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MOLECULAR IDENTIFICATION OF β-LACTAMS AND TETRACYCLINE ANTIBIOTIC RESISTANCE GENES IN *ESCHERICHIA. COLI*

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Abstract

The rising global prevalence of antibiotic resistance (ABR) in bacteria, particularly in food producing animals, poses a substantial threat to both veterinary and human public health. Escherichia coli serves as a crucial indicator for monitoring ABR dissemination. This study aimed to identify β-lactam and tetracycline antibiotic resistance genes in E. coli isolates collected from sheep (n=66) and goats (n=54) in Quetta, Balochistan, Pakistan. Fecal samples (n=120 total) were collected aseptically, and E. coli isolates were identified through standard microbiological methods, including selective plating on EMB agar and Gram staining. Antibiotic Susceptibility Testing (AST) by the Kirby-Bauer disc diffusion method revealed alarmingly high rates of phenotypic resistance. Molecular detection of resistance genes by Polymerase Chain Reaction (PCR) further confirmed these phenotypic findings. Five genes were targeted: uidA (E. coli species marker), tetA, tetB (tetracycline resistance), blaTEM, and blaCTXM (β-lactam resistance). The uidA gene was detected in 79.2% (95/120) of all isolates. Resistance genes were highly prevalent: tetA was found in 76.6% (92/120) of isolates, tetB in 73.3% (88/120), blaTEM in 69.1% (83/120), and blaCTXM in 62.5% (75/120). This study underscores the significant burden of β-lactam and tetracycline resistance genes in E. coli. These results provide crucial data for implementing targeted antimicrobial management programs and efforts to manage the spread of antimicrobial resistance.

Keywords: *Antimicrobial resistance, blaCTXM, blaTEM, Balochistan, ESBL, Escherichia coli, tetA, tetB, Livestock, Zoonotic transmission*

INTRODUCTION

It is widely recognized that antibiotic resistance has become one of the greatest threats to global health in the 21st century. The World Health Organization estimates that due to antimicrobial resistance (AMR), in 2019 alone, approximately 1.27 million people died directly and AMR was a contributing factor to nearly 4.95 million deaths globally (1). The evolving selective pressure on bacterial populations is driven by the widespread and often unsupervised use of antibiotics in both human and veterinary medicine, which hastens the accumulation of resistance gathered across ecological niches (2). *Escherichia coli*, a Gram-negative commensal bacterium found in the gastrointestinal tract of humans and almost all warm blooded animals, is widely acknowledged as a sentinel organism for AMR monitoring trends in the clinical and agricultural environment (3). While typically non harmful but virulent strains of *E.* coli trigger life threatening illness of the urinary tract gastroenteritis and sepsis (4, 5). Importantly, *E. coli* is a major vehicle of horizontal gene transfer for resistance genes between animal and human populations by virtue of their rapid acquisition and dissemination through mobile genetic elements (e.g., plasmids, integrons, transposons) (6, 7).

Extended spectrum β-lactamase (ESBL) including *blaTEM* and *blaCTXM* are the major determinants for the resistance of *E. coli* to penicillins and cephalosporins, while *tetA* and *tetB* genes confer tetracycline efflux pump activity (8). The *blaTEM* gene was one of the first to be discovered and still majorly spread in animal *E. coli*, and *blaCTXM* has come into global predominance starting from early 2000s (9, 10) They account for 37% of total veterinary antimicrobial sales in Europe, and the widespread use of this class has led to high levels of tetracycline resistance primarily driven by efflux pump genes *tetA* and *tetB* in animal origin *E. coli* (8).

Unregulated antibiotics use, absence of formal stewardship systems and few surveillance setups make conductive situation for resistance emergence. This research provides a systematic molecular data for the β-lactam and tetracycline resistance reasons in *E. coli* that is found in sheep and goat of Quetta Balochistan.

The objective of this research include isolating an *E. coli* from sheep and goat fecal samples collected at Quetta, examining antibiotic resistance shown in phenotypes for β-lactam, tetracycline aminoglycoside and also fluoroquinolone antibiotics and using a PCR for molecular identification of resistance genes *blaTEM*, *blaCTXM*, *tetA* or *tetB* with *E. coli* marker *uidA*.

MATERIALS AND METHODS

STUDY AREA AND DURATION

Research was carried out at the Biotechnology and Physiology Laboratory, at Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta, Pakistan from October 2024 to March 2025.

SAMPLE COLLECTION

The fecal samples were collected aseptically from sheep (n=66) and goat (n= 54) using sterile cotton swabs, sampling sites including of local farms in nearby Spini Road and small scale livestock markets in Quetta, Balochistan. Sampling was done at the farm level, and animals were selected randomly. Each sample was place in sterile container transported in ice boxes to ensure bacterial viability.

ANTIBIOTIC SUSCEPTIBILITY TESTING

Antibiotic susceptibility testing (AST) was performed by Kirby-Bauer disc diffusion method on Mueller-Hinton agar media following CLSI M02 guidelines (2024) (11). Bacterial lawns were prepared from overnight cultures adjusted to reach an 0.5 McFarland turbidity standard. The antibiotics discs tested were amoxicillin (AMC, 30µg), cefaclor (30 µg, CEC), cephalothin (KF, 30 µg), cephradine (CE 30 µg), oxacillin (1 µg, OX), tetracycline (TE, 30 µg), an amikacin (AK, 30 µg), neomycin (N, 10 µg), gentamicin (10 µg CN), norfloxacin (NOR, 10 µg), moxifloxacin (5 µg, MXF), ofloxacin (OFX, 5 µg), fosfomycin (FOS, 50µg). Then, plates were kept in 37°C for 24 hours Zones of inhibition got measured in millimeter's and categorized as resistant, intermediate or susceptible with CLSI breakpoints.

INNOCULATION AND IDENTIFICATION OF *E. COLI*

Fecal samples were inoculated into a nutrient broth and the MacConkey broth for incubation at 37°C, which lasted for between 24 and 48 hours. Samples showing turbidity were streaked on eosin methylene blue (EMB) agar, which was also incubated at 37°C for 24 hours. Colonies showing a green metallic sheen were selected. Gram staining produced results matching Gram-negative rod-like forms which were typical for the *E. coli*.

DNA EXTRACTION

Extraction of the genomic DNA was done with boiling method (12). Cultures were centrifuged at 14,000 rpm for ten minutes. The pellets were re -suspended in 200 µL of a phosphate-buffered saline (PBS), boiled at 100°C for a ten minutes, cooled and centrifuged at 9,200 ×g for ten minutes. DNA mostly stayed in supernatant, which was kept at −20°C until later use.

DETECTION OF ANTIBIOTIC RESISITNACE GENES

PCR was performed to amplify five target genes: *blaTEM*, *blaCTXM*, *tetA*, *tetB* (resistance genes) and *uidA* (species-specific marker for *E. coli*). Primer sequences, amplicon sizes, and annealing temperatures are listed in Table I; thermocycler conditions and reaction mixtures are detailed in Table II. Reactions for resistance genes were performed in 25 µL (10 µL master mix, 1 µL each primer, 5 µL DNA template, 8 µL nuclease-free water); *uidA* reactions in 20 µL (10 µL master mix, 1 µL each primer, 3 µL DNA template, 5 µL nuclease-free water). Each run included a positive control (reference *E. coli* with known genotype), a negative control (nuclease-free water replacing template), and a no-template control. A 1,500 bp DNA ladder was used as a molecular size reference.

Table I. Primer sequences, amplicon sizes, and annealing temperatures for PCR detection of *E. coli* and antibiotic resistance genes

Gene	Amplicon size	Annealing temp.	Forward primer (5′→3′)	Reverse primer (5′→3′)
blaTEM	793 bp	56°C	GGTTCACTCGAACGACGTCA	CTGTCCGACAGTTGCATGA
blaCTXM	592 bp	55°C	CCTCAGCTTCTCACGCGTG	GCACCTTGCTGATGACTCTT
tetA	577 bp	57°C	CATTTCCGTGTCGCCCTTAT	TCCATAGTTGCCTGACTCCC
tetB	634 bp	55°C	ATGTGCAGTACCAGTAARGTKATGGC	TGGGTRAARTARGTSSACCAGAAYSAGCGG
uidA	623 bp	52°C	CCAAAAGCCAGACAGAGT	GCACAGCACATCAAAGAG

Table II. Thermocycler conditions and reaction mixture for gene amplification

Genes	Thermocycler conditions	Reaction mixture	Reference
blaTEM, *blaCTXM*, *tetA*, *tetB*	Initial denaturation: 95°C/5 min; 30 cycles: denaturation 95°C/1 min → annealing at gene-specific temp. (Table I)/30 sec → extension 72°C/1 min; final extension: 72°C/10 min	25 µL: 10 µL master mix + 1 µL each primer (F+R) + 5 µL DNA + 8 µL nuclease-free water	13
uidA	Initial denaturation: 95°C/5 min; 35 cycles: denaturation 94°C/1 min → annealing 52°C/30 sec → extension 72°C/1 min; final extension: 72°C/7 min	20 µL: 10 µL master mix + 1 µL each primer (F+R) + 3 µL DNA + 5 µL nuclease-free water	14

*Positive and negative controls included in each run. A 1,500 bp DNA ladder used as molecular size reference

GEL ELCTROPHORESIS

PCR products were determined on 1.2% agarose gel (0.6g agarose in 50 ml of 1 TAE buffer) stained with ethidium bromide (0.5 µg/ml). A 1500 bp DNA ladder served as a size reference. Electrophoresis was conducted at 90-110 V for 45 minutes. DNA bands were visualized and documented under UV transiluminator.

RESULTS

ISOLATION AND IDENTIFICATION OF ECOLI

Of the 120 fecal samples processed (sheep n=66, goats n=54) presumptive *E. coli* colonies displaying characteristic green metallic sheen were identified on EMB agar after 24 hours at 37°C (Fig. 1a). Gram staining revealed pink rod shaped gram negative bacilli (Fig. 1b).

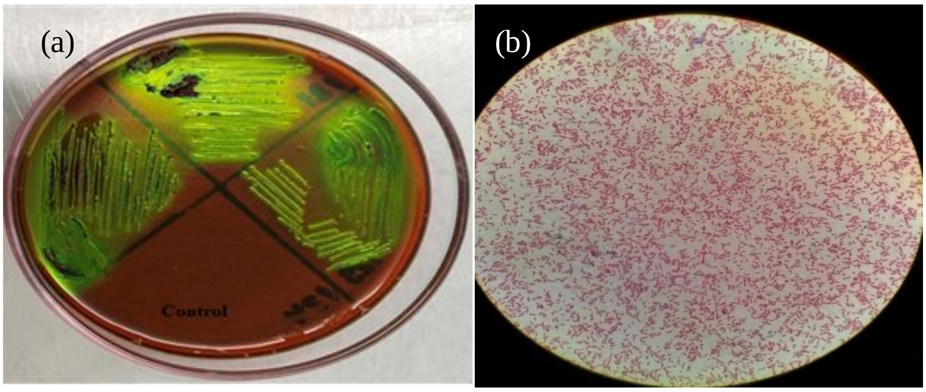

Fig. 1(a). Green metallic sheen colonies of *E. coli* on eosin methylene blue EMB agar after 24-hour incubation at 37°C; **(b)**. Gram-stained *E. coli* showing pink, rod-shaped gram-negative *bacilli* under

ANTIBIOTIC SUSCEPTIBILITT TESTING

The antibiotic susceptibility profile was determined by Kirby-Bauer disc diffusion and interpreted according to CLSI (2024) breakpoints (Table III). None of the isolates were inhibited by any of the tested β-lactam antibiotics (amoxicillin, cefaclor, cephalothin, cephradine, oxacillin) and tetracycline as no inhibition zone was observed (0 mm) for all the discs as shown in Fig. 2 (a, b). The CLSI susceptibility thresholds for gentamicin, norfloxacin, moxifloxacin, ofloxacin, and fosfomycin were exceeded with results of (19 mm), (20 mm), (22 mm), (18 mm), (19 mm), Amikacin and neomycin (15 mm) and (16 mm) respectively Fig. 3 (a, b).

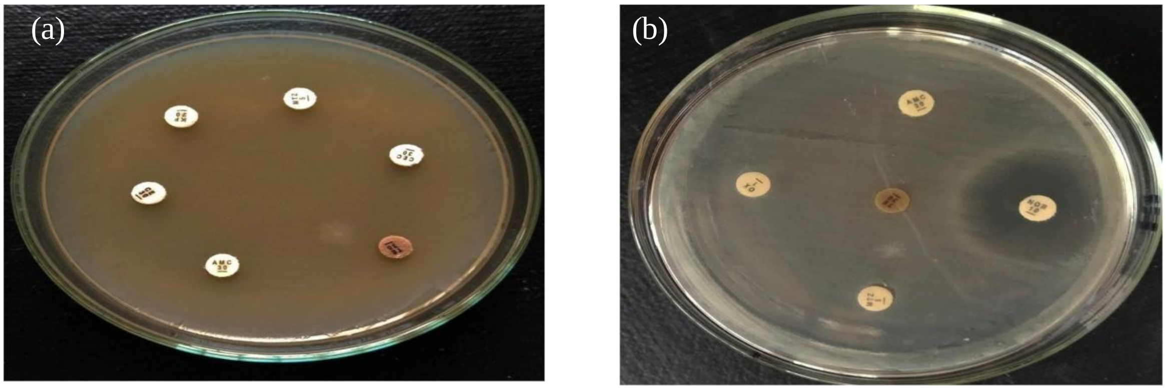

Fig. 2 (**a**). No zones of inhibition observed around the disc containing, AMC -30 (amoxicillin), CEC-30 (cefaclor), KF- 30 (Cephalothin), CE- 30 (Cephradine), and TE 30 (tetracycline) confirming resistance; **(b).** Absence of inhibition zone around 0X -1 (oxacillin), showing resistance to penicillin class beta- lactams

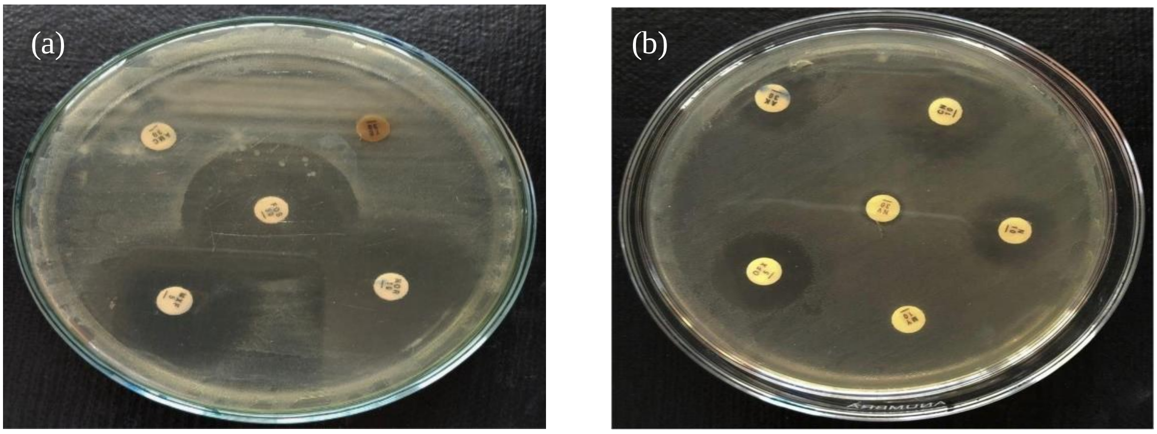

Fig 3 (a). Zones of inhibition around MXF-5 (moxifloxacin), NOR-10 (norfloxacin) and FOS -50 (Fosfomycin) showing susceptibility of *E. coli* isolates; **(b).** Clear Inhibition zones around OFX -5 (ofloxacin), AK-30 (amikacin), N10 (neomycin), and CN-10 (gentamicin) confirming aminoglycoside activity

Table III. Antibiotic resistance and susceptibility profile

S. No.	Antibiotic	Class	Clear zone	CLSI guide (mm)	Results
1	Amoxicillin	Penicillin	0	Works if ≥ 18	Resistant
2	Cefaclor	Cephalosporin	0	Works if ≥ 18	Resistant
3	Cephalothin	Cephalosporin	0	Works if ≥ 18	Resistant
4	Cephradine	Cephalosporin	0	Works if ≥ 18	Resistant
5	Oxacillin	Penicillin	0	Works if ≥ 18	Resistant
6	Tetracycline	Tetracycline	0	Works if ≥ 19	Resistant
7	Amikacin	Aminoglycoside	15	Works if ≥ 17	Susceptible
8	Neomycin	Aminoglycoside	16	Works if ≥ 18	Susceptible
9	Gentamicin	Aminoglycoside	19	Works if ≥ 18	Susceptible
10	Norfloxacin	Fluoroquinolone	20	Works if ≥ 17	Susceptible
11	Moxifloxacin	Fluoroquinolone	22	Works if ≥ 20	Susceptible
12	Ofloxacin	Fluoroquinolone	18	Works if ≥ 16	Susceptible
13	Fosfomycin	Phosphonic acid	19	Works if ≥ 16	Susceptible

DNA EXTRACTION AND CONFIRMATION BY GEL ELECTROPHORESIS

Genomic DNA was extracted using boiling method and confirmed by agarose gel electrophoresis, as shown in Fig. 4a. High molecular weight DNA bands were observed under UV light.

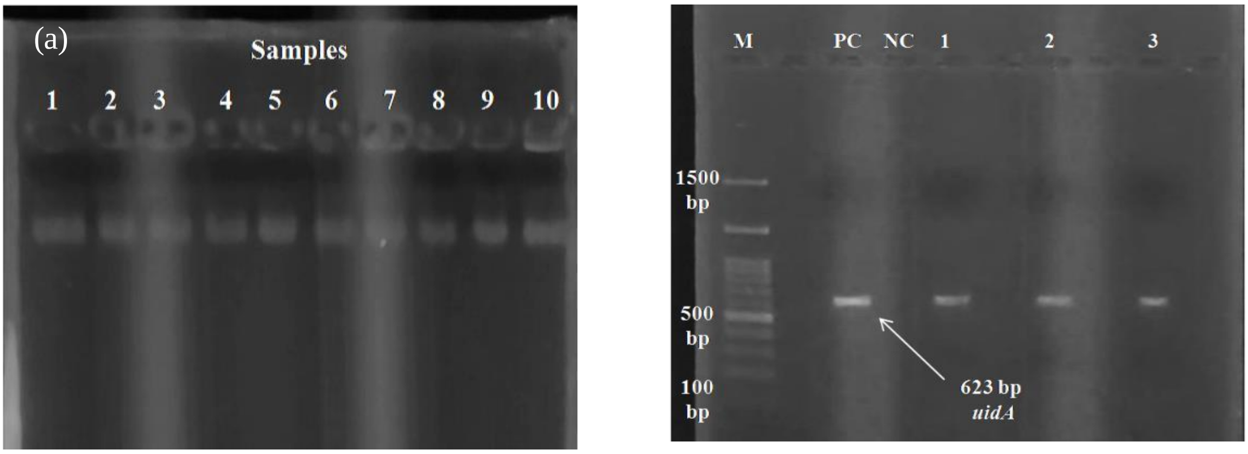

Fig. 4 (a). DNA extracted from *E. coli* isolates showing visible bands on agarose gel, lanes (1-8) confirmed presence of DNA; **(b).** PCR amplification of *uidA* gene (623 bp). Lane M: DNA ladder (1500 bp); lane PC positive control; lane NC negative control, lanes 1,2 and 3 PCR product of *uidA* confirming the presence *E. coli*.

E. COLI SPECIFIC MARKER IDENTIFICATION BY PCR

Molecular identity as *E. coli* based on the species-specific *uidA* marker was identified in 95/120 (79.2%) total isolates (sheep; 53/66, 80.3%; goats: 42/54, 77.7%) on 623 bp (fig 4b).

DETECTION OF RESISITANCE GENES BY PCR

PCR results for the four targeted genes identified in the *E. coli* isolates. Resistance determinants were most commonly *tetA* (92/120; 76.6%), at 577 bp (Fig. 5a), followed by *tetB* (88/120; 73.3%) at 634 bp (Fig. 5b). β-lactamase genes were present in 83/120 (69.1%) isolates for *blaTEM* at 793 bp (Fig. 5c) and 75/120 (62.5%) isolates for *blaCTXM* at 592 bp (Fig. 5d).

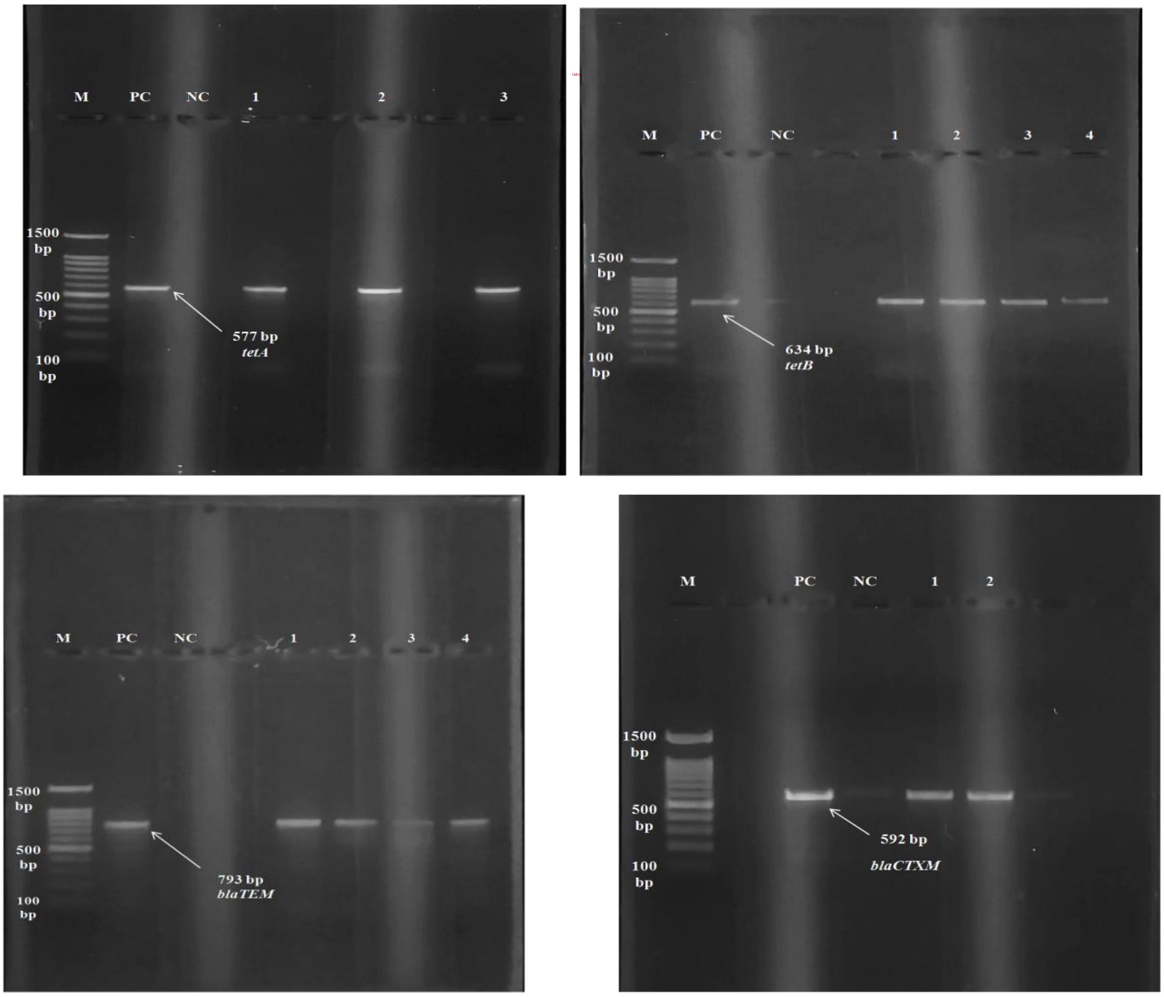

Fig. 5a. PCR amplification of *tetA* gene (577 bp). Lane M: DNA ladder 1500 bp; lane PC Positive control; lane NC negative control; **(b).** PCR amplification of *tetB* gene (634 bp) conferring tetracycline resistance; **(c).** PCR amplification of *blaTEM* gene (793 bp); **(d).** PCR amplification of *blaCTXM* gene (592bp)

Table IV. PCR- based detection genes in *E. coli* isolates

Gene	Sheep (n =66)	Goat (n = 54)	Total positive isolates (n=120)
uidA	53/66 (80.3%)	42/54 (77.7%)	95/120 (79.2%)
tetA	51/66 (77.2%)	41/54 (75.9%)	92/120 (76.6%)
tetB	49/66 (74.2%)	39/54 (72.2%)	88/120 (73.3%)
blaTEM	45/66 (68.2%)	38/54 (70.3%)	83/120 (69.1%)
blaCTXM	40/66 (60.6%)	35/54 (64.8%)	75/120 (62.5%)

DISCUSSION

This study presents the molecular characterization of β-lactam and tetracycline antibiotic resistance gene in *Escherichia coli* isolated from sheep and goats in Quetta, Balochistan, Pakistan. Alarmingly high rates of phenotypic resistance to β-lactam antibiotics and tetracycline were documented, alongside molecular confirmation of corresponding resistance gene, while aminoglycosides and fluoroquinolones retained broad activity.

Resistance to all tested β-lactam antibiotics exceeded 86% in both sheep and goat isolates. This was confirmed molecularly by the detection of *blaTEM* in 69.1% and *blaCTMX* in 62.5% of isolates. These findings align with published data from Pakistan. Ejaz et al (15) detected *blaCTMX* in 73.9% of farm animal *E. coli* isolates. Kumar *et al*., (16) reported co-occurrence of *blaTEM* alongside virulence gene in sheep and goat *E. coli* in India, while Zhao et al (17) found that 64.2% of diarrheic sheep *E. coli* in China carried more than one ESBL gene. The near total β-lactam resistance observed likely reflects the indiscriminate, often unsupervised use of amoxicillin and penicillin in smallholder farming in Balochistan, where these antibiotics are frequently purchased over the counter without veterinary prescription.

Tetracycline resistance exceeded 89% phenotypically in both host species, supported by molecular detection of *tetA* (76.6%) and *tetB* (73.3%). The predominance of *tetA* over *tetB* mirrors findings from Bangladesh (*tetA* at 84.4%) (18). Both genes encode active efflux pumps their co-occurrence in isolates indicates robust, multi mechanism resistance likely to persist even under reduced antibiotics pressure.

The high resistance rates documented here are not surprising given Balochistan's livestock farming context. β-Lactams and tetracycline are widely available without prescription, administered without laboratory guidance, and often used at sub therapeutic doses for extended periods classic conditions for resistance selection. These structural factors align with patterns identified globally by Herawati et al (19) in their systematic review of AMR in small ruminants which identified South Asia as a high-risk region.

In contrast, aminoglycosides (susceptibility 83.3-87.0%) and fluoroquinolones (87.8-90.7%) retained broad efficacy, likely reflecting comparatively lower pressure from less frequent use of these classes in local livestock farming. Similar patterns have been reported in China documented by Bello et al. (20). However, plasmid mediated quinolone resistance genes have been co-detected with ESBL genes in livestock *E. coli*, (20), threatening against assuming this susceptibility will persist if use of these antibiotics increases.

Our findings are consistent with previous studies confirming that resistant *E. coli* in Balochistan's small ruminants is part of a broader South Asian AMR tend, with shared risks of zoonotic transmission through food, environmental contamination, and direct animal human contact.

CONCLUSION

This study confirms widespread antibiotic resistance in *Escherichia coli* isolated from sheep and goats in Quetta, Balochistan. Complete phenotypic resistance to all tested β-lactam antibiotics and tetracycline was observed, with PCR based detection confirming high prevalence of *blaTEM* (69.1%), *blaCTXM* (62.5%), *tetA* (76.6%), and *tetB* (73.3%) resistance genes. Aminoglycosides and fluoroquinolones retained activity. These findings highlight the critical need for regulated antibiotic use, veterinary stewardship, and routine AMR surveillance in livestock populations across Pakistan to curb zoonotic transmission and safeguard public health.

Conflict of interest:

All authors declare no conflict of interest.

Acknowledgment:

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Authors' contribution:

KA Conceived the study, performed molecular detection of resistance genes and drafted the manuscript; ZUD Supervised the research; ISS Assisted in bacterial isolation, DNA extraction and interpretation of resistance gene profiles; FN Conducted antimicrobial susceptibility testing; MT Performed gel electrophoresis; SA Analyzed sequence data for gene variants and curated the resistance gene database; MN Contributed to statistical analysis of gene prevalence and phenotypic resistance correlation; IM Helped in sample collection, E. coli culture and biochemical identification; AD Participated in data validation and literature review on tetracycline resistance mechanisms; SE Managed laboratory logistics, quality control strainsand reagent preparation for molecular work.

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