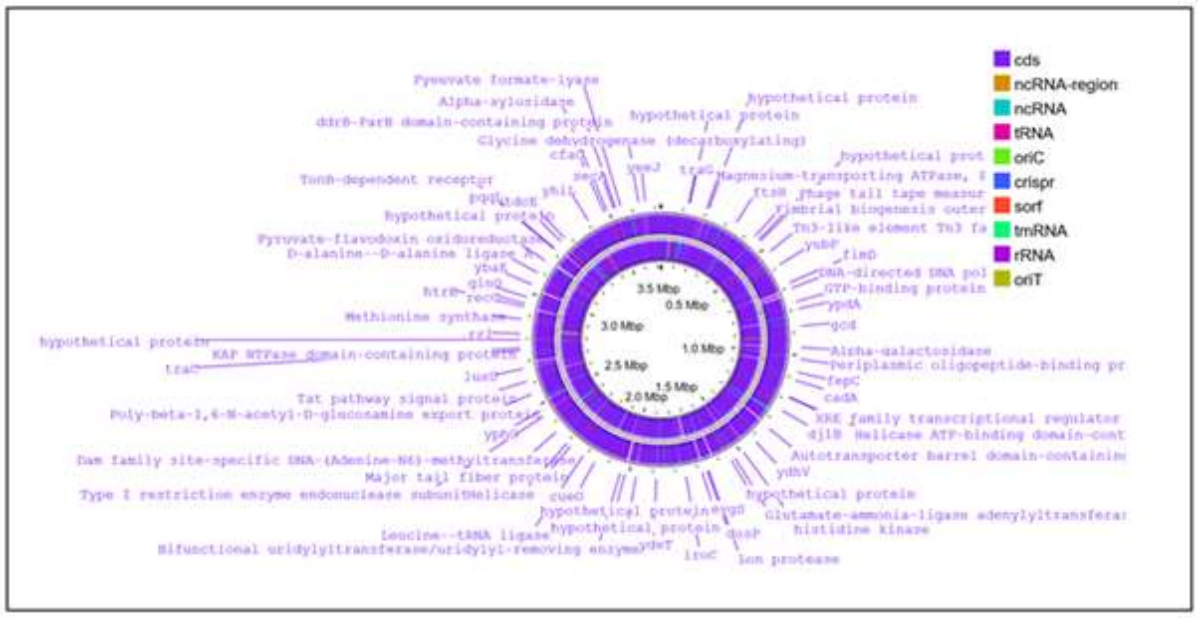

Fig. 1. Whole genome map of representative MDR isolate from a broiler cloacal swab. Visualized using Proksee (Grant *et al.*, 2023).

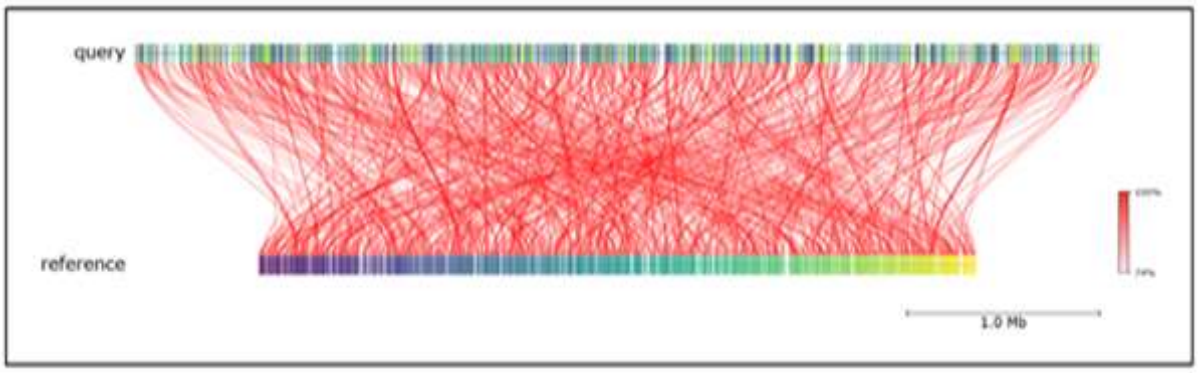

Fig. 2. Average nucleotide identity of query sequence against a reference genome from NCBI GenBank

The isolate was further confirmed to be *Escherichia coli* based on Average Nucleotide Identity (ANI) analysis against reference genomes available in the NCBI GenBank database. The ANI value exceeded the accepted species delineation threshold of 95-96%, confirming the taxonomic identity of the isolate as *E. coli*. Fig. 2 presents the Average Nucleotide Identity comparison of the

query genome against the closest reference genome from NCBI GenBank.

E. coli is a very ubiquitous organism inhabiting many environments and niche. Poultry is almost ubiquitously colonized by *Escherichia coli*, with many strains carrying multiple resistance genes and posing a zoonotic risk.

The dominant pathogenic variants are Avian Pathogenic *E. coli* (APEC), an ExPEC pathotype causing colibacillosis, though poultry can also carry other ExPEC or enteric strains.

Based on annotated whole genome of the isolate, there is a presence of the *cvaC* gene (Fig. 3) which encodes Colicin V (or Microcin V), a plasmid-encoded peptide bacteriocin (toxin) produced under stress conditions. It functions as a virulence factor, aiding in colonization and survival. It is frequently associated with APEC and Urinary Tract Infection (UTI) strains. The *cvaC* gene serves as a marker for Avian Pathogenic *E. coli* strains and is a critical component for the competitive fitness of pathogenic *E. coli* in various hosts. Furthermore, analysis of the genome using the CARD-RGI database revealed multiple resistance-coding genes. Table 2 shows all perfect and strict hits in the genome and their corresponding mechanisms against different families of antibiotics based on protein homolog models. Genes coding, especially for resistance against tetracycline were detected including *marA, evgA* and *H-NS* genes. crucial, often conserved, global regulatory proteins in *Escherichia coli* that coordinate antimicrobial resistance (AMR), particularly through the modulation of efflux pumps (such as AcrAB-TolC) and the reduction of membrane permeability (Morales-Durán *et al.*, 2025; Ayele *et al.*, 2024). These three genes often act in concert. For example, they are frequently identified together in high-risk lineages (e.g., ST131, ST405) that demonstrate high levels of resistance to fluoroquinolones, tetracycline, and beta-lactams. They are considered core elements of the *E. coli* resistome that enable the bacterium to adapt to antibiotic stress.

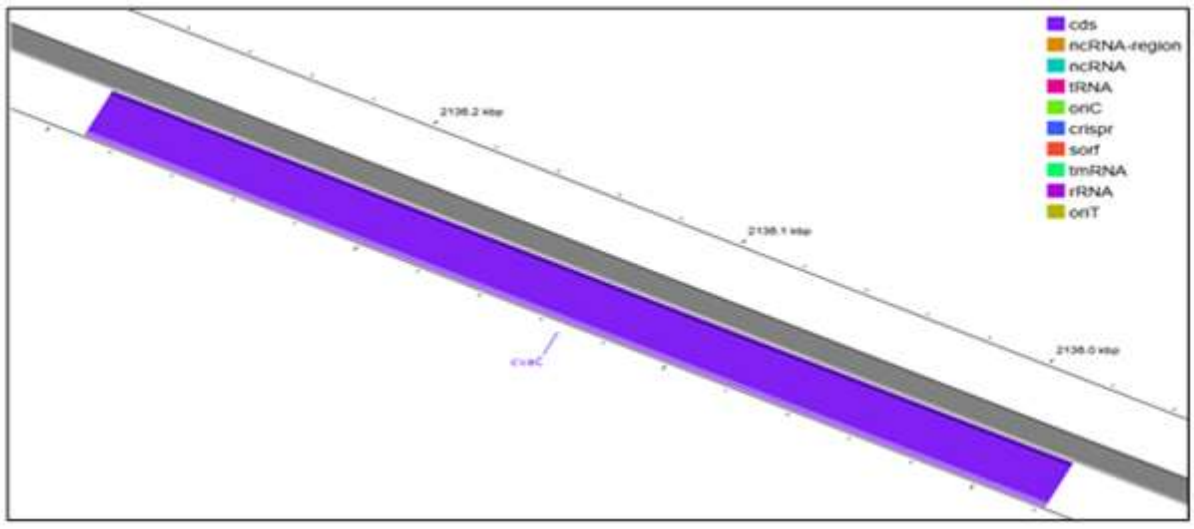

Fig. 3. Annotated *cvaC* gene from the representative MDR isolate, providing insight on the pathogenicity of the APEC isolate

The genes *marA, evgA*, and *H-NS* mostly code for Major facilitator superfamily (MFS) antibiotic efflux pump and Resistance-nodulation-cell division (RND) antibiotic efflux pump. The RND superfamily is the most effective efflux pump family facilitating antibiotic resistance in Gram-negative bacteria meanwhile, MFS transporters play critical roles in host-pathogen communication, particularly adhesion, invasion, intracellular survival, and biofilm formation. The overexpression of these pumps, often

regulated by mutations, allows bacteria to survive multiple, unrelated types of antibiotics, contributing to "superbug" development. Due to their critical role, RND and MFS pumps are major targets for developing efflux pump inhibitors (EPIs) to restore the efficacy of existing antibiotics.

Table 2. Antimicrobial resistant genes are annotated based on Protein Homolog Model

ARO Term	AMR gene family	Drug class	Resistance mechanism	Percent (%) identity of matching regions	RGI Criteria
mdtE	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide, Fluoroquinolone, Penicillin beta-lactam	Antibiotic efflux	100	Perfect
dfrA14	Trimethoprim resistant dihydrofolate dfr	Diaminopyrimidine	Antibiotic target replacement	100	Perfect
marA	Resistance-nodulation-cell division (RND) antibiotic efflux pump; General Bacterial Porin with reduced permeability to beta-lactams	Fluoroquinolone, Monobactam, Carbapenem, Cephalosporin, Glycylcycline, Penicillin beta-lactam, Tetracycline, Rifamycin, Phenicol, Disinfecting agents, & antiseptics	Antibiotic efflux, reduced permeability to antibiotics	100	Perfect
evgA	Major facilitator superfamily (MFS) antibiotic efflux pump, Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide, Fluoroquinolone, Penicillin beta-lactam, Tetracycline	Antibiotic efflux	100	Perfect
H-NS	Major facilitator superfamily (MFS) antibiotic efflux pump, Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide, Fluoroquinolone, Cephalosporin, Penicillin beta-lactam, Tetracycline	Antibiotic efflux	100	Perfect
mdtN	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone	Antibiotic efflux	99.42	Strict
rsmA	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Nucleoside antibiotics, disinfecting agents and antiseptics	Antibiotic efflux	85.25	Strict
emrB	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone	Antibiotic efflux	99.61	Strict
evgS	Major facilitator superfamily (MFS) antibiotic efflux pump, Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide, Fluoroquinolone, Penicillin beta-lactam, Tetracycline	Antibiotic efflux	96.57	Strict
emrK	Major facilitator superfamily (MFS) antibiotic efflux pump	Tetracycline	Antibiotic efflux	98.86	Strict
emrY	Major facilitator superfamily (MFS) antibiotic efflux pump	Tetracycline	Antibiotic efflux	99.61	Strict
gadX	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide, Fluoroquinolone, Penicillin beta-lactam	Antibiotic efflux	94.78	Strict
GadW	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide, Fluoroquinolone, Penicillin beta-lactam	Antibiotic efflux	97.11	Stict
AcrS	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone, Cephalosporin, Glycylcycline, Penicillin beta-lactam, Tetracycline, Rifamycin, Phenicol, Disinfecting agents and antiseptics	Antibiotic efflux	98.18	Strict
AcrE	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone, Cephalosporin, Penicillin beta-lactam,	Antibiotic efflux	99.22	Strict
E. coli mdfA	Major facilitator superfamily (MFS) antibiotic efflux pump	Tetracycline, Phenicol, Disinfecting agents and antiseptics	Antibiotic efflux	96.83	Strict
vanG	Glycopeptide resistance gene cluster, Van ligase	Glycopeptide	Antibiotic target alteration	38.23	Strict
CRP	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Nucleoside antibiotics, disinfecting agents and antiseptics	Antibiotic efflux	99.52	Strict
PmrF	pmr phosphoethanolamine transferase	Peptide antibiotics	Antibiotic target alteration	99.65	Strict

PHM identify genes that are generally resistant based on similarity to a reference protein sequence. They detect functional homologs regardless of specific, small-scale mutations.

Table 3. Antimicrobial resistant genes are annotated based on Protein Variant Model

ARO Term	SNP	AMR gene family	Drug class	Resistance mechanism	Percent (%) identity of matching regions	RGI criteria
E. coli EF-Tu mutants conferring resistance to pulvomycin	R234F	Elfamycin resistant EF-Tu	Elfamycin antibiotic	Antibiotic target alteration	100	Strict

PVM are specifically designed to detect mutations within known resistance genes or drug targets (e.g., specific SNPs in gyrA or ribosomal proteins) that lead to resistance, even if the overall sequence is highly similar to a susceptible one.

Majority of the resistance genes detected code for antibiotic efflux pumps. Formation of efflux pumps is one of the six major mechanisms of resistance to antibiotics: (a) limiting the uptake of the antibiotic by altering the cellular permeability, (b) modifying the antibiotic target site, (c) target site protection, (d) enzymatic inactivating of the antibiotic, (e) active antibiotic efflux pumps, and (f) target bypass (Gaurav *et al.*, 2023).

On the other hand, Tables 3 & 4 highlight AMR genes based on protein variant models and protein overexpression models. PVM detected mutations that confer resistance to different antibiotics, in particular, the gene *E. coli* EF-Tu. Moreover, mutations in the marR gene and *soxR* and *soxS* further exacerbated its ability to expel antibiotics in particular, Fluoroquinolone, Cephalosporin, Glycylcycline, Penicillin beta-lactam, Tetracycline, Rifamycin, Phenicol, Disinfecting agents and antiseptics. Moreover, these mutations and overexpression confer alteration to antibiotic targets, rendering antibiotic treatments ineffective.

The *marR* encodes a repressor of the *marA* gene. Mutations in *marR* (e.g., Y137H, G103S) prevent it from binding to the operator, leading to overexpression of *marA*. *Mar A* acts as a global regulator that activates efflux pumps and reduces porin expression (*OmpF*), decreasing antibiotic uptake (Webber and Piddock, 2001). These mutations (including ramR and acr R) are known to cause the upregulation of *acrB*, a core component of efflux systems in APEC. These mutations can occur in clinical isolates, allowing the bacteria to survive, particularly under oxidative stress and in the presence of antibiotics (FàBrega *et al.*, 2009).

Table 4. Antimicrobial resistant genes annotated based on protein overexpression model

ARO Term	SNP	AMR gene family	Drug class	Resistance mechanism	Percent (%) identity of matching regions	RGI criteria
E. coli AcrAB-TolC with *marR* mutations conferring resistance to ciprofloxacin and tetracycline	Y137H, G103S	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone, Cephalosporin, Glycylcycline, Penicillin beta-lactam, Tetracycline, Rifamycin, Phenicol, Disinfecting agents and antiseptics	Antibiotic target alteration, antibiotic efflux	97.6	Strict
E. coli soxR with mutation conferring antibiotic resistance		ATP-binding cassette (ABC) antibiotic efflux pump, Major facilitator superfamily (MFS) antibiotic efflux pump, Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone, Cephalosporin, Glycylcycline, Penicillin beta-lactam, Tetracycline, Rifamycin, Phenicol, Disinfecting agents and antiseptics	Antibiotic target alteration, antibiotic efflux	98.7	Strict
E. coli soxS with mutation conferring antibiotic resistance		ATP-binding cassette (ABC) antibiotic efflux pump, Major facilitator superfamily (MFS) antibiotic efflux pump, Resistance-nodulation-cell division (RND) antibiotic efflux pump, General Bacterial Porin with reduced permeability to beta-lactams	Fluoroquinolone, Monobactam, Carbapenem, Cephalosporin, Glycylcycline, Penicillin beta-lactam, Tetracycline, Rifamycin, Phenicol, Disinfecting agents and antiseptics	Antibiotic target alteration, antibiotic efflux, reduced permeability to antibiotics	100	Strict
baeR		Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminoglycoside, Aminocoumarin antibiotics	Antibiotic efflux	96.23	Strict

POM detects mutations in regulatory genes that result in the overexpression of efflux complexes (e.g., mutations in mexR leading to overexpression of mexAB-oprM). Unlike PVMs which focus on the target enzyme, POMs focus on the regulatory control of expression levels.

The results of the resistance gene identifier highlight a significant concern on the occurrence and prevalence of AMR avian pathogenic E. coli in the poultry industry. This is highly concerning in particular, with regards to biosecurity measures employed at the farm-level operations and in the context of both animal health and food safety.

Avian Pathogenic *Escherichia coli* (APEC) causes avian colibacillosis, a major, often fatal, systemic disease in poultry, characterized by airsacculitis, perihepatitis, and pericarditis. APEC acts as both a primary pathogen and an opportunistic secondary invader, significantly impacting poultry welfare, egg production, and meat quality (Nawaz *et al.*, 2024,

Kathayat *et al.*, 2021). Furthermore, antimicrobial resistance APEC) within poultry production systems is a major public health and food safety crisis, fueled by excessive and indiscriminate antibiotic use. APEC strains often exhibit high levels of multidrug resistance, threatening both poultry health through increased mortality and food safety due to the transmission of resistant pathogens to humans (Tilahun and Ofa, 2026a, Nawaz *et al.*, 2024).

Farm-level practices and data correlation

Based on the responses gathered from the four sampling sites, all farms do not use antibiotics, nor include additives in their feeding practice. Moreover, all farms also conduct several biosecurity measures in their overall farm operations such as having a restricted access to poultry area, vehicle and equipment sanitation, regular health checks for birds, and quarantine procedures for new stock. All the sampled farms also implement vaccination programs for their respective broilers. The results of the AST assay however, present a clear picture of a potential oversight on these currently employed practices in the farms. With almost all tested isolates across all farms throughout the collection period recorded resistant to at least two classes of antibiotics, it is therefore imperative to investigate potentially calibrating and validating biosecurity measures, feeding practices, and health management in the poultry farms.

Furthermore, several factors can still play in which may explain why resistant bacteria are still prevalent in the farms despite the observed above-mentioned protocols. Plasmid-mediated horizontal gene transfer among gut bacteria in broiler chickens is a major mechanism driving the spread of antimicrobial resistance. By acquiring resistance-bearing plasmids from commensals such as *Escherichia coli*, formerly susceptible pathogens like *Salmonella* can become drug-resistant (Oladeinde *et al.*, 2021, Vinayamohan *et al.*, 2022). Moreover, human and vehicle movement are also major pathways for spreading antimicrobial resistance within broiler production, acting as key routes that transport antimicrobial-resistant bacteria and antimicrobial resistance genes (ARG) across farm environments, between facilities, and into slaughterhouses. Transport, especially in open crates disperses contaminated feces and aerosols, and weak foot-traffic biosecurity (for example, no dedicated footwear) permits the mechanical transfer of bacteria between sites (Jung *et al.*, 2023, Hazards *et al.*, 2022).

Other factors that may be considered as contributors to the spread of AMR in broiler operations include improper litter management, vector transmission, and high stocking density (Farkas *et al.*, 2025). Environmental factors and seasonality are also contributory factors where higher rates of AMR have been associated with specific locations (high-density, hilly/mountainous areas) and seasons (winter/spring). Cold-humid environments can also encourage the persistence of resistant microbes (Wang *et al.*, 2022).

To address the problem, farms should move beyond policy statements to targeted, evidence-based actions: systematically validate and audit on-farm biosecurity with particular emphasis on transport protocols, crate sanitation, and foot-traffic controls, review breeder and supplier histories and reinforce quarantine and sourcing controls, improve litter and waste management practices, reduce stocking density when possible, and implement robust vector control; and strengthen training and documented compliance for farm workers and transport crews (dedicated footwear/clothing, hand hygiene, vehicle sanitation). Surveillance findings should be used to design focused operational changes (for example, cleaning of identified hot spots and modified transport procedures) and to inform a longer-term antimicrobial stewardship and risk-reduction plan.

In conclusion, the AST results indicate that AMR is already established in these farm environments despite the absence of declared on-farm antibiotic use; therefore, a coordinated program of enhanced surveillance, molecular investigation of resistance mechanisms (especially plasmid-mediated transfer),

rigorous auditing of biosecurity in practice (not just on paper), and targeted operational interventions, particularly around transport, litter/waste handling, and human/vehicle movement is essential to identify transmission routes and reduce the burden of resistant bacteria in these broiler farms.

CONCLUSION

The following conclusions are drawn based on the results of the analysis of all cloacal swab samples collected through August to October 2025. Using standard culture and biochemical methods, isolates from cloacal swabs were predominantly Escherichia coli and *Salmonella* spp. recovered from registered broiler farms in Solana, Peñablanca, and Pamplona. Kirby-Bauer susceptibility testing showed nearly all isolates were resistant to at least two antibiotic classes, with especially high resistance to tetracycline and trimethoprim–sulfamethoxazole and notable resistance to ciprofloxacin and certain cephalosporins. The antimicrobial resistance patterns did not correlate with reported absence of on-farm antibiotic use or routine biosecurity/vaccination measures. MDR isolates were widespread across farms suggesting alternative transmission routes (e.g., plasmid transfer, transport-related contamination, litter/waste, human/vehicle movement). Furthermore, whole-genome sequencing of a representative MDR isolate (*E. coli*) revealed a complex resistome and virulence repertoire, including plasmid-associated resistance genes and virulence markers (e.g., *cvaC*), multiple efflux pump components, and regulatory mutations (e.g., *marR*, *soxRS*) consistent with plasmid-mediated spread and chromosomal adaptation. These findings establish a regional baseline that demonstrates entrenched AMR in broiler operations and supports the need for routine molecular surveillance, targeted biosecurity audits (particularly transport and waste management), and strengthened antimicrobial stewardship policies.

RECOMMENDATIONS

The following are recommended in order to deepen and widen the methodological rigor of this study. First, expand and deepen sampling design by conducting longitudinal, multi-season sampling across more farms and supply-chain points (breeders, hatcheries, transport crates, slaughterhouses) and include environmental (litter, water, feed), human, and vehicle swabs to better identify reservoirs and transmission hotspots. Scale up molecular investigations by performing whole-genome sequencing on multiple isolates per farm, plasmid replicon/typing and conjugation assays, and shotgun metagenomics of gut and environmental samples to resolve resistome/mobilome dynamics and horizontal-transfer events. Furthermore, test targeted interventions experimentally. Implement and evaluate controlled before–and after or randomized interventions (improved transport/crate sanitation, dedicated footwear protocols, enhanced litter management, reduced stocking density) with microbiological endpoints to establish causal impact on AMR prevalence. Lastly, standardize metadata, build local capacity, and translate findings. Collect harmonized farm management and biosecurity metadata, adopt standardized AST/WGS reporting, invest in local laboratory and training capacity for routine surveillance, and engage policymakers/stakeholders to convert evidence into antimicrobial stewardship and biosecurity policy.

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