Research Article:

ANTIBIOGRAM AND GENES CONTRIBUTING TO VIRULENCE IN *E. coli* ISOLATED FROM CLINICAL CASES OF COLIBACILOSIS IN BROILERS

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ABSTRACT

Escherichia coli causes colibacillosis in poultry, leading to significant economic losses through reduced productivity, high mortality, and carcass condemnation. The frequent and often indiscriminate use of antibiotics in poultry production has promoted the emergence of multidrug-resistant (MDR) bacterial strains, complicating their treatment. This study aimed to determine the antibiogram and virulence genes of *E. coli* isolated from clinical cases of colibacillosis. A total of 102 *E. coli* isolates were cultured on Eosin Methylene Blue (EMB) agar and subjected to antibiotic susceptibility testing using the Kirby–Bauer disc diffusion method with 16 antibiotics from 8 different classes. Isolates resistant to three or more antibiotic classes were classified as MDR. Polymerase chain reaction (PCR) was employed to detect six virulence-associated genes, namely *iss, iroN, iucC, cvaC, tsh* and *yjj*. High resistance was observed against enrofloxacin (99%) followed by cefoxitin (90.2%), tetracycline (86.3%), erythromycin (79.4%), amoxycillin (77.5%), doxycycline (76.5%) and ciprofloxacin (75.5%) while greater susceptibility was noted to azithromycin (67.6%), followed by ceftriaxone (65.7%), norfloxacin (53.9%) and amikacin (51%). Overall, 82.3% of isolates were detected to be MDR. Among virulence genes, *yjj* (58.82%) was the most prevalent. Besides, *iss*, *iroN*, *iucC*, *tsh* and *cvaC* were PCR positive in 48.04%, 45.10%, 31.37%, 19.61% and 10.78%, respectively. The coexistence of multiple virulence-associated genes in Avian Pathogenic *Escherichia coli* (APEC) isolates with a high multiple antibiotic resistance (MAR) index indicates high selection pressure on broiler farms. This could be a cause for the antibiotic therapy failure, commonly encountered in the broiler farms of the sampling areas.

Keywords: Antibiotic resistance, broilers, colibacillosis, genes, virulence

INTRODUCTION

The avian pathogenic *E. coli* (APEC) causes a variety of generalised and localised infections, including pericarditis, air sacculitis, perihepatitis, peritonitis, omphalitis, egg peritonitis, coligranuloma, and other extraintestinal infections (De Carli et al., 2015; Kathayat et al., 2021). The APEC are genetically heterogeneous and carry diverse combinations of virulence-associated genes (VAGs) involved in iron acquisition, cytotoxicity, adhesion, invasion and immune evasion (Cummins et al., 2019). The number and combination of virulence-associated

genes determine the pathogenicity of *E. coli* strains. However, the particular genes or their combination which would define Avian Pathogenic *E. coli* is yet poorly defined. Wang et al. (2015) categorised *E. coli* into highly pathogenic strains with at least 8 to 13 virulent genes and 51 different combination patterns from these genes. Further, intermediate pathogenic strains contain at least 5 to 8 virulence-related genes and 36 different combination patterns from the genes. In some publications, the isolates with more than 4 virulent determinants are designated as APEC (Kim et al., 2020). The VAGs present in intestinal *E. coli* pose the risk for extraintestinal survival and pathogenicity to cause systemic diseases (Kemmett et al., 2013). The major virulent genes linked with APEC include *cva/cvi*, *papC*, *hlyE/F*, *iucD*, *iroN*, *irp2*, *iutA*, *iss*, *ompT*, and *tsh* (Cummins et al., 2019; Subedi et al., 2018). Some other studies suggest *hlyF*, *iss*, f*imH*, *iutA*, *iroN*, *ompT*, *kpsMTII*, *iucD*, *traT*, *tsh*, *cvi/cva*, and *aerJ* as predominant virulence genes associated with avian colibacillosis (Silveira et al., 2016; de Oliveira et al., 2020).

This study aims to investigate the prevalence of avian pathogenic *E. coli*, its phenotypic antibiotic resistance and genes contributing to the virulence. The findings will support better management and therapeutic interventions for the treatment of the infection in broilers.

RESEARCH METHODS

This study was conducted on the *E. coli* isolated from clinical cases of colibacillosis in broilers from Dang, Chitwan, Kaski and Makwanpur districts of Nepal during 2023-2025 AD. The organisms were preserved in 15% glycerol brain heart infusion (BHI) broth at -80°C. A subset of 102 selected *E. coli* isolates was revived from the frozen state at the biorepository of the National Animal Health Research Centre, Khumaltar, Lalitpur, Nepal.

Preparation of isolates

Frozen vials were thawed in an incubator, and a loopful of the broth was inoculated into freshly prepared Luria Bertani (LB) broth to incubate overnight at 37℃. After overnight incubation, the broth was then streaked onto an eosin methylene blue (EMB) agar plate using a sterile inoculation loop and incubated at 37℃ for 24 hours to obtain pure isolated colonies. Bacterial colonies with a green metallic sheen were the *E. coli*. Pure colonies were transferred to LB broth via sterile inoculation loop, incubated overnight at 37℃, and then duplicated to use for molecular analysis. One of the duplicates was stored at 4℃ for short-term storage until antimicrobial susceptibility analysis.

Antibiotic susceptibility test

Phenotypic antibiotic resistance was studied via the Kirby-Bauer disc diffusion method as described (Humphries & Simner, 2020). Isolates were tested against 16 commonly used antibiotics, cotrimoxazole (COT, 25 mcg), florfenicol (FFC, 30 mcg), gentamicin (GEN, 10 mcg), ceftriaxone (CTR, 30 mcg), enrofloxacin (EX, 10 mcg), amikacin (AK, 30 mcg), amoxicillin (AMX, 10 mcg), doxycycline (DO, 30 mcg), azithromycin (AZM, 15 mcg), norfloxacin (NX, 10 mcg), tetracycline (TE, 30 mcg), erythromycin (E, 15 mcg), cefoxitin (CX, 30 mcg), chloramphenicol (C, 30 mcg), levofloxacin (LE, 5 mcg), and ciprofloxacin (CIP, 5 mcg). The diameter of inhibition zones was measured (in mm) using a vernier calliper and compared with Clinical and Laboratory Standards Institute breakpoints (CLSI, 2024) to classify as susceptible, intermediate or resistant isolates to corresponding antibiotics.

Multiple antibiotic resistance index

The multiple antibiotic resistance (MAR) index for each isolate was calculated using the AST data by dividing the number of antibiotics to which it was resistant by the total number of antibiotics tested. The MAR index for each isolate was calculated using the formula described by Krumperman (1983) as follows. The MAR Index ranges from 0 (susceptible to all antibiotics) to 1 (resistant to all 16 antibiotics).

$$\text{MAR index} = \frac{\text{Number of antibiotics to which the isolate showed resistance}}{\text{Total number of antibiotics tested}}$$

DNA extraction and PCR

Bacterial genomic DNA was extracted from overnight LB broth cultures using the DNeasy Blood and Tissue Kit (Qiagen®, Cat. No. 69504) according to the manufacturer's instructions. The DNA quality and concentration were assessed using a NanoDrop spectrophotometer, and purity was determined by the A260/A280 ratio. Extracted DNA was eluted in TAE buffer and stored at -20°C until analysis.

Six major virulent genes (including *iucC*, *iroN*, *yjj*, *iss*, *cvaC* and *tsh*) of avian pathogenic *E. coli* were identified using conventional endpoint PCR. Uniplex PCR amplification was done for *iucC*, *iroN* and *yjj* genes, whereas multiplex PCR amplification was done for *iss*, *cvaC* and *tsh* genes. QIAGEN® Multiplex PCR Kit, which contains optimised concentrations of HotStarTaq DNA polymerase and $MgCl_2$, plus dNTPs and an innovative PCR buffer specially developed for multiplex PCR, was used to amplify the *iss*, *cvaC* and *tsh* genes. QIAGEN® Taq PCR Master Mix was used for the uniplex amplification of *iucC*, *iroN*, and *yjj* genes.

For uniplex PCR, the reaction mixture consisted of 12.5µl master-mix, 1µl (10mmol/µl concentration) each of the forward and reverse primer, 3µl of template DNA and 7.5 µl of nuclease-free water, summing up to a final reaction volume of 25µl. For the multiplex PCR, the reaction volume consisted of 12.5µl master-mix, 0.5µl (10mmol/µl concentration) each of the forward and reverse primers for the three genes, 3µl template DNA and 6.5µl of nuclease-free water, summing up to a final reaction volume of 25µl. The primer sequence used in this study is described in Table 1 below.

For amplification of the *iucC*, *yjj*, and *iroN* genes, the uniplex PCR cycling conditions consisted of 94℃ for 4 min followed by 35 cycles of 94℃ for 30 sec, 60℃ for 1 min, 68℃ for 2 min, and a final extension at 72℃ for 7 min. For multiplex PCR (*iss*, *cvaC*, *tsh*), the cycling condition was 94℃ for 3 min followed by 25 cycles of 94℃ for 30 sec, 58℃ for 30 sec, 68℃ for 3 min, and a final extension at 72℃ for 10 min. Finally, 5 µl PCR reaction mixture was loaded onto a 1.2% agarose gel and electrophoresed for 1 hour at 96V, 500mA in TAE buffer containing 0.5% GelRed DNA dye. The gel after electrophoresis was transferred to a gel documentation system (Gel Imager), visualised, and interpreted.

Table 1. Primers used for virulent markers for APEC

Target Gene	Primer Sequence (5'-3')	Amplicon (bp)
cvaC	F-CACACACAAACGGGAGCTGTT R- CTTCCCGCAGCATAGTTCCAT	679
iroN	F- AAGTCAAAGCAGGGGTTGCCCG R- GACGCCGACATTAAGACGCAG	667
Iss	F- CAGCAACCCGAACCACTTGATG R- AGCATTGCCAGAGCGGCAGAA	323
iucC	F- CGCCGTGGCTGGGGTAAG R- CAGCCGGTTCACCAAGTATCACTG	541
Tsh	F- GGGAAATGACCTGAATGCTGG R- CCGCTCATCAGTCAGTACCAC	420
Yjj	F- AATGGTTGTCAGCACTATGGC R- GGTCAGTCAGGCAGGATAATCC	1693

Data analysis

Descriptive analysis of the data and correlation between antibiotic resistance and APEC virulent genes was calculated using Graphpad Prism version 09.

RESULTS AND DISCUSSION

During revival, all the bacterial cultures on EMB agar that showed a green metallic sheen were presumptively identified as *E. coli*.

Antibiotic susceptibility test

Seven different classes of antibiotics: aminoglycosides (gentamicin, amikacin), amphenicoles (florfenicol, chloramphenicol), cephalosporines (ceftriaxone, cefoxitin), fluoroquinolones (ciprofloxacin, norfloxacin, enrofloxacin, levofloxacin), macrolides (azithromycin, erythromycin), penicillin (amoxicillin), sulfonamides (trimethoprim-sulphamethoxazole) and tetracyclines (doxycycline, tetracycline) were used for the disc diffusion test.

The isolates showed the highest resistance to enrofloxacin (99%), followed by cefoxitin (90.2%), and tetracycline (86.3%). In others, the resistance was below 80%. Azithromycin was the most effective drug with 67.6% isolates being susceptible, followed by ceftriaxone (65.7%), norfloxacin (53.9%) and amikacin (51%). In this study, 46.1% isolates showed intermediate susceptibility to florfenicol. Overall, the APEC isolates were more resistant to the class fluoroquinolones (enrofloxacin-99% and ciprofloxacin-75.5%) than other classes of antibiotics, while aminoglycosides (amikacin and gentamicin) were still effective compared to other classes of antibiotics (Fig. 1).

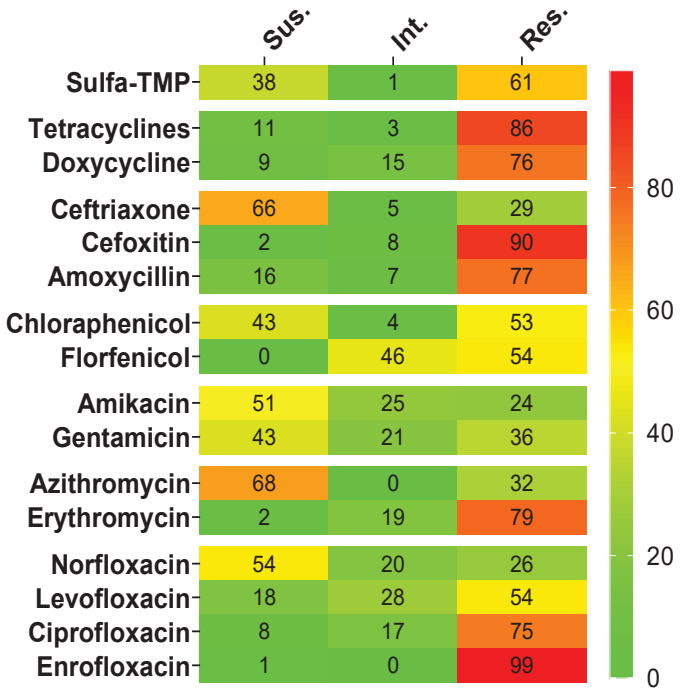

Fig. 1. Antibiogram of *E. coli* (N=102) isolated from cases of colibacillosis in broilers

Colibacillosis is one of the most common infectious bacterial diseases of poultry in Nepal. Traditionally, APEC was considered to cause secondary infection in birds following antecedent viral or Mycoplasma infection or poor management stress. Recently, APEC has been identified as a primary pathogen in avian hosts (Collingwood et al., 2014; Mehat et al., 2021). The infection leading to a significant decrease in egg and meat production, a decrease in hatchability and increased condemnation at slaughter leads to huge economic loss in the poultry industry (Morgan & Prakash, 2006; Azam et al., 2019; Kathayat et al., 2021; Bhattarai et al., 2024).

With very few studies on the pathogenicity of *E. coli* in poultry, this study correlates the virulence pattern of the organism with antimicrobial resistance in Nepalese broiler farming conditions. High resistance to enrofloxacin (99%) was observed. Antibiotics have been used extensively for the prevention and treatment of diseases, aiding the emergence of antimicrobial resistance in APEC infection (Acharya & Wilson, 2019). The infection has been very hard to treat due to high multidrug resistance shown by the bacteria (Hasan et al., 2011; Ranabhat et al., 2024). The elevated resistance level in the present study could be attributed to the excessive and unregulated use of fluoroquinolones in Nepalese poultry farms, often without veterinary prescription, poor farm biosecurity, and continuous use of antibiotics in compound feed at sub-therapeutic levels (Khanal et al., 2017). The results of this study showed huge variations when compared to the global scenario. Such variations are the reflection of the differences in antibiotic administration practices, regional exposure, and regulatory control in poultry production systems. Earlier reports from Nepal suggest that inadequate knowledge among poultry producers leads to inappropriate antibiotic use beyond therapeutic purposes. Poor biosecurity practices and stressors are major factors associated with increased antibiotic usage (Adhikari et al., 2025). Continuous use of antibiotics in compound feed (though it is banned by the law!) at sub-therapeutic levels, which is a common practice in Nepalese poultry production, could positively contribute to the increased degree of resistance (Khanal et al., 2017; Mandal et al., 2022). These findings highlight the pressing need for antibiotic stewardship and stricter regulation of antimicrobial use in poultry production.

MAR index

Upon calculation, it was shown that 6 isolates were categorised as low risk (MAR index between 0.1 and 0.2), and 96 isolates were categorised as high risk (0.3 and 1.0). The "mode" MAR index value was 0.8 (n = 21) with a negatively skewed (the 'tail' pointing toward the lower values, with 'bulk' on the right) distribution. Almost 77% (n = 79) of the isolates were grouped within these higher values of MAR (Fig. 2).

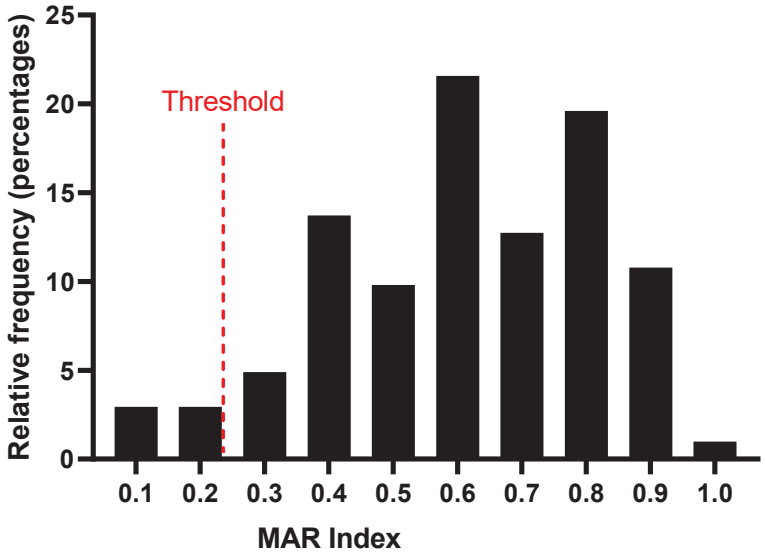

Fig. 2. Multiple antimicrobial resistance (MAR) index of *E. coli* isolates

A MAR index value >0.2 indicates that the isolate originated from a high-risk environment where antibiotics are frequently used or misused (Krumperman, 1983). In this study, six isolates were categorised as low risk (MAR index between 0.1 and 0.2), and 96 isolates were categorised as high risk (0.3 and 1.0), which showed that 94% of the isolates came from a high-risk source. The "mode" MAR index value was 0.8 (n=21), suggesting that the most common resistance profile in our sample was very high (resistance to ≥80% of the antibiotics used). Similarly, a negatively skewed (the 'tail' pointing toward the lower values, with 'bulk' on the right) distribution indicated that the source population was under intense selective pressure. The isolates are not just 'slightly' resistant; they are progressively moving toward MDR. The MAR Index values between 0.5 and 1.0 represent extreme MDR. Our data showed that almost 77% (n = 79) of the isolates are grouped within these values of MAR. This suggests that the source of these isolates is an environment with heavy antibiotic exposure, which is true in our case, as these isolates were derived from the clinical cases of colibacillosis that were non-responsive to antibiotic treatment at the broiler farms.

PCR for virulent genes of APEC

Virulent genes (*yjj*, *iss*, *iroN*, *iucC*, *tsh* and *cvaC*) were tested in all *E. coli* isolates. The most common virulent gene present in the isolates was the *yjj*, detected in 58.82% of organisms. Others like *iss*, *iroN*, *iucC*, *tsh* and *cvaC* were PCR positive in 48.04%, 45.10%, 31.37%, 19.61% and 10.78% of the isolates, respectively (Fig. 3). In this study, 3.92% (n = 4) isolates were positive for all six virulent genes, while 20.58% (n = 21/102) did not harbour any genes.

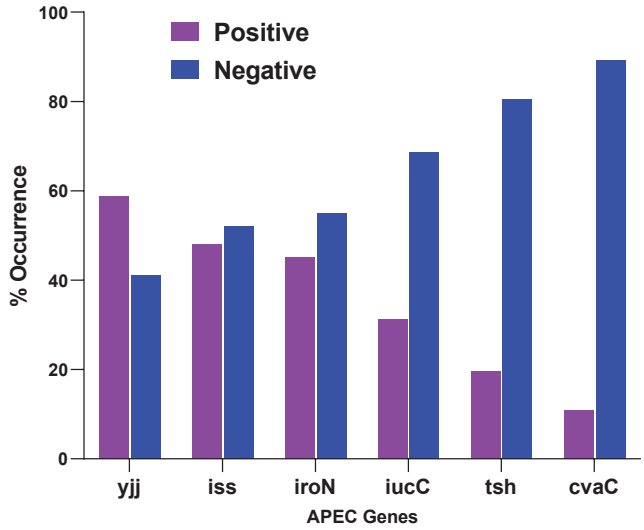

Fig. 3. Frequency of virulence genes in *E. coli* isolates

Molecular detection revealed that *yjj*, *iss*, and *iroN* were the most frequently identified virulence genes, detected in 58.82%, 48.04%, and 45.10% of isolates, respectively, while *iucC*, *tsh*, and *cvaC* were found at lower frequencies (31.37%, 19.61%, and 10.78%). Earlier studies have reported that *iss* and *iroN* are among the most prevalent APEC-associated genes (Zhao et al., 2005; Afayibo et al., 2022; Joseph et al., 2023; Patel et al., 2024; Ranabhat et al., 2024), justifying our premise that the *E. coli* circulating on our broiler population is indeed APEC, so it must be considered as a primary pathogen rather than a secondary pathogen. The *iss* gene contributes to capsule synthesis, which masks surface antigen, inhibiting complement-mediated killing and membrane attack complex formation (Biran et al., 2021). Its contribution in capsule synthesis could prevent antibiotic penetration (Slack & Nichols, 1982) and repel cationic antimicrobial peptides due to the capsule's negative charge (Gao et al., 2024; Bansal et al., 2015). The *iroN* gene, responsible for iron acquisition, is essential for bacterial growth and metabolic activity within iron-limited host environments (Caza et al., 2008). Iron also plays a crucial role in biofilm formation, which increases bacterial tolerance to antibiotics by reducing drug penetration through the biofilm matrix (Afrasiabi et al., 2025; Tang et al., 2025). In contrast, the *tsh* and *cvaC* genes were the least frequently detected in this study, similar to Joseph et al (2023), but in contrast to Zhao et al., 2005, Afayibo et al., 2022 and Gambi et al., 2022, who reported a higher prevalence of these genes. Such discrepancies are the usual result of differences in host species, geographic regions, or the genetic diversity of circulating APEC strains.

Gene-antibiotic correlation

Pearson's correlation (Fig. 4) analysis between antibiotic resistance and virulence genes revealed that *E. coli* resistance to tetracycline, doxycycline, and amoxicillin showed a weak positive correlation with all virulence genes ($0 < r < 0.3$). In addition, resistance to norfloxacin, levofloxacin, and enrofloxacin demonstrated weak positive correlations ($r = 0.1 – 0.2$) with virulence genes, except for *iucC*, *yjj*, *cvaC*, and *tsh* in norfloxacin; *yjj* and *cvaC* in levofloxacin; and *cvaC* and *tsh* in enrofloxacin, where no correlation was observed.

Resistance to azithromycin exhibited a weak negative correlation with all virulence genes. Similarly, antibiotic resistance showed a weak negative correlation ($0>r>–0.4$) with all virulence genes, except *iroN* for amikacin; *iroN*, *iss*, and *tsh* for gentamicin; and *iucC* and *yjj* for ceftriaxone, for which no correlation ($r = 0$) was observed.

Virulence genes *iss* and *iroN* showed the strongest positive alignment (up to 0.3) with higher MAR on average, while *iucC* and *cvaC* contribute little to none. In the multivariable model, *iss* retained the most positive coefficient; others were small or near zero once adjusted for co-occurrence.

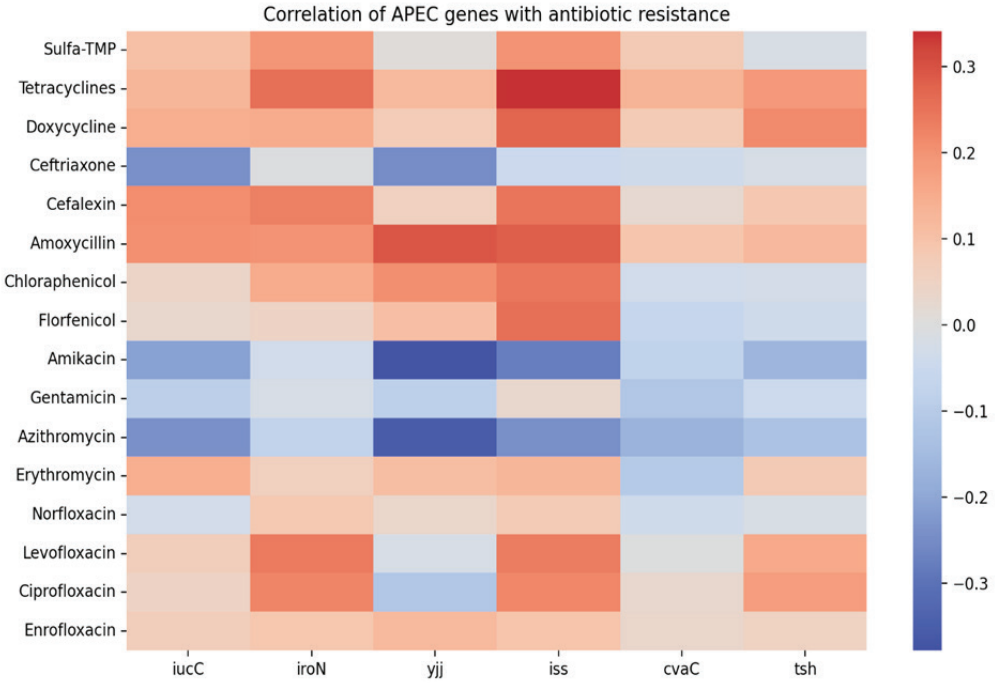

Fig. 4. Heatmap showing Pearson correlations between virulent gene and antibiotic resistance. Warmer colours showed a higher positive association. *ISS* & *iroN* showed the strongest positive alignment with higher MAR on average. *iucC* & *cvaC* contribute little to none to resistance

Correlation between APEC virulent genes and antibiotic class

Classwise, *iss* aligned most with higher antimicrobial resistance towards tetracyclines and penicillins, with moderate signals in sulfonamides and amphenicols. The genes *yjj*, *iroN* and *iucC* revealed a weak positive correlation (r = 0.1- 0.3) with antimicrobial resistance to some classes of the antibiotic. However, *tsh* and *cvaC* exhibited no or a negative relation with the resistance to antibiotic classes under study (Fig. 5).

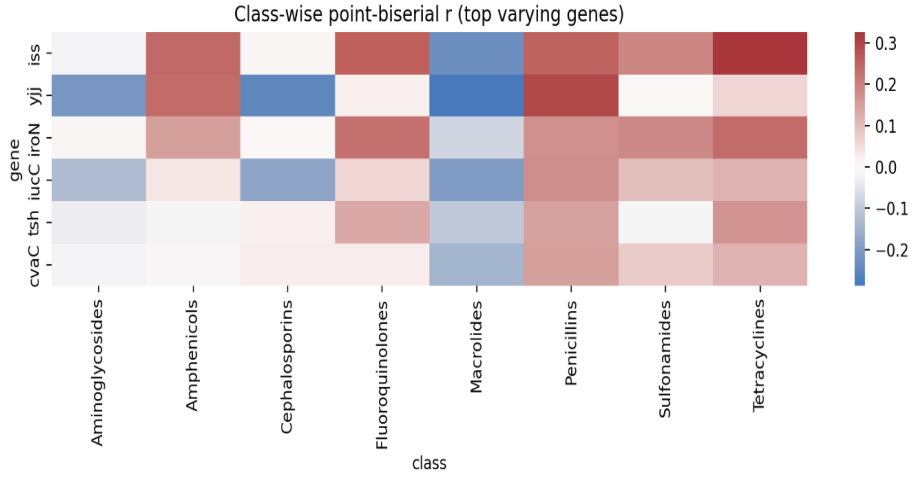

Fig. 5. Biserial correlation between APEC virulent genes and antibiotic class

Correlation analysis revealed that *iss* and *iroN* showed a positive association with higher MAR indices, particularly in tetracycline and penicillin groups. This suggests that virulent strains may have enhanced survival under antibiotic pressure, possibly due to co-localisation of virulence and resistance genes on mobile genetic elements (plasmids, integrons). Several resistance plasmids have been described in Enterobacteriaceae to carry virulence factors, such as bacteriocins, siderophores, cytotoxins, or adhesion factors, and virulence plasmids have been described to carry resistance genes (Cepas et al., 2020). However, overall correlations between virulence genes and antimicrobial resistance were weak or non-significant, indicating that the acquisition of virulence determinants does not necessarily confer antibiotic resistance.

CONCLUSION

The study confirmed the presence of multidrug-resistant *E. coli* strains carrying multiple virulence genes. This reflects that a majority of the cases of clinical colibacillosis in Nepalese broilers are caused by APEC with an extremely high MAR index. Genes such as *yjj*, *iss*, and *iroN* contributed to pathogenicity and extraintestinal survival, but not directly to antibiotic resistance. The high resistance patterns in the antibiogram and the skewed MAR index reflect extensive selection pressure, possibly due to unregulated antibiotic use in broiler production.

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AUTHOR CONTRIBUTIONS

NP: Conceptualization, Resources, Funding acquisition, Supervision; **SB:** Investigation, Writing – original draft; **LP:** Investigation, Writing – original draft; **NT:** Investigation; **RM:** Project administration, Writing – original draft, Supervision; **RP:** Investigation.

CONFLICTS OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICAL APPROVAL AND PERMITS

This research did not involve any regulated materials, and therefore, ethical approval or permits are not necessary.

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