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RESEARCH ARTICLE

Antimicrobial Resistance and *tet*A Gene Mediated Tetracycline Resistance in Avian Pathogenic *Escherichia coli* from Broiler Farms in Multan, Pakistan

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ABSTRACT

Colibacillosis, caused by *Escherichia coli* (*E. coli*), has been steadily reducing broiler efficiency and output in the poultry sector. Present research aimed to describe antimicrobial resistance (AMR) and *tet*A gene mediated tetracycline resistance in *E. coli* from broiler chicken samples after testing their drug susceptibility and molecular validation. Fifty specimens of liver were retrieved from broiler chicks and *E. coli* was isolated and purified using MacConkey agar (MCA) and Eosin Methylene Blue agar (EMB) followed by culturing at 37°C for 24 hours. Several biochemical procedures such as catalase and oxidase tests were performed to confirm bacterial isolates following Gram's staining presenting morphological assessment. The susceptibility of *E. coli* was investigated using Amoxicillin (AX) 25 µg, Ciprofloxacin (CIP) 5 µg, Enrofloxacin (ENR) 5 µg, Sulfamethoxazole + Trimethoprim (SXT) 23.75+1.25 µg, Gentamicin (CN) 10 µg, Tetracycline (TE) 30 µg and Cephalexin (CL) 30 µg antibiotic disks placed aseptically at Muller Hinton agar (MHA) plates. The results revealed that 67%, 70%, 39%, 39%, 78%, and 91% of *E. coli* samples were found resistant for AX, CIP, ENR, SXT, CN and TE, respectively while CL found highly effective. The cultural percentage of isolated *E. coli* was 92% and a clear depiction of antibiotic-resistant *tetA* strain of *E. coli* was validated using polymerase chain reaction (PCR) with 89% resistant percentage.

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INTRODUCTION

The poultry industry, a sub-sector of Pakistan agriculture, contributes 1.3% to the country's gross domestic product (GDP) by providing employment to 1.5 million people. The sector's investment is currently above 750 billion rupees with 8-10% increase annually. From 2020 to 2021 poultry meat production was 1.80 million tons (Hamza *et al*., 2025). The poultry sector has faced several disasters over the years due to various health issues, especially colibacillosis, fowl cholera, salmonellosis, Newcastle disease and coccidiosis.

Avian pathogenic *E. coli* (APEC) responsible for colibacillosis in chickens is characterized by air sac inflammation, pericarditis, perihepatitis, peritonitis, salpingitis and synovitis (Ahmed *et al*., 2025). Colibacillosis causes economic losses in the poultry sector because of excessive death rates and reduction of growth in infected birds (Hafez and Shehata, 2024). Additionally, bacteria are recognized as substantial public health concern

due to their zoonotic potential (Abebe *et al*., 2020). Effective APEC control measures are essential in contemporary poultry farming and consumption, as antimicrobial drugs are frequently administered to animals, particularly broilers, either as antimicrobial growth promoters (AGPs) or as preventative measure in many geographical regions (Alam *et al*., 2023).

Antimicrobial resistance (AMR) has been well-investigated in human, animal and poultry products. AMR has become major worldwide health concerns as bacteria mutate for years (Pinheiro *et al*., 2020). This could be due to vertical/horizontal transfer of genes processing in mobile genetic cassets, wherein plasmids and transposons are integral components (Salam *et al.,* 2023; Bhat *et al.,* 2023). AMR bacteria and their determinants are directly transferable to people from food animals and their products originating in the environment of these animals (Muloi *et al.,* 2018). It is more common and severe in low-middle income countries (LMIC) relative to high-income ones and is a burden for resource-limited countries (Laxminarayan *et al.,* 2016).

One Health is an integrated, holistic approach bringing together human health, animal health and the environment using a multi-layered strategy of mitigation, prevention and surveillance. One Health's main goal is to achieve sustainability and the best health status in people, animals, and environment (Prata *et al.,* 2022). This method is valuable in updating AMR burden planning and management (Mudenda *et al.,* 2023). A recent systematic review of One Health interventions found associations between interventions aiming to reduce antibiotic use in food-producing animals and their role on animal AMR (Hoelzer *et al.,* 2017). The knowledge and patterns of the AMR in isolates from food-producing animals has importance for establishing tailored treatment programs to decrease the prescription of antibiotics. Polymerase chain reaction (PCR) is a method used to amplify DNA segments for molecular, biological and clinical applications (Ghannam and Varacallo, 2023). The presence of *E. coli* bacteria in liver samples from broiler chicken and the existence of various disease genes in APEC population were detected by PCR method. Abd *et al*., (2015) investigated the impact of antibiotics application on the distribution of antibiotics and strains among commensal *E. coli* sourced from the intestines of broiler chickens.

Tetracycline (TE) serves as a broad-spectrum antibiotic, effectively impeding microbial protein synthesis by inhibiting the binding of aminoacyl-tRNA to the microbial ribosome. TE functions as the primary pharmacological agent employed in conjunction with anti-mycoplasmal medications in poultry industry to mitigate the complications arising from Mycoplasma and multiple bacterial infections. The phenomenon of antibiotic resistance is mediated by one or more of the 36 *tet* genes that have been identified at present. These genes facilitate resistance through one of three distinct mechanisms: the operation of an efflux pump, the provision of ribosomal protection, or the direct enzymatic inactivation of the antibiotic compound. Efflux mechanisms appear to be more common in Gram-negative bacteria, while ribosomal protection appears to predominate in Gram-positive bacteria (Balasubramaniam *et al.,* 2014).

The study included tetracycline resistance, due to usage in the poultry industry and previous studies indicating high levels of historical resistance. It was suggested that the *tet*A gene can spread more easily within environment than the *tet*B. Both humans and animals acquire AMR through various modes such as direct/indirect contact, food/feed intake, and environment. This scenario provides ample possibilities of emergence of resistance genes which would be vertically and horizontally transmitted among different pathogens (Alam *et al.,* 2023; Balasubramaniam *et al.,* 2014).

The efficacy of various vaccines in preventing colibacillosis in broilers means that some likely vaccine candidates have shown promising results but there are problems and limitations that need to be considered. The killed and subunit vaccines are safe with animals, since only pathogen fragments are given to the bird, but they have a limited scope of protection and need to be administered accordingly in birds. In contrast, while live attenuated vaccines can be applied broadly into the field by convenient channels, such as drinking water or spray, strict safety assessment and long-term studies on side effects are still required. The development of an effective vaccine for colibacillosis in chickens is hampered by the significant heterogeneity of *E. coli* isolates, elucidated disease mechanisms, and the lack of definitive markers for pathogenic isolates (Ghunaim *et al*., 2014; Kathayat *et al*., 2021). The complexity is apparent in restricted number of vaccines available in commercial market, accompanied by inconsistent reports regarding their effectiveness (Abd El-Mawgoud *et al.,* 2020; Elbestawy *et al.,* 2021).

The published literature reported the antibiotic resistance in Diarrheagenic *E. coli* (DEC) in broiler feces and meat samples from the slaughtering shops located in open markets of major cities in south Punjab with lack of *tet*A gene mediated tetracycline resistance estimation (Amir *et al.,* 2021). The other study by Amir *et al.,* (2019) reported spread of antibiotic-resistant *E. coli* from broiler to human populations through isolation of pathogen from fecal samples and identification of both *tet*A and *tet*B genes.

The present research focused on investigating AMR and the *tet*A gene-mediated tetracycline resistance in APEC isolates from freshly deceased broiler chickens in Multan. Current research aims to offer comprehensive guidelines regarding molecular epidemiological investigation and the characteristics of resistant *E. coli* isolates, with an emphasis on the presence of *tet*A gene. This current research presents the first definitive evidence of *tet*A-mediated TE resistance at farm-level APEC strains in broilers in Multan, addressing the gap neglected by previous market-centered investigations. The results reinforce regional surveillance of AMR and provide practical recommendations for enhancing management and controlling disease in poultry production.

MATERIALS AND METHODS

Materials: The antibiotic disks were obtained from Bioanalyse® (Turkey) and Mueller Hinton Agar (MHA) acquired from Accumix® (India). The cetrimide agar (CA), nutrient broth (NB), MacConkey agar (MCA) and Eosin Methylene Blue agar (EMB) were procured from Conda Lab (Spain). All reagents and compounds were used in accordance with the manufacturer's specifications.

Sample collection: A total of 50 liver samples were collected from freshly deceased broilers at different farms in Multan division, Pakistan using the convenient sampling method. Five broiler farms located at Multan, Shujabad, Jalalpur and Alipur were determined (Fig. 1), the fresh deceased birds with clinical signs of diarrhea and ages between 3 to 4 weeks were sampled for ten livers per homestead. The samples were obtained under aseptic conditions. To avoid risk of any contamination during the whole process, all liver samples were carefully collected under strict guidelines. Each bird was manipulated with single-use sterile gloves, scalpel, and forceps; instruments changed or re-sterilized between specimens to avoid any potential cross-contamination. The liver tissues were immediately placed into sterile sampling bags with each bag marked for identification. The birds were handled in such a manner that contact with outside surfaces, feathers or workers was avoided. The samples were sent to the laboratory in an ice box held at 4°C and tested immediately.

Isolation and recognition of *E. coli*: *E. coli* was isolated according to methods described by (Zaki *et al.,* 2025). All media such as NB, MCA and EMB were made fresh and sterilization was performed by autoclaving prior to use. All maceration, inoculation and sub-culturing was carried out in a Class II biosafety cabinet using flame-sterilized inoculating loops to create an aseptic working environment. All testing followed the protocol and proceeded through doffing in accordance with PPE adaptation. Liver samples from broilers were softly macerated, combined with nutrient broth and incubated overnight at 37°C. A loopful of bacterial suspension was cultured onto MCA and incubated aerobically for 24 hours at 37°C. Throughout the incubation period, the plates and broths were meticulously sealed to avert any potential environmental contamination. The uninoculated media plates served as negative control, incubated concurrently with test samples to ensure the absence of any inadvertent contamination. To purify the growth, the pink-colored presumptive *E. coli* colonies were cultivated in triplicate on EMB agar. Purified colonies exhibiting metallic green sheen on EMB were Gram's stained to confirm Gram Negative, pinkish, rod-shaped morphology and subjected to biochemical assays, including catalase and oxidase test, as per National Committee for Clinical Laboratory Standards (NCCLS) guidelines.

Antimicrobial susceptibility testing: *E. coli* isolates were examined for antibiotic susceptibility by using the disk diffusion test on MHA. Antimicrobial discs containing specific drug concentrations as mentioned in Table 1 were used aseptically at 0.5 McFarland standard cultured growth of *E. coli*, following (Carvalho *et al.,* 2015) guidelines. Plates were incubated at 37 °C for 24 hours and zones of inhibition were determined in millimeters (mm) using a digital vernier caliper.

Table 1: Antibiotic disks groups and concentrations

Antibiotic groups	Antibiotic	Concentration/disk (µg)
Beta lactams	Amoxicillin	25
Cephalosporin	Cephalexin	30
Aminoglycosides	Gentamicin	10
Tetracyclines	Tetracycline	30
Quinolones	Ciprofloxacin	5
Fluoroquinolone	Enrofloxacin	5
Sulfonamide	Sulfamethoxazole+ Trimethoprim	23.75+1.25

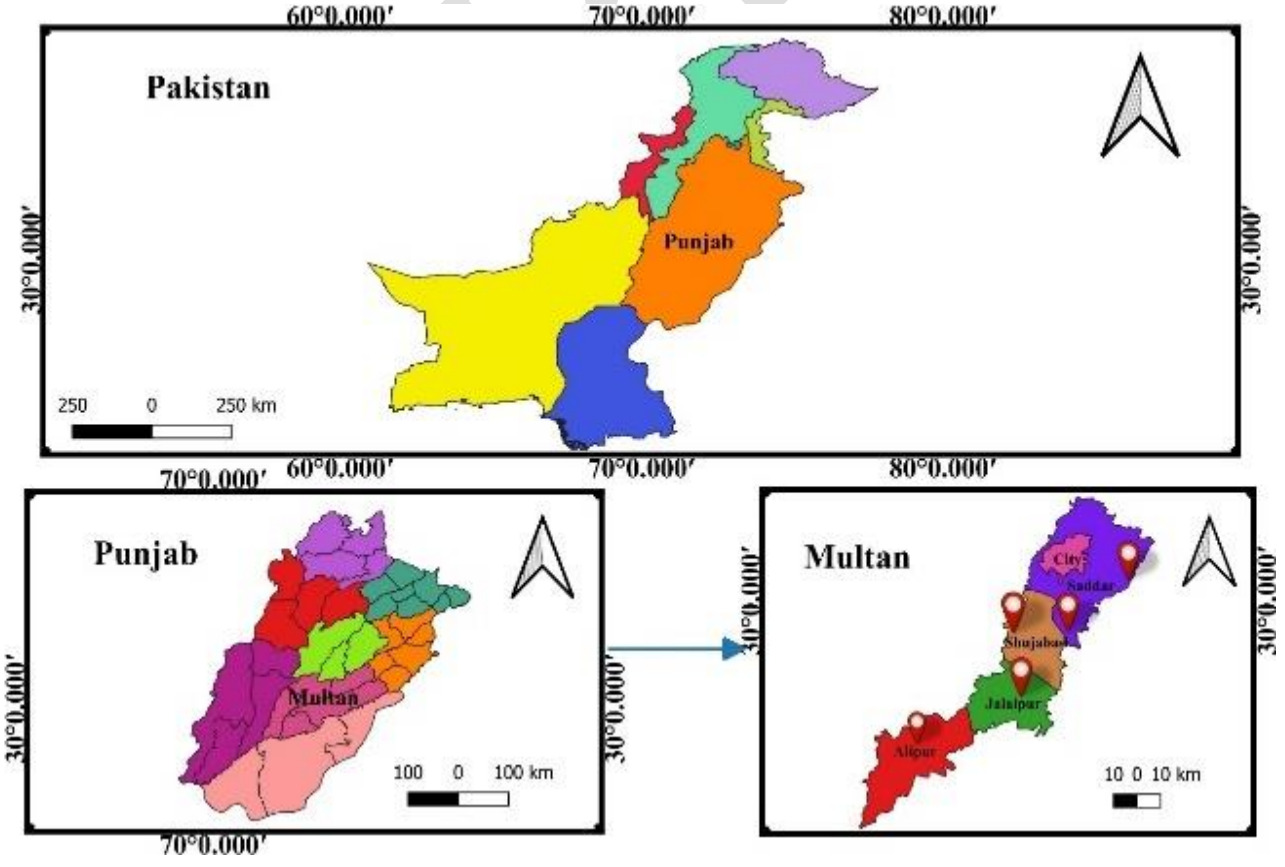

Fig. 1: Broiler liver samples collection sites from various sites of Multan, Punjab, Pakistan.

Molecular characterization of resistant *E. coli*

DNA Extraction and Polymerase Chain Reaction (PCR): DNA was extracted using a GeneJet Genomic DNA purification kit (Ref: K0721; LOT 01238798) by Thermo Scientific according to protocol provided by the manufacturer. The quality and quantity of DNA was checked by NanoDrop 1000 spectrophotometer. The extracted DNA was stored at -20°C till further use. PCR was performed on 46 positive resistant *E. coli* isolates from broiler liver. For the identification of the TE resistance gene of *E. coli*, a 576-bp fragment of the *tet*A gene was optimized using *tet*A-F (5'-GGTTCACTCGAACGACGTCA-3') and *tet*A-R (5'-CTGTCCGACAAGTTGCATGA-3') primers (Radwan *et al*., 2020). PCR was performed in 20 µl of reaction mixture containing 6 µl of pyrogen-free water (biowest, MS016S 1004) and 6 µl of ready-to-use 2X premaster mix of Wizbio Sol. (W1401. 3D0619-07), 2 µl of each forward and reverse primer and 4 µl of extracted DNA of *E. coli*. The PCR was carried out in a BIORAD C1000 machine with the following conditions, initial denaturation 94°C for 5 min, second denaturation 94°C for 30 sec, annealing 55°C for 40 sec, extension 72°C for 45 sec, cycles 35 followed by final extension 72°C for 10 min. After PCR, aliquots (6 µl) of individual amplicons were observed by agarose gel electrophoresis (1.5% gels in 0.5x TAE buffer [20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA]). Gels were stained with Ethidium bromide, visualized under a UV-Illuminator (GEP-UV1, Jinan, China) and photographed.

Statistical Analysis: Descriptive statistics were employed to evaluate the prevalence of *E. coli*, the percentages of antibiotic sensitivity and resistance, as well as the positivity of *tet*A gene. The values of the inhibition zones for each antibiotic were presented as mean ± SD. All analysis was performed considering $P<0.05$.

RESULTS

Cultural and morphological characteristics of isolated *E. coli:* Among 50 liver samples collected from five randomly selected broiler farms in the Multan division, 46 samples (92%) were positive for *E. coli*. On MCA, the colonies were bright pink-colored, whereas on EMB agar colonies exhibited a yellow-green metallic sheen (Fig. 2a–c). On NA, colonies appeared circular, smooth, and colorless (Table 2). All 46 positive isolates were catalase-positive, confirming their biochemical identity as *E. coli* (Fig. 2d). The remaining 4 samples (8%) did not show positive *E. coli* growth. The farm-level positivity ranged from 80% to 100% (Fig. 3 and Table 3), while 4 samples (8%) were negative.

Antimicrobial susceptibility test: The *in-vitro* antimicrobial susceptibility of isolated *E. coli* was evaluated using disk diffusion assay. The zones of inhibition were assessed and compared with CLSI 2012 standards for respective antibiotics.

For selected antibiotic disks the maximum inhibition zone diameters (mm) of enrofloxacin (ENR) , tetracycline (TE), ciprofloxacin (CIP), gentamicin (CN), amoxicillin (AX), sulfamethoxazole + trimethoprim(SXT) and cephalexin (CL) calibrated as 19.62±1.8, 14.08±1.45, 31.38±3.07, 21.13±1.41, 24.7±1.46, 20.83±2.06 and 22.48±1.75 mm, while least zones as 7.19±0.71, 0.0±0.0, 7.23±0.55, 6.14±0.64, 3.85±0.36, 0.0±0.0 and 6.42±0.83 mm, respectively as depicted in Fig. 4a. The isolated *E. coli* showed the highest sensitivity percentage to CL, SXT, ENR as 87, 61 and 61%, moderate sensitivity towards AX, CIP and CN as 33, 30 and 22%, while least for TE as 9%, respectively. The highest resistance percentage in *E. coli* was found for TE, CN, CIP and AX as 91, 78, 70 and 67% while intermediate towards ENR and SXT as 39 and 39%, and similarly least for CL as 13%, separately shown in Fig. 4b. The degree of resistance percentage to TE, CN, CIP, and AX was consistently high across all farms. The detailed description of the number of sensitive and resistant samples for relative antibiotics is presented in Table 4.

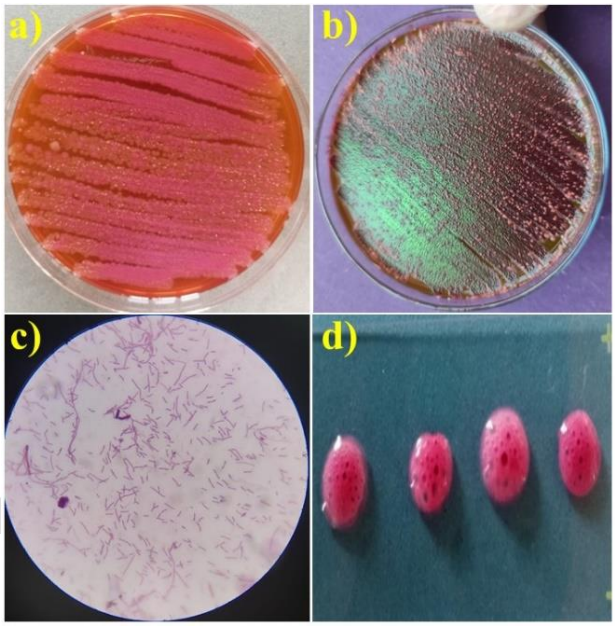

Fig. 2: Cultural characteristics of *E. coli* on (a) MacConkey agar (b) Eosin Methylene Blue agar (c) Gram staining (d) and Catalase test.

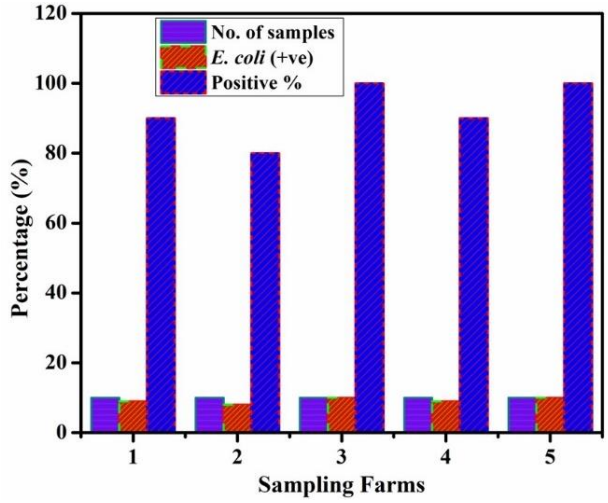

Fig. 3: Positive percentage of *E. coli* isolated from broiler farms.

Table 2: Cultural and morphological characteristics of *E. coli*

Media	Bacteria colony characteristics	Morphology
MacConkey agar	Bright, pink-colored apparent smooth enhanced colony.	Gram-negative, pink, small rod-shaped, arranged in single or appeared short
EMB agar	Yellow green colored metallic sheen	
Nutrient agar	Circular, smooth, colorless appearance of colonies	

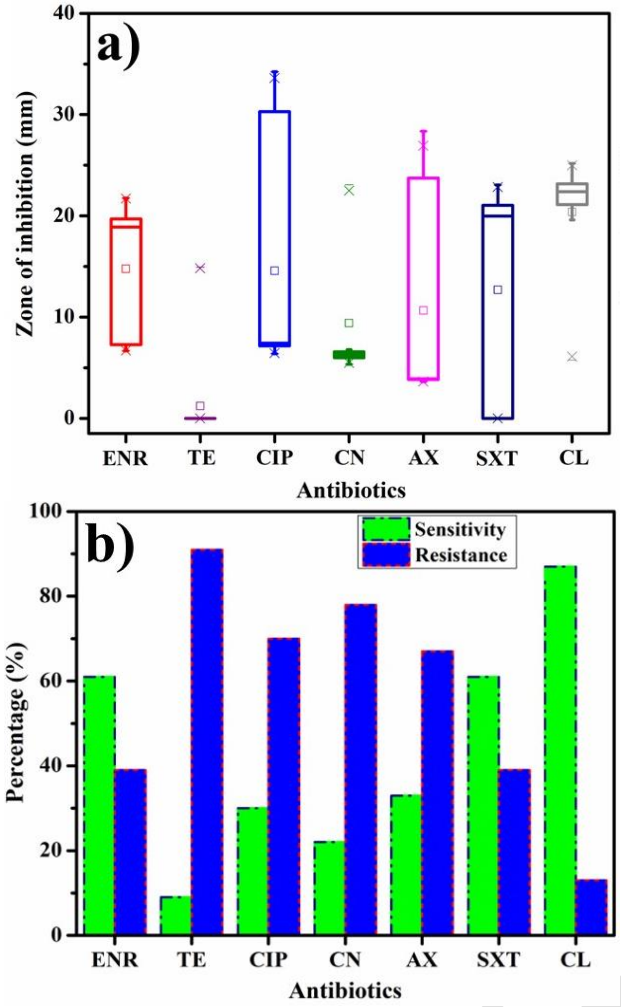

Fig. 4: (a) Antibiotic susceptibility of isolated APEC, Boxplots indicating least and maximum zones of inhibition with mean values **(b)** sensitivity and resistance percentage of selected antibiotics towards isolated *E. coli* from broiler farms.

Table 3: Cultural percentage of *E. coli* in selected broiler farms

No. of farms	Sample	No. of Samples	*E. coli* (+ve)	% age
5	Liver	10	9	90
		10	8	80
		10	10	100
		10	9	90
		10	10	100
Total		50	46	92

Table 4: Prevalence percentage of antibiotic sensitive and resistant samples.

Bacteria	Antibiotics	Sensitive Samples	percentage	Resistant Samples	percentage
E. coli	ENR	28	61	18	39
	TE	4	9	42	91
	CIP	14	30	32	70
	CN	10	22	36	78
	AX	15	33	31	67
	SXT	28	61	18	39
	CL	40	87	6	13

Molecular prevalence of *tetA* gene in *E. coli* isolates: PCR revealed a 576 bp match amplicon, specifically for *tetA* gene of resistant *E. coli* (Fig. 5), in screened broiler liver specimens obtained from Multan division. All 46 positive *E. coli* specimens proceeded to PCR, that indicated existence of *tetA* gene in 41 (89%) samples. Cohen's kappa ($\kappa \approx 0.89$) demonstrated nearly ideal concordance between phenotypic resistance and the existence of *tet*A, thereby affirming that *tet*A is the primary factor contributing to tetracycline resistance among these isolates.

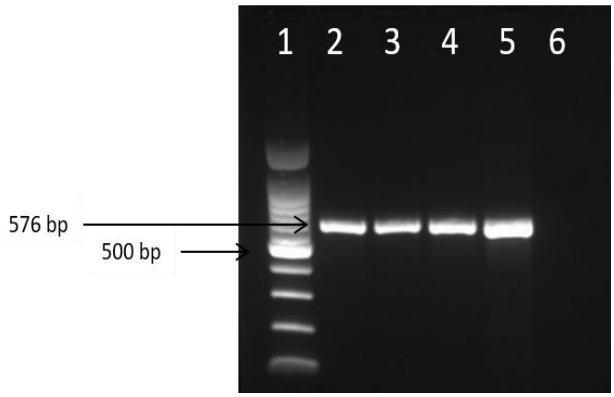

Fig. 5: Molecular identification of resistant *E. coli*. Lane: 1 stands for the 100 bp DNA marker; lanes 2-4 indicate broiler liver positive samples for *tet*A gene. While L5 and 6 depict the control positive and control negative, respectively.

DISCUSSION

E. coli is a significant health issue in poultry, resulting in substantial economic losses, illness, and mortality in newborn chicks, broilers, and layers. The manifestations of sepsis are linked to a significant mortality rate, together with infectious diseases marked by air-sacculitis and simultaneous inflammatory conditions impacting the pericardium, abdomen, and liver (Radwan *et al*., 2020).

The present research found 92% of positive *E. coli* samples across different broiler farms in Multan division, which is comparable to another study reporting 89.2% prevalence of APEC in Pakistan (Azam *et al*., 2019). Contemporary studies in various regions have identified an existence of *E. coli* in chickens, with prevalence rates attaining 94% in Nepal (Koju *et al*., 2022).

The identified 46 isolates were analyzed for susceptibility against different antibiotics such as Enrofloxacin, Tetracycline, Ciprofloxacin, Gentamicin, Amoxicillin, Sulphamethoxazole + Trimethoprim, and Cephalexin. The resistance rate to ENR was found to be 39%, which closely align with 37.74% and 32% reported by (Talebiyan *et al*., 2014 and Nhung *et al*., 2017), correspondingly.

TE constitutes a class of broad-spectrum antibiotics, including chlortetracycline and oxytetracycline, which are employed in treating bacterial, chlamydial, rickettsial, and protozoal infections (Eliopoulos *et al.,* 2003). These compounds rank among the most widely utilized antibiotics in poultry farming; however, their application extends into human medicine owing to their effectiveness, affordability, and minimal adverse effects. TE is one of the most widely used first line antibiotics in poultry which has been applied even before the acquisition of resistance traits by the pathogens (Miles *et al.,* 2006). The isolated *E. coli* showed 91% resistance to TE, which was found similar to the resistance reported by (Nhung *et al*., 2017), while 96.50% and 97.3% by (Latif *et al*., 2019 and Azam *et al*., 2019) separately. The observed pattern of resistance is marginally lower than findings from recent reports in Pakistan indicating 100% TE resistance (Liaqat *et al*., 2022; Zainab *et al*., 2022).

The obtained isolates exhibited resistance for CIP as 70%, which closely aligns with 71.80%, 72%, and 67% reported by (Latif *et al*., 2019; Azam *et al*. 2019 and Nhung *et al*., 2017), respectively.

The isolates presented 78% resistance to CN in contrast with 55% and 45.5% documented by (Amer *et al*., 2018 and FR *et al*., 2019), separately while, higher resistance of 92.5% reported by Radwan *et al*., 2020. The National Central Drug Plan of Portugal indicates that the application of TE, quinolones, and sulfonamides in veterinary medicine has led to the development of AMR (Liaqat *et al*., 2022).

Resistance to AX appeared in 67% of investigated isolates, while resistance levels of 80% and 89.4% were reported by (Nhung *et al*., 2017 and Latif *et al*., 2019), respectively. In contrast, a 100% and 97.5% resistance to AX were documented by (FR *et al*., 2019 and Radwan *et al*., 2020) correspondingly.

The isolated *E. coli* exhibited 39% resistant to SXT, found similar as 39.62% reported by (Talebiyan *et al*., 2014). Meanwhile higher resistance of 68%, 77.5% and 77.6% achieved by (Azam *et al*., 2019; Radwan *et al*., 2020 and Latif *et al*., 2019), respectively.

CL exhibited 87% sensitivity in contrast with 81% resistance observed in Nepal (Khanal *et al*., 2017).

The current study found the presence of *tet*A gene in isolated *E. coli* as 89%, which aligns closely with previous studies reporting 90% (Tawakol & Younis, 2019). Similar results were reported in India, where 84% of samples found positive for *tet*A gene (Jaiswal *et al*., 2024) and similarly 100% in Egypt (FR *et al*., 2019; Radwan *et al*., 2020). The detection of 576 bp amplicon corresponding to *tet*A gene in broiler liver samples indicates a significant presence of TE-resistant *E. coli* (FR *et al*., 2019; Tawakol & Younis, 2019; Radwan *et al*., 2020). Strong associations were observed between AMR phenotypes and genotypes in the tested *E. coli* isolates as have been previously reported (Hossain *et al.,* 2022). The objective of the study was therefore to describe phenotypic and genotypic characteristics of AMR, plasmid profiling, and virulence genes associated with a multidrug-resistant *E. coli* strain (S3) obtained from a poultry farm in Enugu State Nigeria. Genome analysis indicated a total size of 5.33 Mb; this was organized in five contigs including one chromosome and four plasmids. The strain harboured multiple antibiotic resistance genes (ARGs) including *bla*CTX-M-15, *bla*OXA-1, *bla*TEM-1, aac(6')-Ib-cr, aadA5, aph(3'')-Ib, sul1/sul2, *tet*(A), dfrA17 and mph(A) and they were found co-localised on plasmids that might provide mobility mechanism of horizontal gene transfer (HGT). The plasmids classes included Col156, IncF and two replication clusters (Ejikeugwu *et al.,* 2025).

This research was conducted with convenient sampling from clinically positive birds, therefore the resistance frequencies obtained may not be extrapolated to larger broiler population. Besides, the investigation was restricted to disk diffusion and a single resistance gene (*tet*A), limiting the molecular analysis of resistance characterization.

The intentional implementation of strategies is necessary to optimize the efficacy of limited resources and target the most critical weaknesses, including improving visibility for key constituencies and enforcing policies to ensure thoughtful use of antimicrobials. In a comparable manner, the incorporation of AMR into the nation's overall health, development, and agricultural transformation strategies will be essential to yield confronting advantages (Qiu *et al.,* 2024).

Conclusions: In this work we observed a high prevalence of *E. coli* (92%) and *tet*A gene (89%) in broiler chicken liver samples, suggesting a widespread occurrence of TEs resistance was also detected. Cephalexin remained a highly efficient treatment; however, testing for antimicrobial susceptibility indicated considerable resistance to commonly utilized antibiotics, particularly tetracycline, enrofloxacin, ciprofloxacin, amoxicillin, sulfamethoxazole + trimethoprim, and gentamicin. These results show that AMR is a major problem in chicken production and emphasize the necessity of using antibiotics carefully implementing biosecurity protocols and raising farmer knowledge to stop the development of new resistance. Responsible antimicrobial techniques are crucial for preserving the health and welfare of poultry as well as for ensuring food safety and reducing hazards to public health. Effective efforts for reducing AMR in the poultry industry must be guided by ongoing surveillance of resistant *E. coli* and their resistance genes, as well as more extensive and representative sampling.

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Data Availability statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflict of interest: 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.

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