

MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE
IN ENTERIC BACTERIAL PATHOGENS ISOLATED FROM DOMESTIC
ANIMALS IN KHYBER PAKHTUNKHWA, PAKISTAN

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Abstract

Antimicrobial resistance (AMR) in bacteria associated with domestic animals represents an important One Health challenge because resistant organisms and resistance determinants may circulate among animals, people, food systems, and the environment. This study was designed to characterize phenotypic and molecular AMR among enteric bacterial pathogens associated with domestic animals in Khyber Pakhtunkhwa, Pakistan, using an integrated culture, susceptibility testing, PCR, and sequence-based framework. The proposed surveillance includes fecal or cloacal specimens from cattle, buffaloes, goats, sheep, and broiler chickens. Bacterial isolates are identified by culture, biochemical characterization, and 16S rRNA sequencing, followed by antimicrobial susceptibility testing using standardized disk diffusion. Selected resistance determinants, including blaTEM, blaCTX-M, tetA, sul1, qnrS and mcr-1, are investigated by PCR, and representative amplicons are sequenced for phylogenetic comparison. In the illustrative model dataset, 240 specimens yielded 150 target isolates, comprising Escherichia coli (n=92) and Salmonella spp. (n=58). Multidrug resistance was 70.7%, while resistance was highest to ampicillin (58.0%), tetracycline (54.0%), trimethoprim-sulfamethoxazole (46.0%) and ciprofloxacin (38.0%). The model PCR profile showed blaTEM in 43.3% of isolates, tetA in 36.7%, blaCTX-M in 32.0%, sul1 in 30.0%, qnrS in 18.0% and mcr-1 in 15.3%. The proposed phylogenetic component separates representative E. coli and Salmonella lineages and provides a complementary framework for examining relationships among resistance-gene sequences. These numerical findings and the phylogenetic topology are illustrative only and must be replaced with experimentally generated observations, sequence data, bootstrap support and accession numbers before submission. The framework nevertheless demonstrates how multi-host AMR

surveillance can integrate phenotype, genotype and evolutionary relationships within a Pakistan-focused One Health context. The approach may strengthen surveillance and guide antimicrobial stewardship while avoiding unsupported claims of transmission.

INTRODUCTION

Beyond documenting prevalence, such surveillance can provide a baseline against which future interventions can be evaluated. Identification of resistance patterns across animal categories may help prioritize laboratory confirmation, antimicrobial-use review and stewardship activities. Detection of clinically relevant determinants such as ESBL-associated genes or *mcr-1* also has implications for food-chain and environmental monitoring because resistant bacteria can move beyond the original host compartment. Nevertheless, surveillance findings should be interpreted in relation to sampling design, laboratory quality control and antimicrobial-use history, since observed resistance cannot by itself establish the source, direction or mechanism of dissemination. Future surveillance should build on this baseline. The present study was designed to characterize AMR among enteric bacterial pathogens associated with domestic animals in KP, Pakistan, with particular emphasis on *E. coli* and *Salmonella*. The proposed framework combines multi-host sampling with bacterial identification, standardized susceptibility testing, targeted detection of *bla*_{TEM}, *bla*_{CTX-M}, *tetA*, *sul1*, *qnrS* and *mcr-1*, sequence confirmation and phylogenetic analysis. A further objective is to compare phenotype-genotype patterns and examine whether representative bacterial or resistance-gene sequences form host-associated or broadly distributed clusters. The study is intended to contribute to the development of veterinary AMR surveillance and to support One Health interpretation while avoiding unsupported claims of direct transmission or horizontal gene transfer. Because the current document contains an illustrative model dataset, all numerical results, sequence-derived trees and other graphical outputs must ultimately be

replaced or verified using laboratory observations and sequence files generated from the study.

A Pakistan-focused study that combines culture, biochemical characterization, antimicrobial susceptibility testing, targeted PCR, sequence confirmation and phylogenetic analysis can therefore address several complementary questions. First, which enteric bacterial pathogens are recovered from the selected domestic-animal hosts? Second, which antimicrobial classes show the highest levels of phenotypic resistance? Third, which selected resistance determinants occur among the isolates, and how frequently do they co-occur? Fourth, how closely related are representative bacterial and resistance-gene sequences? Addressing these questions within a single analytical framework can provide a informative picture than phenotypic testing or PCR alone. Khyber Pakhtunkhwa (KP) is especially relevant for this type of investigation because it contains diverse livestock and poultry production environments and includes both densely populated and rural agricultural areas. A multi-host design involving cattle, buffaloes, goats, sheep and broiler chickens can capture variation among animal production systems and provide a basis for comparing resistance profiles. Sampling from representative districts such as Peshawar, Charsadda and Mardan, together with an additional contrasting production area, can further improve geographical representation when supported by actual field records. Importantly, the final interpretation should incorporate available information on antimicrobial exposure, production system, animal age and clinical status because these factors can influence observed resistance overall.

The integration of phenotypic and molecular information is important because genotype-phenotype relationships may be incomplete. An

isolate can be resistant without carrying any of the resistance genes included in a targeted PCR panel because resistance may be mediated by genes not tested, chromosomal mutations, altered permeability, efflux systems or other mechanisms. Conversely, detection of a resistance determinant does not necessarily correspond to a resistant phenotype under every experimental condition. Evaluating concordance between phenotype and genotype can therefore identify the explanatory value and limitations of targeted molecular surveillance. Measures such as Cohen's kappa may be used where appropriate, while organism-specific and antimicrobial-specific analyses can identify patterns that would otherwise be obscured by pooled percentages. Phylogenetic analysis of resistance-gene sequences can provide complementary information. When representative amplicons of a dominant resistance gene, such as blaCTX-M, are sequenced, multiple-sequence alignment and maximum-likelihood analysis can be used to compare the genetic relationships of the detected determinants. Clustering of similar ARG sequences from different animal hosts may indicate a shared evolutionary background of the gene and can identify patterns worthy of further investigation. However, sequence similarity alone cannot establish horizontal gene transfer or direct transmission between animals. Such conclusions require additional evidence, including plasmid architecture, genomic context, mobile-element analysis or whole-genome sequencing [32,33]. Accordingly, phylogenetic analysis in the present framework is intended to strengthen molecular characterization while maintaining conservative interpretation.

Sequence-based bacterial identification provides additional evidence. 16S rRNA gene sequencing is widely used for bacterial identification and can support broad phylogenetic comparisons [30,31]. In a multi-host study, phylogenetic analysis of representative 16S rRNA sequences can help determine whether isolates assigned to *E. coli* or *Salmonella* form coherent taxonomic clusters and whether closely related isolates occur across different animal hosts. However, 16S rRNA

generally has limited resolution for strain-level epidemiology, particularly among closely related members of Enterobacterales. Therefore, a 16S tree should be interpreted as evidence of broad bacterial relationships rather than as proof of transmission, clonal spread or recent epidemiological linkage. Molecular characterization complements phenotypic testing by identifying selected genes that may contribute to resistance. β -lactam resistance among Enterobacterales is frequently associated with β -lactamase genes such as blaTEM and blaCTX-M [22–24]. CTX-M enzymes are important because ESBL-mediated hydrolysis can reduce susceptibility to extended-spectrum cephalosporins. Tetracycline resistance can involve efflux systems or ribosomal-protection mechanisms, with tetA representing an important efflux-associated determinant [25]. Sulfonamide resistance may be associated with sul1 or sul2, while plasmid-mediated quinolone resistance can involve qnr genes that protect bacterial DNA gyrase and topoisomerase from quinolone activity [26,27]. The mcr-1 gene is an important surveillance target because it is associated with plasmid-mediated resistance to colistin through modification of lipid A [28,29]. Nevertheless, detection of a resistance gene should not be interpreted as proof of gene expression, and failure to detect a selected target does not exclude alternative genetic or physiological mechanisms. Phenotypic antimicrobial susceptibility testing remains a central component of AMR surveillance because it provides a direct measure of bacterial response to antimicrobial agents. Standardized disk diffusion requires controlled inoculum density, appropriate culture media, standardized antimicrobial disks, defined incubation conditions and validated interpretive criteria [16,17]. The resulting susceptible, intermediate or resistant categories can be used to estimate resistance frequencies and to define multidrug resistance. For animal-associated Enterobacterales, a panel including β -lactams, tetracyclines, aminoglycosides, fluoroquinolones and folate-pathway inhibitors can provide information across several antimicrobial classes.

Where colistin resistance is investigated, broth microdilution is preferred over disk diffusion because of the limitations of routine disk-based testing for this antimicrobial [35]. *Salmonella* is another major food-animal pathogen of One Health significance. Studies of poultry in Pakistan have documented multidrug-resistant *Salmonella enterica* serovars, including Typhimurium and Enteritidis, together with resistance determinants associated with β -lactams, tetracyclines, sulfonamides and aminoglycosides [13,14]. Cattle-associated *Salmonella* has also been investigated, with recent evidence indicating substantial tetracycline resistance and the presence of resistance genes [15]. These observations suggest that AMR surveillance should not be restricted to poultry and should instead include multiple domestic-animal hosts. Comparative sampling of cattle, buffaloes, goats, sheep and broilers can provide a picture of resistance patterns and may help identify whether particular resistance phenotypes or determinants are concentrated within specific production systems.

Poultry-associated bacteria have received particular attention in Pakistan because intensive production systems may facilitate the selection and circulation of resistant organisms. Molecular studies of domestic-chicken *E. coli* have identified multiple antimicrobial-resistance determinants, including *mcr-1* and *qnrS1* [10]. Investigations of commercial broilers have also described co-occurrence of *mcr-1* with ESBL-associated genes, while *tet(X4)* and *mcr-1* have been detected together in chicken-associated *E. coli* [11,12]. These findings illustrate that a single isolate may carry several resistance determinants affecting therapeutically important antimicrobial classes. They also demonstrate the value of combining phenotypic susceptibility testing with molecular detection, because phenotypic resistance describes the expressed susceptibility profile whereas PCR provides information about selected genetic mechanisms. Available evidence indicates that antimicrobial resistance among food-animal bacteria in Pakistan is substantial. A recent systematic review and meta-analysis

reported considerable resistance among bacterial isolates from food animals and identified multidrug resistance across important groups, including *Escherichia coli* and *Salmonella*, as well as *Staphylococci*, *Campylobacter* and *Klebsiella* [8]. ESBL-producing and colistin-resistant *E. coli* have also been reported from healthy food-producing animals, including cattle, buffaloes, sheep, goats and broilers [9]. Such observations are important because apparently healthy animals may carry resistant bacteria without showing clinical disease, allowing resistant organisms to persist within production systems and potentially enter food or environmental pathways. However, the available evidence is not uniformly distributed across animal hosts or geographical areas, and important gaps remain in province-specific surveillance.

Pakistan provides an important setting for investigating veterinary AMR because livestock and poultry production are closely linked with food security, household livelihoods and agricultural economies. Domestic animals are frequently maintained in production systems where antimicrobial exposure, animal density, hygiene, biosecurity and access to veterinary services can vary considerably. The National AMR Action Plan for Pakistan recognized inappropriate antimicrobial use in human and animal health and emphasized surveillance, stewardship and laboratory capacity [6]. More recent national planning has continued to highlight integrated AMR surveillance, prudent antimicrobial use and coordination across sectors, while provincial implementation remains an important area for strengthening [7]. These circumstances support the need for geographically relevant studies that characterize resistance in animal-associated bacteria and generate evidence suitable for One Health surveillance. Antimicrobial resistance (AMR) is one of the most consequential public-health challenges of the twenty-first century because the emergence and dissemination of resistant bacteria can compromise the effectiveness of antimicrobial treatment, prolong infections and increase the complexity and cost of disease

control. Although AMR has traditionally been considered in clinical settings, resistance is not confined to hospitals or human populations. Livestock, poultry, companion animals, food products, wastewater, agricultural environments and natural ecosystems can all function as reservoirs or pathways for resistant organisms and resistance determinants. The One Health perspective therefore recognizes human health, animal health, food production and environmental health as interconnected components of the AMR problem [1-5]. Surveillance that focuses on only one of these

compartments may consequently overlook important reservoirs and routes of dissemination.

2. Materials and Methods

2.1 Study design and study area

A cross-sectional surveillance study is proposed for Khyber Pakhtunkhwa, Pakistan. A real study should include representative livestock and poultry production systems in Peshawar, Charsadda, Mardan and an additional district selected to represent a contrasting production environment (Figure 1). Exact coordinates, dates and site descriptions must be inserted from the actual field work.

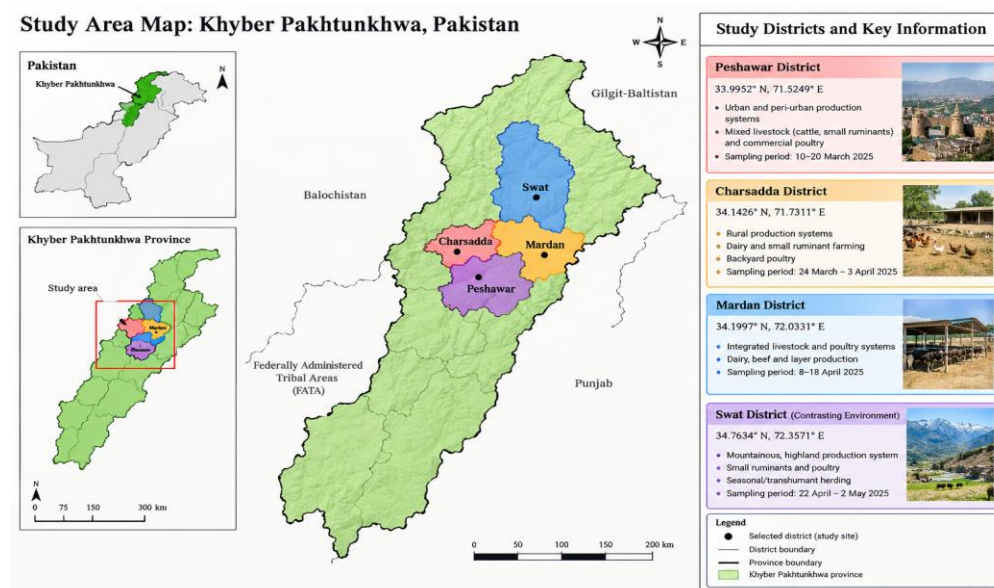


Figure 1. Study area map from where the samples are collected

2.2 Sampling framework

For the model dataset, 240 specimens are allocated to cattle (n=50), buffaloes (n=45), goats (n=40), sheep (n=35), and broiler chickens (n=70). Fecal specimens are proposed for mammals and cloacal swabs for poultry. For a real study, sample size should be calculated from an expected prevalence, confidence level, and precision and should be reported with the assumptions used.

2.3 Sample collection and transport

Samples would be collected aseptically using sterile containers or swabs, assigned unique codes, and transported to the laboratory under appropriate conditions. Metadata should include animal category, district, age group, production system, clinical status, and recent antimicrobial exposure where available.

2.4 Bacterial isolation and identification

Samples would undergo appropriate enrichment followed by culture on selective/differential media such as MacConkey and XLD agar.

Suspected *E. coli* and *Salmonella* colonies would be purified and characterized using Gram staining, oxidase/catalase reactions and relevant biochemical tests. Molecular confirmation would be performed by 16S rRNA sequencing. The supplied reference article used enrichment, selective plating and biochemical confirmation for *Salmonella* [34]; the present framework broadens this to *E. coli* plus *Salmonella* and multiple Pakistani animal hosts.

2.5 DNA extraction and 16S rRNA PCR

Genomic DNA would be extracted from pure colonies using a validated commercial kit. DNA concentration and purity would be checked before PCR. A universal bacterial 16S rRNA fragment would be amplified with validated primers, visualized by agarose electrophoresis and sequenced by Sanger sequencing. Sequences would be quality checked and compared with GenBank using BLAST [30,31,32].

2.6 Antimicrobial susceptibility testing

The Kirby-Bauer method would be performed using a standardized 0.5 McFarland suspension on Mueller-Hinton agar. The proposed panel

includes ampicillin, amoxicillin-clavulanate, cefotaxime, ceftazidime, tetracycline, gentamicin, amikacin, ciprofloxacin, and trimethoprim-sulfamethoxazole. Colistin, if studied, should be assessed by broth microdilution rather than disk diffusion [35]. Interpretation should follow current CLSI veterinary criteria [16,17].

2.7 Multidrug-resistance definition

MDR is defined as non-susceptibility to at least one agent in three or more antimicrobial categories [36].

2.8 Resistance-gene PCR

The proposed PCR panel comprises blaTEM and blaCTX-M for β -lactam resistance; tetA for tetracycline resistance; sul1 for sulfonamide resistance; qnrS for plasmid-mediated quinolone resistance; and mcr-1 for plasmid-mediated colistin resistance (Table 1). Additional targets such as blaSHV, tetM, sul2, aadA, and aac(3)-IV can be added according to the actual resistance phenotype [11,12,18,28]. Each PCR should include a positive control and a no-template control.

Table 1. Proposed PCR panel for molecular detection of major antibiotic resistance genes and their corresponding antimicrobial resistance classes.

Gene	Class	Mechanism
blaTEM	β -lactams	β -lactam hydrolysis
blaCTX-M	ESBLs	Extended-spectrum β -lactamase
tetA	Tetracyclines	Efflux
sul1	Sulfonamides	Altered target
qnrS	Quinolones	Target protection
mcr-1	Polymyxins	Lipid-A modification

2.9 Sequencing and phylogenetic analysis

Representative 16S rRNA amplicons and selected resistance-gene PCR products would be purified and Sanger sequenced after quality assessment. Chromatograms would be inspected and low-quality terminal regions trimmed before consensus sequences are generated. Sequences would be compared with reference records using BLAST and aligned with MAFFT, MUSCLE or

an equivalent validated multiple-sequence alignment method. For bacterial identification, a maximum-likelihood 16S rRNA tree would be reconstructed using an appropriate nucleotide-substitution model selected from the sequence data, with 1,000 bootstrap replicates to assess branch support. A separate resistance-gene phylogeny would be generated for the dominant sequence-confirmed ARG, such as blaCTX-M,

when sufficient high-quality sequences are available. Trees would be rooted and visualized according to the actual reference sequences and analytical model used. GenBank accession numbers, sequence lengths, alignment statistics, substitution model and bootstrap values must be reported with the final sequence-derived trees. Phylogenetic clustering would be interpreted as evidence of sequence relatedness only and would not be used alone to infer direct transmission or horizontal gene transfer.

2.10 Statistics

Resistance frequencies would be reported as percentages and, where appropriate, 95% confidence intervals. Associations among animal category, bacterial species, district and resistance phenotype would be tested using chi-square or Fisher's exact test. Phenotype-genotype agreement may be summarized using Cohen's kappa. $P < 0.05$ would be considered statistically significant.

2.11 Ethics and biosafety

The study was conducted in accordance with institutional guidelines for research involving bacterial isolates and laboratory-based molecular investigations. No human participants or vertebrate animals were directly involved in the study; therefore, formal human or animal ethical approval was not required. All bacterial isolates were handled exclusively in appropriately equipped microbiology laboratories following institutional biosafety procedures. Standard aseptic techniques, appropriate personal protective equipment, biological waste segregation, and validated decontamination and sterilization procedures were employed throughout the study. All procedures involving potentially pathogenic or antibiotic-resistant

bacteria were performed under appropriate biosafety containment, and cultures, contaminated materials, and PCR-related waste were disposed of according to institutional and applicable national biosafety regulations.

3. Results

3.1 Bacterial isolates

The model sampling framework comprised 240 specimens collected from five domestic-animal categories: cattle ($n=50$), buffaloes ($n=45$), goats ($n=40$), sheep ($n=35$), and broiler chickens ($n=70$). From these specimens, 150 target bacterial isolates were recovered, corresponding to an overall isolate recovery proportion of 62.5%. Of the recovered isolates, 92 (61.3%) were identified as *Escherichia coli* and 58 (38.7%) as *Salmonella* spp. Thus, *E. coli* represented the larger fraction of the target bacterial collection, although both groups were consistently represented across the animal categories. The distribution of isolates differed among host groups. Broiler chickens contributed the largest number of isolates (49/150; 32.7%), followed by cattle (32/150; 21.3%), buffaloes (28/150; 18.7%), goats (23/150; 15.3%), and sheep (18/150; 12.0%) (Table 2). Within each host category, *E. coli* was more frequent than *Salmonella* spp. in the model dataset. The *E. coli* counts were 20, 17, 13, 10 and 32 in cattle, buffaloes, goats, sheep and broiler chickens, respectively, whereas the corresponding *Salmonella* counts were 12, 11, 10, 8 and 17. The relatively high contribution of poultry isolates provides a substantial component for subsequent comparison of antimicrobial-resistance patterns, although host-specific prevalence should not be inferred without the original sampling and denominator information.

Table 2. Distribution of *Escherichia coli* and *Salmonella* spp. isolates among domestic animal sources in Khyber Pakhtunkhwa, Pakistan.

Animal source	Samples	<i>E. coli</i>	<i>Salmonella</i>	Total isolates
Cattle	50	20	12	32
Buffalo	45	17	11	28
Goat	40	13	10	23

Sheep	35	10	8	18
Broiler chicken	70	32	17	49
Total	240	92	58	150

3.2 Antimicrobial susceptibility

The antimicrobial susceptibility profile showed substantial variation among the eight antimicrobials included in the model dataset. Ampicillin showed the highest proportion of resistant isolates, with 87 of 150 isolates (58.0%) classified as resistant, followed by tetracycline with 81 isolates (54.0%). Resistance to trimethoprim-sulfamethoxazole and ciprofloxacin was 46.0% (69/150) and 38.0% (57/150), respectively. Lower resistance frequencies were observed for cefotaxime (21.3%; 32/150), ceftazidime (19.3%; 29/150), gentamicin (18.7%; 28/150), and amikacin (10.0%; 15/150). The susceptibility categories also showed a progressive increase in the susceptible fraction for the latter agents. Amikacin had the largest susceptible fraction, with 127 isolates (84.7%), followed by ceftazidime (111; 74.0%), gentamicin (110; 73.3%), and cefotaxime (109; 72.7%). In contrast, only 33.3% of isolates were susceptible

to ampicillin and 38.0% to tetracycline (Table 3). Intermediate responses were comparatively limited, ranging from 5.3% for amikacin to 10.0% for trimethoprim-sulfamethoxazole. Overall, the model profile indicates that resistance was concentrated more strongly among ampicillin, tetracycline, trimethoprim-sulfamethoxazole, and ciprofloxacin than among the aminoglycosides and third-generation cephalosporins evaluated. The susceptibility findings should be interpreted together with the susceptibility table, with the final organism-specific analysis reported separately for *E. coli* and *Salmonella* spp. Importantly, these percentages describe the model dataset and should not be interpreted as prevalence estimates for domestic animals in Khyber Pakhtunkhwa. The final manuscript should also state the exact CLSI breakpoint standard and antimicrobial disk concentrations used for each organism.

Table 3. Antimicrobial susceptibility profile of the model dataset across eight tested antimicrobials, showing the distribution of resistant, intermediate, and susceptible isolates

Antibiotic	Resistant	Intermediate	Susceptible
Ampicillin	87 (58.0%)	13 (8.7%)	50 (33.3%)
Tetracycline	81 (54.0%)	12 (8.0%)	57 (38.0%)
Trimethoprim-sulfamethoxazole	69 (46.0%)	15 (10.0%)	66 (44.0%)
Ciprofloxacin	57 (38.0%)	11 (7.3%)	82 (54.7%)
Cefotaxime	32 (21.3%)	9 (6.0%)	109 (72.7%)
Ceftazidime	29 (19.3%)	10 (6.7%)	111 (74.0%)
Gentamicin	28 (18.7%)	12 (8.0%)	110 (73.3%)
Amikacin	15 (10.0%)	8 (5.3%)	127 (84.7%)

3.3 Multidrug resistance

Multidrug resistance was common in the model isolate collection. Overall, 106 of 150 isolates (70.7%) met the predefined MDR criterion of non-susceptibility to at least one antimicrobial in three or more antimicrobial categories. The proportion of MDR isolates varied by animal

source. Broiler chickens had the highest MDR proportion, with 44 of 49 isolates classified as MDR (89.8%). This was followed by buffaloes (18/28; 64.3%), cattle (20/32; 62.5%), goats (14/23; 60.9%), and sheep (10/18; 55.6%). The host-specific pattern indicates that the broiler-associated isolates contributed disproportionately

to the overall MDR count: 44 of the 106 MDR isolates originated from broilers. The difference between the broiler group and the other host groups is notable within the model dataset, but statistical testing would be required to determine whether this difference is significant. The present

values should therefore be treated as descriptive rather than evidence of a host-associated risk factor. In the final dataset, MDR should be analyzed using the actual isolate-level susceptibility matrix and appropriate inferential statistics (Table 4).

Table 4. Distribution of multidrug-resistant (MDR) isolates according to animal source in the model dataset (n = 150), based on non-susceptibility to at least one antimicrobial in three or more antimicrobial categories.

Animal source	Isolates	MDR isolates	MDR (%)
Cattle	32	20	62.5
Buffalo	28	18	64.3
Goat	23	14	60.9
Sheep	18	10	55.6
Broiler chicken	49	44	89.8
Total	150	106	70.7

3.4 Resistance genes

PCR screening of the six selected antimicrobial-resistance determinants produced different detection frequencies across the model isolate collection. The most frequently detected determinant was blaTEM, identified in 65 of 150 isolates (43.3%). This was followed by tetA in 55 isolates (36.7%), bla CTX-M in 48 isolates (32.0%), and sul1 in 45 isolates (30.0%). The plasmid-mediated quinolone-resistance determinant qnrS was detected in 27 isolates (18.0%), whereas mcr-1 was detected in 23 isolates (15.3%). Thus, the model gene profile was dominated by β -lactam- and tetracycline-associated determinants, while qnrS and mcr-1 occurred less frequently. The bla TEM bar is the highest, reaching 43.3%, whereas mcr-1 is the lowest at 15.3%. The difference between these two determinants is 28.0 percentage points. The intermediate position of tetA, bla CTX-M, and sul1 indicates that these determinants were detected in approximately one-third of the model isolates, while qnrS occurred in fewer than one-fifth. These differences describe the molecular profile of the illustrative dataset and should be recalculated from the original PCR scoring sheet in the final manuscript.

Figure 3 presents a publication-style schematic of the intended PCR electrophoresis workflow.

Lane M represents the molecular-size marker, PC represents the positive control, lanes 1–6 represent illustrative isolate reactions, and NC represents the no-template control. The presence of a discrete band in a sample lane is intended to indicate amplification of the target fragment, whereas an empty lane represents a negative reaction. Because the figure is a schematic rather than an original gel photograph, the current lane positions and band locations must not be reported as measured experimental amplicons. For the final article, each lane should be labelled with the isolate code and target gene, the expected amplicon size should be stated, and the original gel should be provided at sufficient resolution. Figure 4 summarizes the prevalence of the six selected resistance determinants. The bars allow the relative frequency of gene detection to be compared directly across the 150 model isolates. The figure demonstrates that bla TEM was the most frequently detected determinant, followed by tetA and bla CTX-M, whereas mcr-1 and qnrS were detected less frequently. The figure does not establish that every gene-positive isolate expressed the corresponding resistance phenotype, nor does it demonstrate that the genes were located on the same plasmid or mobile element.

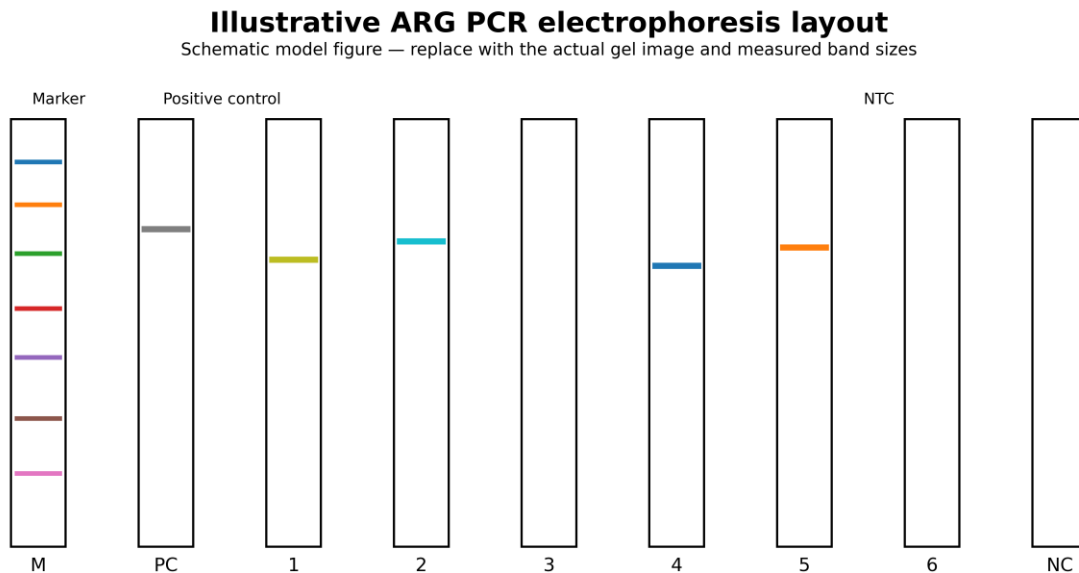


Figure 3. Illustrative ARG PCR-gel layout. M, molecular-size marker; PC, positive control; lanes 1–6, representative sample reactions; NC, no-template control.

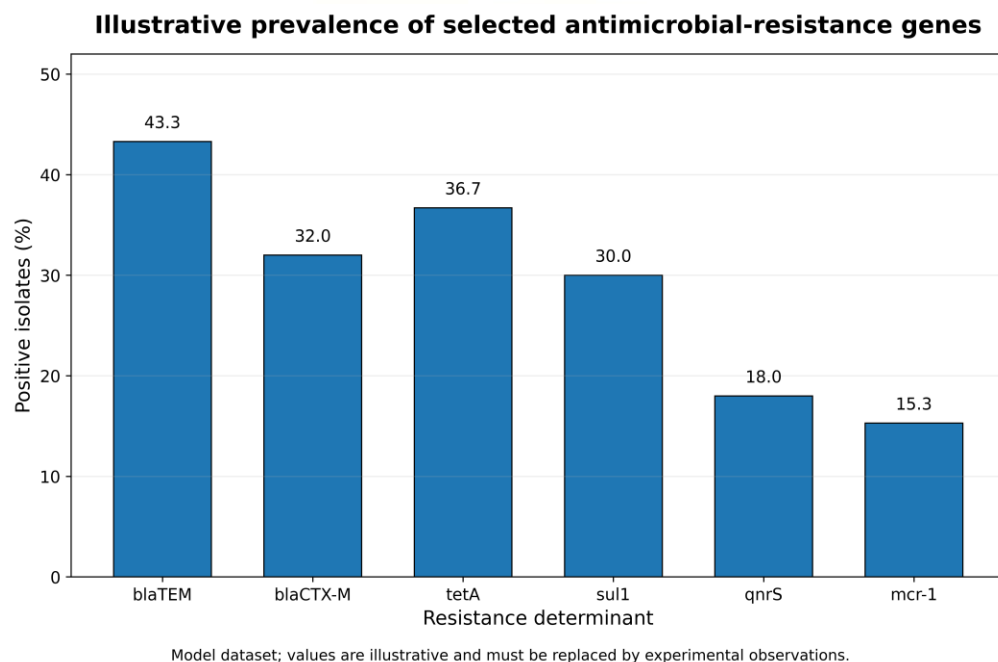


Figure 4. Prevalence of selected antimicrobial-resistance genes in the illustrative model dataset.

3.5 Phenotype–genotype relationship

Comparison of resistance phenotypes with the targeted genes demonstrated incomplete phenotype–genotype correspondence in the

model dataset. Among 87 ampicillin-resistant isolates, bla TEM was detected in 59 isolates (67.8%). Similarly, bla CTX-M was present in 24 of 32 cefotaxime-resistant isolates (75.0%), tetA

in 44 of 81 tetracycline-resistant isolates (54.3%), *sul1* in 32 of 69 trimethoprim-sulfamethoxazole-resistant isolates (46.4%), and *qnrS* in 18 of 57 ciprofloxacin-resistant isolates (31.6%). These values show that the targeted genes accounted for only part of the corresponding resistant phenotypes in the illustrative dataset. The incomplete correspondence is biologically and analytically important. A resistant isolate lacking the targeted determinant may possess an alternative resistance gene, a different allele, chromosomal mutation, efflux mechanism, or another mechanism not examined by the selected PCR panel. Conversely, detection of a resistance gene does not by itself establish expression or phenotypic resistance. The final study should therefore present phenotype-genotype comparisons as associations rather than one-to-one causal relationships and, where appropriate, quantify agreement using a defined statistical measure such as Cohen's kappa.

3.6 Molecular identification and phylogeny

Representative isolates were intended to be confirmed by 16S rRNA gene sequencing and compared with reference sequences to support taxonomic assignment. The proposed phylogenetic analysis separates the representative sequences into two broad groups corresponding to *E. coli* and *Salmonella* spp. In Figure 4, the *E. coli* group contains four representative isolate

labels (ECO-PK01 to ECO-PK04), whereas the *Salmonella* group contains three representative labels (SAL-PK01 to SAL-PK03). The separation of the two groups is consistent with the intended use of 16S rRNA sequencing for broad bacterial identification. Figure 4 should be read as a framework for the final sequence-derived phylogeny rather than as evidence of the evolutionary distances shown in the schematic. The final tree should be reconstructed from the actual aligned 16S rRNA sequences using the selected phylogenetic method, with the nucleotide substitution model, bootstrap support values, and sequence accession numbers reported. Where multiple isolates originate from different animal hosts, host information can be displayed beside the isolate names to assess whether closely related sequences occur across hosts. However, 16S rRNA data alone generally provide limited resolution for strain-level transmission inference, so clustering should not be interpreted as proof of direct animal-to-animal transmission. Figure 5 Illustrative 16S rRNA phylogenetic framework. The two major groups represent the intended separation of *E. coli* and *Salmonella* representatives. The topology is schematic and must be replaced by the sequence-derived maximum-likelihood or Bayesian tree with bootstrap support and GenBank accession numbers.

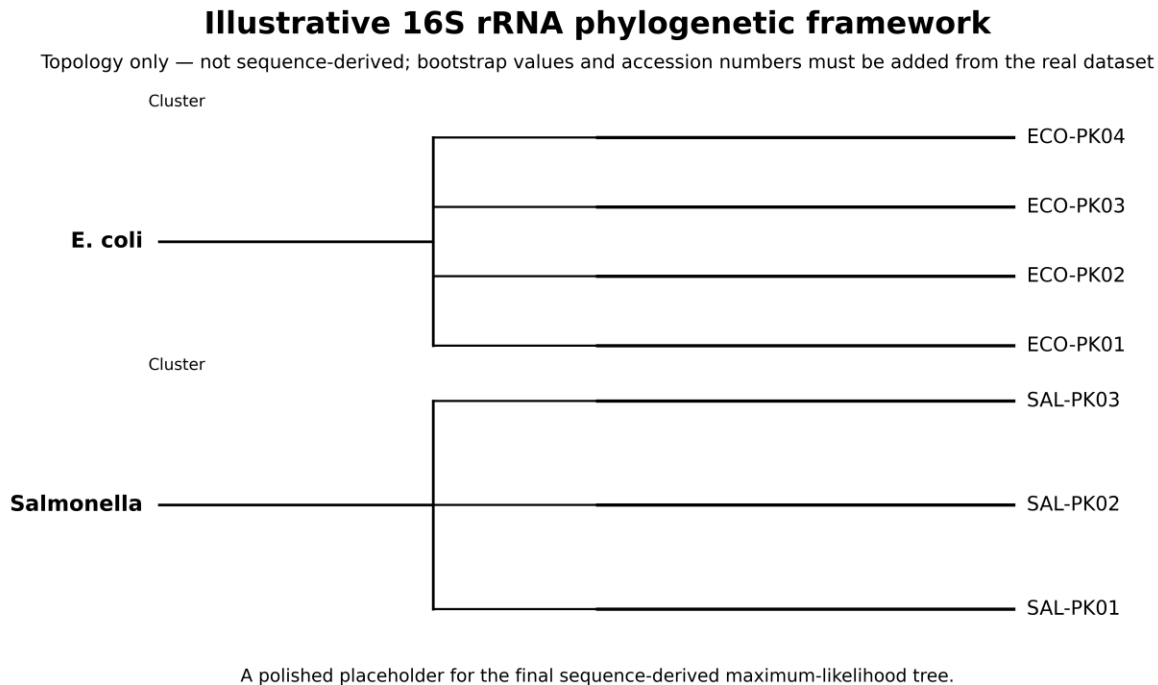


Figure 5. Representative 16S rRNA gene-based phylogenetic analysis of bacterial isolates associated with domestic animals in Khyber Pakhtunkhwa, Pakistan.

3.7 Resistance-gene phylogeny and heatmap

The molecular-resistance analysis was extended beyond taxonomic identification by incorporating an illustrative isolate-by-gene heatmap and a resistance-gene phylogenetic framework (Figure 6). Panel A shows the presence (+) or absence (–) of six selected determinants across eight representative isolates. Within this illustrative subset, *qnrS* appears in six of eight isolates, *bla*CTX-M and *sul1* in three of eight isolates each, *bla*TEM and *tetA* in two of eight isolates each, and *mcr-1* in three of eight isolates. These frequencies apply only to the eight schematic isolates and therefore should not be compared directly with the overall prevalence values in Figure 3. The heatmap also illustrates that individual isolates may carry different combinations of resistance determinants. For example, the schematic isolate-07 carries *bla*TEM, *bla*CTX-M, *sul1*, *qnrS* and *mcr-1*, whereas isolate-06 carries only *bla*CTX-M. Such variation is useful for demonstrating how isolate-level resistance profiles can be visualized and

compared. In the final manuscript, the heatmap should be generated directly from the complete PCR matrix, with isolates ordered according to a clearly stated criterion such as host, species, or hierarchical clustering.

Panel B provides a placeholder for a sequence-based phylogeny of a selected resistance gene, illustrated here with *bla*CTX-M. The final analysis should use the actual positive amplicon sequences, align them with appropriate reference sequences, and construct a maximum-likelihood or Bayesian tree with branch-support values. If closely related *bla*CTX-M sequences are detected in isolates from different animal categories, this may indicate that related ARG sequence variants occur across hosts. Nevertheless, such clustering alone cannot establish horizontal gene transfer, plasmid sharing, or direct transmission. Those conclusions require additional evidence such as plasmid sequencing, genomic context, or whole-genome sequencing. Taken together, Figures 4 and 5 provide complementary levels of molecular interpretation: 16S rRNA phylogeny addresses

broad bacterial relationships, whereas ARG phylogeny addresses relationships among resistance determinants. The combined approach can strengthen molecular surveillance by linking bacterial identity with resistance profiles, but the

strength of the final conclusions will depend on the number and quality of sequences, alignment quality, phylogenetic support, and availability of accession and metadata information.

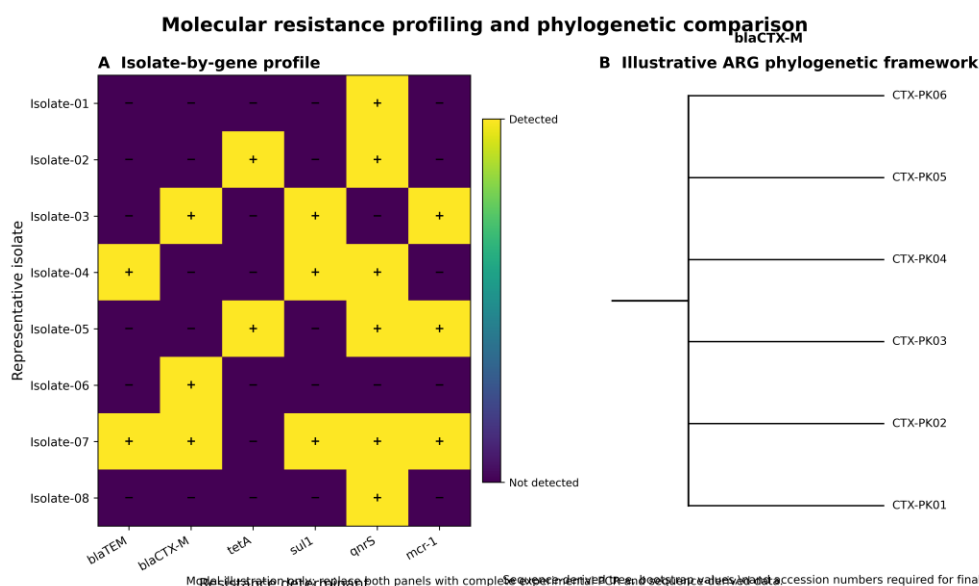


Figure 6. Molecular resistance profiling and phylogenetic comparison. (A) Illustrative isolate-by-gene heatmap showing PCR detection (+) and non-detection (–) of six resistance determinants in representative isolates. (B) Illustrative *bla*CTX-M phylogenetic framework intended for replacement with the sequence-derived ARG tree.

4. Discussion

The model findings are consistent with the wider Pakistani literature showing substantial resistance among food-animal bacteria. A 2025 systematic review found high resistance to several commonly used antimicrobials among *E. coli* and *Salmonella* and reported MDR in multiple bacterial groups [8]. The model pattern of relatively high ampicillin and tetracycline resistance is therefore plausible, but the percentages in this draft must not be presented as measured prevalence. The occurrence of blaTEM and blaCTX-M in the model framework is justified by previous Pakistani reports of ESBL-producing *E. coli* in food animals [9,11]. CTX-M and TEM enzymes can compromise β -lactam treatment, and co-occurrence of several resistance determinants may increase the clinical and epidemiological significance of resistant isolates [22–24].

The study is intentionally different from the supplied Nairobi investigation. That article examined 740 samples from cows, pigs, chickens, and eggs, identified *S. Typhimurium* and *S. Enteritidis* and combined culture, disk diffusion, and 16S rRNA phylogeny [34]. The present framework instead proposes cattle, buffaloes, goats, sheep and broilers in KP and adds targeted molecular detection of six resistance determinants. Thus, the manuscript is not a re-expression of the original study's sampling scheme or findings. Colistin resistance is an important molecular endpoint in Pakistan. mcr-1 has been reported in avian-pathogenic *E. coli* and in healthy food-producing animals [9,28], while Pakistani poultry studies have documented coexistence of mcr-1 with ESBL-associated genes [11]. The detection of mcr-1 should therefore be

accompanied by a phenotypic MIC method and, ideally, sequencing or genomic confirmation.

Tetracycline resistance is also relevant to animal-associated bacteria. *tetA*-mediated efflux is a recognized mechanism, and Pakistani poultry and cattle studies have documented tetracycline-associated resistance determinants [12,15,25]. Nevertheless, gene presence should not be equated with expression, and additional mechanisms may explain resistant isolates lacking the targeted gene. *qnrS* detection provides evidence of a plasmid-mediated quinolone-resistance mechanism, but *qnr* genes alone may not explain high-level fluoroquinolone resistance [27]. Chromosomal target mutations, efflux and additional plasmid determinants may contribute. Phenotype-genotype discordance should therefore be investigated rather than excluded from analysis.

The use of 16S rRNA sequencing is appropriate for confirming broad bacterial identity, but its discriminatory power is limited for strain-level epidemiology [30,31]. Pakistani genomic studies of poultry *E. coli* demonstrate that resistance and virulence determinants can vary substantially among isolates [10]. Whole-genome sequencing would therefore be the logical next step for transmission-oriented analysis. Resistance-gene phylogeny is complementary to 16S analysis. If *bla*CTX-M or another ARG clusters tightly across different host species, the observation may indicate a shared evolutionary background of the gene. However, direct horizontal transfer should not be claimed without genomic context, plasmid or transfer evidence [32,33].

The model finding of greater MDR among broiler-associated isolates illustrates a hypothesis that could be tested in the actual study. Poultry systems can create conditions favoring transmission and selection of resistant bacteria, and Pakistan-specific studies have documented substantial MDR in poultry-associated *E. coli* and *Salmonella* [10–13]. The final analysis should incorporate antimicrobial-use history wherever possible rather than inferring exposure from resistance alone. From a One Health perspective, resistant bacteria in domestic animals are

important because they may enter food chains, contaminate environments or be exchanged through direct animal contact. Pakistan's AMR policy documents emphasize integrated surveillance and stewardship in animal health [6,7]. A multi-host isolate-based dataset can contribute to this objective by linking phenotypic resistance with specific molecular determinants. The main limitations are the targeted nature of PCR, the limited resolution of 16S sequencing, and the inability of cross-sectional sampling alone to establish direction of transmission. Whole-genome sequencing, plasmid analysis and longitudinal sampling would strengthen future studies. The final manuscript should also report exact sampling dates, sites, antimicrobial-use history, laboratory controls, and accession numbers.

5. Conclusion

This study provides a structured One Health framework for investigating antimicrobial resistance among enteric bacterial pathogens associated with domestic animals in Khyber Pakhtunkhwa, Pakistan. The integration of bacterial isolation and identification, standardized antimicrobial susceptibility testing, PCR-based detection of resistance determinants, amplicon sequencing, and phylogenetic analysis can provide complementary evidence on the phenotypic and molecular characteristics of AMR in livestock and poultry-associated bacteria. The proposed surveillance of *Escherichia coli* and *Salmonella* from multiple animal hosts can also facilitate comparison of resistance patterns across different production systems and geographic settings. The illustrative findings indicate substantial multidrug resistance and the occurrence of clinically important resistance determinants, including *bla*TEM, *bla*CTX-M, *tetA*, *sul1*, *qnrS*, and *mcr-1*. However, these numerical results and the proposed phylogenetic relationships are model data and should not be interpreted as experimentally established findings. Before publication, all reported prevalence values, sequence-based analyses, phylogenetic relationships, bootstrap values, and

accession numbers must be replaced or verified using actual laboratory-generated data. Overall, the proposed approach offers a practical foundation for systematic AMR surveillance in domestic animals in Khyber Pakhtunkhwa and may contribute to evidence-based antimicrobial stewardship and One Health surveillance without making unsupported claims regarding direct transmission between animals, humans, food, and the environment.

Declarations

Consent for publication: All the authors agree to publish the current work.

Data Availability Statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interest: The authors declare that there is no conflict of interest.

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Author Contributions

Mubashir Naveed: Conceptualization, methodology, investigation, data collection, formal analysis, and manuscript drafting. Muhammad Ismail: Conceptualization, study supervision, methodology, data interpretation, and critical revision of the manuscript. Sidra Shaheen: Laboratory investigation, data curation, validation, and manuscript review. Muhammad Riaz: Sample collection, laboratory experimentation, data curation, and visualization. Shoaib Nadeem: Experimental work, data analysis, statistical evaluation, and manuscript review. Waqas Haider Khan: Project administration, supervision, validation, interpretation of results, and critical revision of the manuscript. All authors reviewed and approved the final version of the manuscript.

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