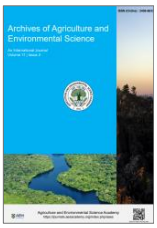
Archives of Agriculture and Environmental Science 11(3): 344–351 (2026)
https://doi.org/10.26832/24566632.2026.1103011

e-ISSN: 2456-6632

This content is available online at AESA

Archives of Agriculture and Environmental Science

Journal homepage: journals.aesacademy.org/index.php/aaes



Original Research Article

Minimum inhibitory concentration of antibiotics to salmonella of poultry origin in selected regions of Nepal

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ARTICLE HISTORY

Received: 25 May 2026
Revised: 01 August 2026
Accepted: 07 August 2026
Published: 25 September 2026

KEYWORDS

Antimicrobial resistance
Minimum inhibitory concentration
Poultry
Salmonella

ABSTRACT

Antimicrobial resistance (AMR) in Salmonella from poultry is an emerging threat to animal and public health, yet quantitative minimum inhibitory concentration (MIC) data from Nepal remain scarce. This study aimed to determine the MIC profiles of Salmonella isolates from layer poultry farms and identify farm-level factors associated with multidrug resistance (MDR). A cross-sectional survey was conducted between March 2020 and March 2023, during which 1,080 cloacal swab and litter samples were collected from 180 layer farms across Kaski, Chitwan, Makawanpur, and Dang districts. Salmonella was detected in 65 samples (6.01%) by culture and PCR targeting the invA gene, and MICs for 15 antimicrobials were determined using E-test strips and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values. High resistance was observed to tetracycline (87.69%), doxycycline (81.54%), ciprofloxacin (78.46%), norfloxacin (69.23%), amoxicillin (53.85%), and chloramphenicol (50.77%), whereas amikacin exhibited the lowest resistance (10.77%). Correlation analysis revealed co-resistance among β-lactam and tetracycline antibiotics, indicating antimicrobial selection pressure within poultry production systems. Poisson regression identified the objective of antibiotic use (IRR = 0.289, p = 0.029), dose measurement practices (IRR = 0.619, p = 0.021), and outcomes of the last antibiotic treatment episode (IRR = 0.284, p = 0.011) as significant predictors of MDR. This study provides the first comprehensive MIC-based evidence of Salmonella AMR in Nepalese layer poultry, emphasizing the need for routine MIC-based surveillance, strengthened antimicrobial stewardship, and evidence-based antibiotic use policies to curb MDR and protect food safety within a One Health framework.

Citation of this article: Adhikari, J., Thapaliya, S., Munakarmi, R., Acharya, P., Basnet, H. B., & Paudyal, N. (2026). Minimum inhibitory concentration of antibiotics to salmonella of poultry origin in selected regions of Nepal. *Archives of Agriculture and Environmental Science*, *11*(3), 344–351. https://doi.org/10.26832/24566632.2026.1103011

INTRODUCTION

Both human and veterinary medicine are increasingly at risk from the rise of antimicrobial-resistant Salmonella strains, especially multidrug-resistant (MDR) isolates, which reduce the available treatment options and increase disease burden (Acharya & Wilson, 2019). Infected flocks may experience increased morbidity and mortality rates; up to 20% have been reported (Pattison *et al.*, 2008). In the Nepalese context, earlier studies have shown that among the total analyzed cloacal swab samples from layer chickens, 48% were positive for Salmonella, with 45% of the organisms multidrug-resistant.

Another study showed that Salmonella isolates exhibited high levels of multi drugs resistance to those of Highest Priority Critically Important Antibiotics (HPCIA) such as tetracycline (77.5%), followed by ciprofloxacin (both 70%), doxycycline (67.5%), norfloxacin (62.5%), erythromycin (60%), amoxicillin (52.5%), ampicillin (40%), enrofloxacin (30%), cefotaxime (25%), and chloramphenicol (22.5%) (Timilsina, 2023). Antibiotic use in Nepalese poultry is largely unmonitored, with practices such as unsupervised administration, lack of withdrawal periods, and widespread use of human-critical antibiotics (Nelson *et al.*, 2020). This type of indiscriminate antibiotic use, often without veterinary prescription, can largely contribute to the emergence of multidrug-resistant (MDR) strains of Salmonella (Acharya & Wilson, 2019).

Salmonella is one of the commonly studied organisms in terms of prevalence and antibiogram in Nepal (Timilsina, 2023). Most of these antibiogram studies employ qualitative disc diffusion, with very few studies reporting the quantitative Minimum Inhibitory Concentrations (MIC) of common antibiotics against *Salmonella*. This is even more stark in the poultry sector, where, despite its prevalence in all types of commercial chicken, there is a dearth of research on the MIC of antibiotics. The paucity of data on the MICs of commonly used antibiotics generally impedes rational therapeutic decisions (Sallam, 2025). Furthermore, evidence on farm-level practices associated with multidrug-resistant (MDR) *Salmonella* in Nepalese layer poultry production is limited. This lack of quantitative susceptibility data and epidemiological evidence restricts evidence-based antimicrobial stewardship and informed treatment decisions. This study minimizes the critical gap by assessing the MICs of commonly used antibiotics against *Salmonella* isolated from layer poultry in Nepal. It also discusses the farmer practices associated with the occurrence of multidrug resistance at the farm level. The findings are expected to provide baseline evidence for strengthening antimicrobial stewardship, guiding prudent antibiotic use, and supporting national AMR surveillance and food safety programs.

MATERIALS AND METHODS

Study design and area

A cross-sectional study was conducted from March 2020 to March 2023 in the four major poultry-producing districts of Nepal: Kaski, Chitwan, Makawanpur, and Dang. These districts were purposively selected due to their high density of commercial layer poultry farms (Figure 1). Farms that had been in operation for at least a year and had a flock size of between 1000 and 5000 birds were eligible and were purposively selected to ensure representation of small- to medium-scale commercial production systems. In this study, the epidemiological unit was defined as the flock, where each flock comprised birds of similar age and management conditions (EFSA, 2018). The birds were apparently healthy with no typical clinical symptoms as evaluated during the sampling visit to the farm premises. Within each selected farm, multiple samples were collected, resulting in a clustered sampling structure.

The initial sample size was calculated using Thrusfield (2018) for estimating prevalence in cross-sectional studies:

$$n=Z^2 \times p\,(1-p)/d^2 n$$

Where Z = 1.96 at 95% confidence level, p = expected prevalence, and d = desired precision (0.05).

Because the study employed a cluster sampling design, the calculated sample size was adjusted using a design effect (DEFF) to account for intra-cluster correlation:

$$n_{adj}=n\times(1+(m-1)\times \text{ICC})$$

Where m represents the average number of samples collected per farm, and ICC is the intra-cluster correlation coefficient. Assuming an average cluster size (m) of 6 samples per farm (five pooled cloacal samples and one litter sample) and an ICC of 0.1, the design effect was estimated to be 1.5. Accordingly, the final adjusted sample size was 1080 samples, ensuring adequate statistical power and representativeness.

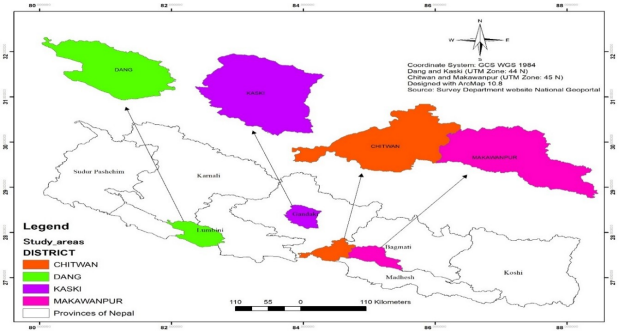

Figure 1. *Study area shown within the map of Nepal.*

A total of 180 farms were included in the study, 45 in Makawanpur, 30 in Chitwan, 46 in Dang, and 59 in Kaski, as described in our previous study (Adhikari *et al.*, 2025). From these farms, 1,080 samples were collected, consisting of: 900 cloacal swab samples: within each farm, birds were stratified by age group, and five birds per group were randomly selected; their cloacal swabs were pooled to form one composite sample per group and another 180 litter samples: five subsamples collected during a transect walk inside each poultry shed were pooled to form one composite litter sample per farm. This multistage cluster sampling strategy enhanced logistical practicality while maintaining the representativeness of the study population.

Sample collection and processing

Cloacal and litter samples were collected aseptically to prevent cross-contamination between farms. Sterile sampling materials, including swabs, shoe covers, bags, and disposable gloves, were used during collection. Cloacal samples were collected from the individual bird and put in a tube separately. For litter sampling, a sterile drag swab pre-moistened with buffered peptone water (BPW) was tied to a long thread and dragged diagonally across the shed floor. Five drag swabs from the same pen were pooled into one composite drag swab sample. The collected swab was then placed in a 50 mL Falcon tube containing 25 mL of BPW and transported to the laboratory in an icebox. Samples pre-enriched in BPW were incubated at 35±2°C for >18 hours. Subsequently, 1 mL of inoculum from the pre-enriched broth was transferred to 9 mL of Rappaport-Vassiliadis (RV10) enrichment broth, followed by incubation at 42°C for 24 hours to enhance Salmonella growth before sub-culturing.

The turbid broth from the enriched media was streaked onto Xylose Lysine Deoxycholate (XLD) agar and MacConkey's agar plates, then incubated at 35±2°C for 24–48 hours. Colonies exhibiting typical Salmonella characteristics (translucent red colonies with black centers or translucent red colonies on XLD agar, and colourless, translucent-to-opaque colonies with irregular edges on MacConkey's agar were presumptively considered Salmonella (Sohail *et al.*, 2021), followed by standard

biochemical tests (TSI, SIM, urease and citrate utilization). Molecular confirmation was performed using PCR targeting the *inv*A gene, a widely accepted genus-specific marker for Salmonella (WOAH, 2024). This combined phenotypic and molecular approach improved specificity and reduced false identification.

Briefly, the primer sequence was 139-F: GTG AAA TTA TCG CCA CGT TCG GGC AA & 141-R: TCA TCG CAC CGT CAA AGG AAC C. The cycling conditions used were initial denaturation at 95°C for 5 minutes; denaturation at 95°C for 30 seconds; annealing at 60°C for 30 seconds; extension at 72°C for 45 seconds, cycled 35 times and a final extension at 72°C for 10 minutes. The amplicons of 294 bp were visualized after staining with GelRed in 1.5% agarose electrophoresis. Once confirmed as *Salmonella*, the confirmed isolates were subjected to the MIC test. Further characterization to the serovar level was performed as recommended by Zhu *et al.* (2015) using a multiplex PCR to identify common poultry serovars, namely *Kentucky*, Heidelberg, Enteritidis and Group 2 (comprised of other *S. enterica* serovars such as Schwarzengrund, Typhimurium, Bareilly).

Minimum inhibitory concentrations (MICs) were determined using commercially available gradient diffusion strips (E-test method). A fresh bacterial suspension equivalent to 0.5 McFarland standard was prepared in Muller Hinton Broth and uniformly inoculated onto Mueller-Hinton agar plates using a sterile swab to obtain a confluent lawn. E-test strips impregnated with predefined antibiotic gradients were aseptically placed on the agar surface inoculated with the test microorganism, allowing antibiotic diffusion and lawn formation. Plates were incubated at 35 ± 2°C for 16–20 hours. MIC values were read at the point where the ellipse of inhibition intersected the scale on the strip (Table 1). This is a validated method and is widely recognized for its reliability, ease of use, and robust results (EUCAST, 2023). Interpretation of MIC values into susceptible and non-wild type categories was performed using epidemiological cut-off values (ECOFFs) as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2023). All tests were performed in duplicate, and any discrepant results were re-evaluated. To ensure accuracy and reproducibility of MIC results, quality control was performed using reference strain *Salmonella* Typhimurium ATCC 14028 in accordance with EUCAST guidelines. MIC values obtained for control strains were verified to fall within the acceptable reference ranges. Each PCR run also included a positive control (Salmonella Typhimurium ATCC 14028), a negative control (non-Salmonella bacterial DNA), and a no-template control (NTC) to ensure specificity and absence of contamination.

Table 1. Quality control ranges for MIC testing using Salmonella Typhimurium ATCC 14028 (EUCAST guidelines).

S. No.	Antibiotic	Class	QC MIC Range (μg/mL)	Observed QC MIC (μg/mL)	Status
1	Gentamicin	Aminoglycoside	0.25 – 1	0.5	Within range
2	Amikacin	Aminoglycoside	0.5 – 2	1	Within range
3	Streptomycin	Aminoglycoside	4 – 16	8	Within range
4	Amoxicillin	Aminopenicillin	2 – 8	4	Within range
5	Ampicillin	Aminopenicillin	2 – 8	4	Within range
6	Ceftriaxone	Cephalosporin	0.03 – 0.12	0.06	Within range
7	Ceftazidime	Cephalosporin	0.12 – 0.5	0.25	Within range
8	Azithromycin	Macrolide	4 – 16	8	Within range
9	Chloramphenicol	Phenicol	4 – 16	8	Within range
10	Ciprofloxacin	Fluoroquinolone	0.004 – 0.015	0.008	Within range
11	Enrofloxacin	Fluoroquinolone	0.015 – 0.06	0.03	Within range
12	Levofloxacin	Fluoroquinolone	0.015 – 0.06	0.03	Within range
13	Norfloxacin	Fluoroquinolone	0.03 – 0.12	0.06	Within range
14	Tetracycline	Tetracycline	2 – 8	4	Within range
15	Doxycycline	Tetracycline	1 – 4	2	Within range

Limitations

Farms were selected purposively based on operational size and accessibility, which may introduce selection bias. However, this approach was justified to ensure inclusion of active commercial farms with consistent production systems. The limitation of non-random sampling is acknowledged.

Data analysis

Data were entered into Microsoft Excel and analyzed using SPSS version 28. Descriptive statistics were used to summarize MIC distributions and resistance patterns. Multidrug resistance (MDR) was defined as resistance to ≥3 antimicrobial classes, which were presented in a squastogram. Pairwise correlations between antimicrobial resistance profiles were assessed using Spearman's rank correlation coefficient. To account for multiple comparisons across antibiotic pairs, p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) correction. Adjusted p-values (q-values) < 0.05 were considered statistically significant.

A Poisson regression model with robust standard errors was used to estimate incidence rate ratios (IRR) for factors associated with multidrug resistance (MDR). Over-dispersion was assessed using the Pearson chi-square test, and no significant over-dispersion was detected. Variables with p < 0.20 in univariable analysis were included in the multivariable model.

RESULTS AND DISCUSSION

Salmonella prevalence

A total of 65 samples tested positive for *Salmonella*, representing a 6.01% (65/1080) sample prevalence by PCR methods focusing on the *inv*A gene typical of the genus (Figure 2). Further characterization to serovar level was performed, and identified serovars included Kentucky, Heidelberg, Enteritidis and Group 2, which comprised other *S. enterica* serovars such as Schwarzengrund, Typhimurium, Bareilly.

MIC Evaluation

The squashtogram (Table 2) shows MIC values of commonly available antibiotics grouped by their classes for salmonella isolates using epidemiological cut-off values (ECOFF) that were tested in the assay. The initial row presents a two-fold serial dilution of the antibiotics, spanning concentrations from 0.002 to 256 μg/mL, thereby offering a comprehensive perspective on their antimicrobial efficacy. The bold vertical line in each row delineates the epidemiological cut-off levels set forth by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), separating the susceptible population from the resistant population. The number of isolates in each MIC concentration is expressed as a percentage. This squashtogram showed that among these 15 molecules tested, classical molecules such as tetracycline, doxycycline, ciprofloxacin, norfloxacin, amoxicillin and chloramphenicol are generally more resistant, with the majority of isolates showing MICs greater than the cut-off values. The high levels of resistance were observed for tetracycline (87.69%) and doxycycline (81.54%), with MIC values frequently exceeding 256 μg/mL, whereas the lowest resistance was observed for amikacin (10.77%). It is of great importance that the majority of the isolates have MICs higher than the cut-off value. In this study, *Salmonella* isolates were tested for MIC against 15 antibiotics using the E-strip. This study revealed that the highest number of isolates were resistant to first-line antibiotics such as tetracycline (87.69%), doxycycline (81.54%), ciprofloxacin (78.46%), norfloxacin (69.23%), amoxicillin (53.85%), chloramphenicol (50.77%) and ampicillin (47.69%). Similarly, streptomycin (30.77%), enrofloxacin (30.77%), ceftriaxone (27.69%), gentamicin (24.62%), ceftazidime (23.08%), levofloxacin (23.08%), azithromycin (15.38%) and amikacin (10.77%). These susceptibility patterns suggest that limited use and effective control by farmers of these compounds could have a positive influence on AMR (WHO, 2023).

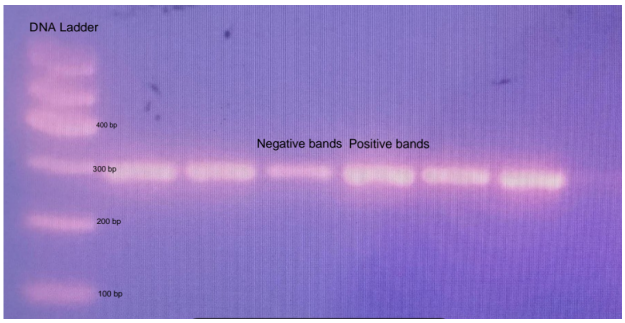

Figure 2. *Representative image of gel electrophoresis for Salmonella (positive bands at 294 bp). The ladder in the far-left lane is 100 bp.*

Correlation between the resistance

Pairwise correlations between antimicrobial resistance profiles were assessed using Spearman's rank correlation coefficient, with p-values adjusted using the Benjamini–Hochberg false discovery rate (FDR). As shown in Figure 3, significant positive correlations were observed between ampicillin and amoxicillin ($r = 0.72$, $q < 0.05$) and between tetracycline and doxycycline ($r = 0.72$, $q < 0.05$). Moderate positive correlations were also observed among fluoroquinolones, including ciprofloxacin, norfloxacin, enrofloxacin, and levofloxacin. In contrast, ceftazidime showed weak negative correlations with ampicillin ($r = -0.58$) and amoxicillin ($r = -0.46$).

Drivers of multidrug resistance (MDR)

Poisson regression analysis revealed that objective of antibiotic usage (IRR=0.289, p=0.029), measurement of dose (IRR=0.69, p=0.021), and outcome of last episode of antibiotic use (IRR=0.284, p=0.011) significantly influence MDR at the farm level (Table 3). This study provides one of the first comprehensive quantitative minimum inhibitory concentration (MIC) profiles of *Salmonella* isolates recovered from commercial layer poultry farms in Nepal using EUCAST epidemiological cut-off values (ECOFFs). Despite the large number of samples collected, the relatively low recovery rate of Salmonella may be attributed to intermittent bacterial shedding, prior antimicrobial use at farm level, and limitations in culture sensitivity. Numerous reports of Salmonella in Nepalese poultry have indicated varying levels of antibiotic resistance. A study on non-typhoidal *Salmonella* showed a prevalence of 9% at various farms in Chitwan (Bhatta *et al.*, 2025). Another study conducted in five different provinces of Nepal reported a higher prevalence of 48% *Salmonella* spp. from cloacal swabs of layer flocks. These variances in the prevalence of salmonellae in poultry depict its dynamic nature and are not uniform throughout multiple regions of Nepal. This variation may be due to the different sampling methods, testing procedures, and the season during which the studies were performed.

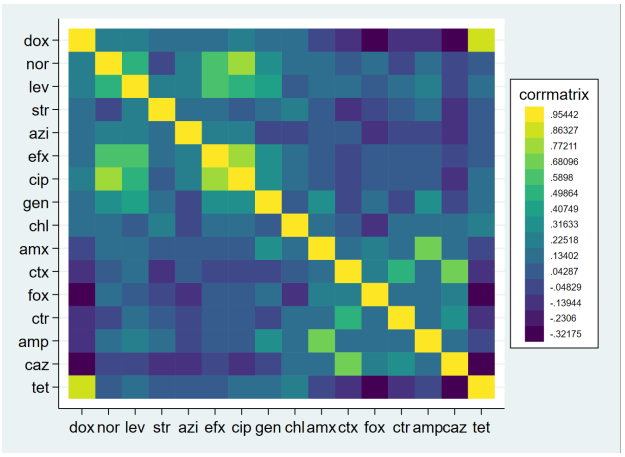

Figure 3. *Correlation heat map of antimicrobial resistance among Salmonella isolates (n = 65), (tet: -tetracycline, dox: -doxycycline, nor: -norfloxacin, lev: -levofloxacin, str: -streptomycin, azi:-azithromycin, gen:-gentamicin, efx:-enrofloxacin, cip:-ciprofloxacin, chl:-chloramphenicol, amx:-amoxicillin, ct:-ceftriaxone, amp:-ampicillin, caz:-ceftazidime) and plotted on heat map.*

Similarly, our study also isolated Kentucky, Heidelberg, and Enteritidis as the most common serovars. The serovars of the Serotype 2 were not identified due to limited resources but these also could be another forum for the future studies. The most notable finding was the exceptionally high MICs observed for tetracycline and doxycycline, with most isolates exceeding the ECOFF threshold, indicating widespread acquisition of resistance determinants within the poultry production system. Similar high resistance to tetracyclines has been reported among poultry-associated *Salmonella* in Nepal, although most previous studies relied on qualitative susceptibility testing rather than MIC determination. Our study showed significantly high levels of resistance for tetracycline (87.69%) and doxycycline (81.54%), with MIC values frequently exceeding 256 μg/mL.

Table 2. Squashtogram showing MIC values of commonly available antibiotics grouped by their classes for salmonella isolates using epidemiological cut-off values (ECOFF).

S.N.	Antibiotic class	Molecule	Resistant	Concentration of the MIC Strip (μg/ml)																			
				0.002	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>256
1	Amino-glycoside	Gentamicin (GEN)	24.62%			1.54		4.62	4.62	15.38	32.31	1.54	7.69	7.69	7.69	9.23	1.54	1.54		4.62
		Amikacin (AK)	10.77%			1.54					6.15	6.15	53.85	21.54	4.62	6.15				
		Streptomycin (STR)	30.77%							1.54	3.08	4.62	27.69	15.38	1.54	15.38	15.38		6.15	9.23
2	Beta-lactamase inhibitors	Amoxicillin (AMX)	53.85%			3.08	1.54	1.54	4.62	21.54	12.31		1.54			9.23	9.23	6.15		29.23
3	Cephems	Ceftriaxone (CRT)	27.69%	0.00	1.54	16.92	7.69	10.77	1.54		4.62	3.08	4.62	1.54	7.69	1.54	12.31			
		Ceftazidime (CAZ)	23.08%		1.54	1.54	10.77	46.15	9.23	1.54				6.15	3.08	1.54	1.54			16.92
4	Macrolide	Azithromycin (AZI)	15.38%		1.54			1.54	4.62	4.62	3.08	3.08	9.23	13.85	32.31	9.23	3.08		12.31	
5	Penicillin	Ampicillin (AMP)	47.69%					1.54		3.08	6.15	12.31	18.46	4.62	3.08	1.54	4.62	7.69		33.85
6	Phenicol	Chloramphenicol (CHL)	50.77%								1.54	1.54	13.85	16.92	15.38	12.31	21.54	4.62	1.54	10.77
7	Quinolone	Enrofloxacin (EFX)	30.77%	3.08	1.54	6.15	7.69	10.77	6.15	4.62	29.23	4.62	9.23	6.15	6.15		4.62			
		Ciprofloxacin (CIP)	78.46%					6.15	12.31		3.08	6.15	21.54	10.77	7.69	9.23	6.15			1.54
		Levofloxacin (LEV)	23.08%	3.08	1.54	9.23	6.15	10.77	9.23	3.08	3.08	12.31	18.46	13.85	6.15		3.08			
		Norfloxacin (NOR)	69.23%				3.08	6.15	12.31	9.23		1.54	6.15	23.08	10.77	9.23	9.23	6.15		3.08
8	Tetracycline	Doxycycline (DOX)	81.54%			3.08		1.54	1.54		6.15		3.08	3.08	1.54	1.54	20.00	3.08		55.38
		Tetracycline (TET)	87.69%			1.54					1.54	3.08	3.08	3.08	3.08		1.54		1.54	81.54

Table 3. Poisson Regression Analysis of Driving of Multiple Drug Resistance (MDR)

Multiple Drug Resistance	Log- IRR	St. Err.	t-value	p-value	95% Con.	Interval	Sig
Objective of antibiotic use	.289	.133	2.18	.029	.029	.55	**
Measurement of dose	.619	.267	2.32	.021	.095	1.143	**
Outcome of the last episode of antibiotic use	.284	.112	2.54	.011	.065	.502	**
Feed type	.002	.133	0.01	.989	-.259	.262	
Constant	1.216	.271	4.48	0	.684	1.747	***
Mean dependent var		8.537		SD dependent var		3.017	
Pseudo r-squared		0.156		Number of obs		41	
Chi-square		32.647		Prob > chi2		0.000	
Akaike crit. (AIC)		186.173		Bayesian crit. (BIC)		194.741	

*** $p<.01$, ** $p<.05$, * $p<.1$

Nelson *et al.* (2020) found that 97% of Salmonella isolates from chicken samples in Chitwan displayed tetracycline resistance, while 84.0% of poultry meat samples from Bharatpur, Nepal (Shrestha *et al.*, 2017) which was similar to our findings. However, a study on salmonella isolated from layer poultry farms in Kaski district, Nepal also presented that 66% of isolates were resistant to tetracycline, and 55% of isolates were resistant to doxycycline (Nepali *et al.*, 2025). The rising prevalence of resistance to tetracyclines, along with the widespread use of doxycycline in Nepalese poultry farms (Adhikari *et al.*, 2025), is concerning, especially given its shared resistance mechanism with other tetracycline-class antibiotics.

Similarly, the low resistance to amikacin in our study (10.77%) is consistent with that of Acharya & Wilson (2019), who reported that 7% of amikacin was resistant to Salmonella in broiler flocks and 5% was resistant in layer flocks. This low resistance to aminoglycosides further supports the role of controlled antibiotic deployment, as injectable drugs like amikacin are seldom used in poultry due to logistical challenges. Moreover, in this study, fluoroquinolones with MIC values of 0.125-256 μg/mL, such as ciprofloxacin (78.46%), norfloxacin (69.23%), enrofloxacin (44%) and levofloxacin (23.08%), showcased high resistance to Salmonella isolates. Some findings show that 40% of the qrnS (64/157) gene was detected in Salmonella isolates that were resistant to ciprofloxacin in Nepal (Nepali *et al.*, 2025). Another study reports on the antimicrobial resistance of Salmonella isolates, showing that the Salmonella isolates were highly resistant to enrofloxacin (100%), followed by ciprofloxacin (36.3%), tetracycline (27.3%), and chloramphenicol (18.2%) (Dhakal *et al.*, 2016). The high resistance rates of antibiotics shown by this study correspond with antimicrobials routinely utilized in the visited farms. Drug misuse, over-the-counter antibiotic sales, and the continuous and widespread use of antibiotics in poultry farms can all account for escalating the prevalence of drug resistance (Upadhyaya *et al.*, 2020). Consequently, resistance to clinically significant classes of antimicrobials will restrict therapy options and result in elevated treatment expenses. Therefore, it is of urgency that the responsible authorities, stakeholders and farmers be concerned about the meticulous and deliberative use of these molecules.

The pairwise correlation analysis of antimicrobial resistance profiles of drugs in this study showed distinct co-resistance patterns among Salmonella isolates, suggesting shared resistance mechanisms and common selection pressures across different antibiotic classes. A strong positive correlation between ampicillin and amoxicillin indicates a high level of co-resistance likely driven by β-lactamase-mediated mechanisms. Both are β-lactam antibiotics of the penicillin group, and similar plasmid-borne genes often encode resistance (Alzahrani *et al.*, 2023). A similarly strong correlation was observed between tetracycline and doxycycline, which is not surprising given that both belong to the tetracycline class and are affected by similar efflux pump mechanisms such as tet(A) and tet(B) genes. Antimicrobial-resistant non-typhoidal Salmonella enterica from Chitwan, Nepal documents high tet(A) prevalence (80%) in poultry isolates, with mobile genetic elements implicated in gene dissemination (Nelson *et al.*, 2020). This high level of resistance likely reflects the strong selection pressure exerted by the widespread use of tetracyclines in Nepalese poultry farming. A multi-district study found that tetracyclines were the second most frequently used antibiotic class, accounting for 19% of reported antibiotic applications in layer chicken farms (Adhikari *et al.*, 2025). Consequently, the elevated minimal inhibitory concentrations (MICs) observed for tetracycline can be interpreted as a direct consequence of sustained sub-therapeutic exposure that selects for resistant bacterial populations. Although these associations are well documented, their presence in the current study supports the consistency of the MIC findings and suggests that similar antimicrobial selection pressures continue to operate in Nepalese layer poultry farms. Conversely, weak or negative correlations between unrelated antibiotic classes indicate that resistance to these agents is likely mediated through different genetic mechanisms rather than widespread co-selection.

The consistent co-resistance within this class suggests that continued use of one member may sustain resistance across the group. Fluoroquinolones such as ciprofloxacin, norfloxacin, enrofloxacin, and levofloxacin showed moderate positive correlations. Fluoroquinolone resistance, particularly to ciprofloxacin, may arise through two major mechanisms: chromosomal mutations and plasmid-mediated quinolone resistance (PMQR). Chromosomal mutations in the quinolone resistance-determining regions (QRDR) of genes such as gyrA and parC lead to structural alterations in DNA gyrase and topoisomerase IV, resulting in high-level resistance and elevated MIC values. In contrast, PMQR mechanisms, including qnr genes, aac(6')-Ib-cr, and efflux pumps such as qepA and oqxAB, typically confer low-level resistance but play a critical role in facilitating the selection of additional chromosomal mutations. These plasmid-mediated mechanisms are of particular concern due to their horizontal transfer potential across bacterial populations. The moderate to high resistance observed in this study may therefore reflect a combination of both mechanisms, potentially driven by antimicrobial selection pressure in poultry production systems. Fluoroquinolone resistance among the isolates can be attributed to either overuse in veterinary practice or potential clonal spread or both (Kibwana *et al.*, 2023). In contrast, ceftazidime showed weak or negative correlations with several antibiotics, including ampicillin and amoxicillin, suggesting distinct resistance mechanisms and limited co-selection. This observation may reflect the lower usage frequency of third-generation cephalosporins in poultry or differences in the genetic context of resistance genes, such as extended-spectrum β-lactamase (ESBL) versus narrow-spectrum β-lactamase activity (Mitman *et al.*, 2022). The majority of the other antibiotics studied showed little to no association with azithromycin and chloramphenicol, suggesting that resistance to these agents arises primarily through independent genetic mechanisms rather than co-selection or shared mobile genetic elements. Resistance to azithromycin in poultry-origin Salmonella is frequently mediated by inactivating enzymes like mph(A) or macrolide-specific efflux pumps like macAB, which are usually unrelated to genes that confer resistance to β-lactams, tetracyclines, or fluoroquinolones (Liu *et al.*, 2016; Shrestha *et al.*, 2017). Similarly, some efflux systems (floR) or chloramphenicol acetyltransferase genes (CAT) are also frequently responsible for encoding chloramphenicol resistance (Liu *et al.*, 2016). Therefore, the lack of substantial pairwise correlations is physiologically feasible and suggests that resistance is driven separately by various selection pressures (e.g., farm-level usage of macrolides vs. phenicols).

Conclusion

This study provides vital insights into the antimicrobial susceptibility profile of *Salmonella* isolates recovered from layer poultry farms in Nepal using minimum inhibitory concentra-

tion (MIC) testing. The findings demonstrated a high prevalence of reduced susceptibility to several critically and commonly used antimicrobials, particularly tetracycline, doxycycline, ciprofloxacin, and norfloxacin, while amikacin exhibited the greatest in vitro activity against the isolates. Furthermore, inappropriate antimicrobial use practices such as misuse of antibiotics, inaccurate dose measurement, and prior treatment failure were significantly associated with the occurrence of resistant *Salmonella* isolates. These findings underscore the importance of integrating MIC-based antimicrobial susceptibility testing in routine veterinary diagnostics to support evidence-based therapeutic decisions. Antimicrobial stewardship, prudent use of antimicrobials at farm level and continuous AMR surveillance are critical to mitigate the emergence and dissemination of antimicrobial-resistant *Salmonella* within the poultry production system and to reduce the potential risk to animal and public health.

DECLARATIONS

Author contribution statement: Writing– original draft, data curation, investigation, methodology: J.A.; Writing– review & editing: S.T.; Data curation, methodology, formal analysis, writing– review & editing: R.M., Writing– review & editing, data curation, methodology, visualization: P.A.; Writing– review & editing: H.B.; Conceptualization, funding acquisition, resources, writing– review & editing, project administration, and supervision: N.P. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest: The authors declare that there are no conflicts of interest regarding the publication of this manuscript. The participants provided their oral informed consent to participate in this study.

Ethics approval: This study was conducted in view of the institutional ethical guidelines and does not harm the human participants. The participants provided their consent to participate in this study.

Consent for publication: All co-authors gave their consent to publish this paper in AAES.

Data availability: The data that support the findings of this study are available on request from the corresponding author.

Supplementary data: No supplementary data is available for the paper.

Funding statement: The author(s) declare that financial support was received for the research and/or publication of this article. The funding for the execution of this study was obtained from the Nepal Agricultural Research Council (NARC) for the project entitled “Monitoring of Antibiotic Resistance in Pathogens Isolated from Food Animals (MARPFA),” LMBIS No 542/2019/20-2024/25 to the corresponding author. The funding for the publication of the manuscript was made possible from the personal funds of the first author.

Additional information: No additional information is available for this paper.

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