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RESEARCH ARTICLE

Molecular Detection of Carbapenemase and *mcr*-1 Resistance Genes in Enterobacterales at the Animal–Human Interface in Pakistan: A One Health Perspective

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ABSTRACT

Carbapenem- and colistin-resistant Enterobacterales (CRE and Col-R) are critical-priority pathogens within the One Health framework and pose a significant public health threat. This study investigated the prevalence and molecular characteristics of resistance across clinical, veterinary, and environmental sources. A total of 600 samples were collected, including clinical (urine and blood), veterinary (poultry cloacal swabs), and environmental (hospital and surface wastewater) specimens. Enterobacterales were identified using culture, biochemical tests and VITEK® 2. Antimicrobial susceptibility testing, MIC determination, Modified Hodge's Test, and PCR-based detection of carbapenemase genes and *mcr-1* were performed. Enterobacterales were detected in 41% of clinical, 100% of veterinary and 98–100% of environmental samples. Carbapenem resistance in clinical isolates was 32.4% in *E. coli* and 33.3% in *K. pneumoniae*, while colistin resistance was 10.8 and 5.6%, respectively. Veterinary *E. coli* showed 12.5% carbapenem resistance and 28% colistin resistance. Environmental isolates exhibited carbapenem resistance in 29.8% of *K. pneumoniae* and 11.1% of *E. coli*, with colistin resistance of 22.2 and 6%, respectively. Co-resistance to carbapenems and colistin was observed in environmental (11.1%), veterinary (4%) and clinical (2.7%) *E. coli*. High meropenem MICs reached 1024µg/mL and colistin MICs 32µg/mL. Genotypically, bla_{NDM} (66.7%) predominated, followed by bla_{VIM} (58.3%), bla_{IMP} (37.5%) and bla_{OXA} (33.3%). The *mcr-1* was detected in all phenotypically colistin-resistant isolates. High MAR indices (>0.5) were most frequent in environmental (68%) and veterinary (39%) isolates. These findings highlight widespread cross-sector transmission and emphasize the urgent need for integrated One Health surveillance and antimicrobial stewardship in Pakistan.

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INTRODUCTION

Antimicrobial resistance (AMR) represents a quintessential One Health challenge, driven by the interconnected dynamics of human, animal, and environmental systems (Al-Khalaifah *et al*., 2025). The widespread misuse and overuse of antimicrobials in clinical medicine, veterinary practice, and agricultural and environmental settings accelerate the emergence and dissemination of resistant bacteria and antimicrobial resistance genes (ARGs) (Estany-Gestal *et al*., 2024). These determinants circulate across sectors through direct contact, food production chains, wastewater discharge, and environmental contamination (Caneschi *et al*., 2023). Consequently, AMR increasingly undermines the effectiveness of antimicrobial therapy, facilitating the spread of difficult-to-treat infections and contributing to rising morbidity and mortality worldwide. In 2019 alone, bacterial AMR was associated with an estimated 4.95 million deaths worldwide, including 1.27 million deaths directly attributable to drug-resistant infections (Murray *et al*., 2022). Recent projections from the Global Research on Antimicrobial Resistance (GRAM) Project suggest that, without effective intervention, more than 39 million deaths could occur globally between 2025 and 2050 due to AMR infections (Naghavi *et al*., 2024). This growing burden is expected to disproportionately affect low- and middle-income countries (LMICs), particularly in South Asia, with older populations at greatest risk (Allel *et al*., 2024). The World Health Organization (WHO) has

designated carbapenem-resistant (CRE) and colistin-resistant (Col-R) Enterobacterales as critical-priority pathogens, reflecting both their limited therapeutic options and their capacity to cause severe, life-threatening infections (Ma *et al*., 2022). Carbapenems, including imipenem, meropenem and ertapenem, are broad-spectrum β-lactam antibiotics widely used for the treatment of serious Gram-negative infections (Finberg and Guharoy, 2021). Colistin, categorized within the WHO Reserve group, is often used against carbapenem-resistant strains (RoyChowdhury *et al*., 2025). The emergence of resistance to both carbapenems and colistin, therefore, represents a profound therapeutic challenge, substantially narrowing effective treatment options and increasing the risk of untreatable infections. The majority of *Escherichia coli* and *Klebsiella pneumoniae* acquired carbapenemase genes, including bla_{NDM}, bla_{VIM} $bla_{\text{IMP,}}$ and bla_{OXA}, while colistin resistance is due to the *mcr-1* gene (De Koster *et al*., 2024). In a study conducted in Pakistan, genomic analysis of carbapenem-resistant *K. pneumoniae* from the clinical setting identified NDM alleles on broad–host-range plasmids, including the first report of $bla_{\text{NDM-7}}$ carried on a hybrid IncFIB/IncHI1B plasmid backbone, a configuration rarely described worldwide (Qamar *et al*., 2025b). First case of colistin-resistant *E. coli* was detected from a 35-year-old male patient suffering from burn-associated wounds in Pakistan in 2016 (Mohsin *et al*., 2017). Moreover, two studies reported the mcr-1 gene in plasmid-mediated colistin resistance in avian-pathogenic *E. coli* from poultry samples (Azam *et al*., 2017; Lv *et al*., 2018). Pakistan ranks among the top ten countries in livestock production and relies extensively on antimicrobials for growth promotion and disease prevention. Antimicrobial use (AMU) in food animals is projected to increase by approximately 44% between 2020 and 2030, with overall consumption levels estimated to exceed 60% (Qiu *et al*., 2024) and environmental compartments further amplify this burden. Hospital effluents and surface wastewater accumulate antibiotic residues, resistant organisms and genetic determinants, creating conditions conducive to horizontal gene transfer (Al-Khalaifah *et al*., 2025). A wastewater-based genomic surveillance study from Pakistan reported widespread carbapenem resistance, identifying bla_{NDM} allele and bla_{OXA} in *E. coli* and *K. pneumoniae* (Sattar *et al*., 2024). Therefore, the One Health framework recognizes the intrinsic interconnectedness of human, animal and environmental health systems. Antimicrobial resistance exemplifies this interdependence, as resistant bacteria and resistance genes disseminate across sectors through globalization, population mobility, food production networks and environmental contamination. In this context, we investigated carbapenem and colistin resistance among Enterobacterales isolated from poultry cloacal swabs, clinical specimens (urine and blood) and hospital and surface wastewater under One Health umbrella in Pakistan.

MATERIALS AND METHODS

Ethical approval of the study: Between August 2024 and September 2025, we conducted a cross-sectional One Health investigation in Faisalabad, Pakistan, encompassing clinical, environmental and veterinary sources. The study was approved by the Ethical Review Committee (ERC) of Government College University, Faisalabad (Ref. No. GCUF/ERC/236). Clinical procedures complied with the Declaration of Helsinki and written informed consent was obtained from all participants, and the patients' information was kept confidential throughout the study.

Samples collection from different sources: A total of 600 samples were collected between August 2024 and September 2025 across veterinary, clinical, and environmental domains in Faisalabad, Pakistan. Poultry cloacal swabs (n=200) were collected from different poultry farms within a 30 km radius of Faisalabad using Amies transport swabs. Farms with active poultry production and no recent disinfection procedures within 48 h prior to sampling were included in the study. Only apparently healthy birds from commercial broiler and layer farms were sampled to ensure representative surveillance of circulating Enterobacterales. All veterinary samples were transported to the laboratory at 4°C for further processing. Clinical sampling included 200 specimens comprising blood (n=100) and urine (n=100) collected from patients admitted to tertiary care hospitals. Inclusion criteria for clinical isolates included patients presenting with suspected bacterial infections and no duplicate samples from the same patient during the study period. The samples were collected before patients' antibiotics were taken. Midstream urine samples were collected using the clean-catch technique in sterile containers and transported to the laboratory at 3–4°C within 2h of collection. Venous blood (5–10mL) was aseptically drawn following skin disinfection with 70% isopropyl alcohol and 2% iodine tincture and inoculated into BD BACTEC blood culture bottles, which were incubated in the BD BACTEC alert system for up to 5 days. Environmental sampling comprises 200 wastewater specimens, including 100 hospital wastewater (HWW) samples collected from drainage outlets of clinical wards and 100 surface wastewater samples (SWW) obtained from canals across the city. Sampling sites were selected based on continuous wastewater flow, accessibility, and proximity to areas with high anthropogenic activity. Samples were collected in sterile screw-cap containers and transported to the laboratory within 2 h at 4–6°C. Across all domains, only properly labeled, non-leaking, and adequately preserved samples were included for microbiological analysis, while contaminated, insufficient-volume, or improperly transported samples were excluded.

Samples processing and bacterial confirmation: Urine specimens were cultured on cystine–lactose–electrolyte-deficient (CLED) agar. Positive blood culture bottles were sub-cultured onto blood and MacConkey agar plates and incubated aerobically at 37°C overnight. Poultry cloacal swabs and 50µL aliquots of hospital and surface wastewater samples were plated on blood and MacConkey agar supplemented with meropenem (4µg/mL) or colistin (4µg/mL) and incubated at 37°C overnight under aerobic conditions. Preliminary identification was based on colony morphology, Gram staining and standard biochemical testing using API 20E. Further, species-level identification

was confirmed using the VITEK® 2 Compact system (bioMérieux, France) with GN identification cards according to the manufacturer's instructions. In brief, bacterial suspensions were standardized to 0.5 McFarland, loaded into GN ID cards, and processed in VITEK 2 system. Identification was assigned by the system software based on kinetic biochemical profiling.

Antimicrobial susceptibility testing of isolates: Antimicrobial susceptibility testing (AST) was performed by disk diffusion method using Mueller–Hinton agar (MHA). Bacterial suspensions standardized to 0.5 McFarland and swabbed on MHA plates. Different classes of antibiotic discs were placed on MHA at distance of 15mM. Plates were incubated at 37°C for 18–24 hrs and inhibition zone diameters were interpreted according to Clinical Laboratory Standard Institute (CLSI) 2024 guidelines. The antibiotic panel included β-lactams (ampicillin, amoxicillin–clavulanate, piperacillin–tazobactam, ceftriaxone, ceftazidime, cefepime, imipenem, meropenem), aminoglycosides (amikacin), fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin), tetracycline (tigecycline), co-trimoxazole, fosfomycin, nitrofurantoin, and polymyxin (colistin) (Qamar *et al*., 2025a). Further, minimum inhibitory concentration (MIC; µg/mL) determination was performed using the VITEK® 2 Compact system (bioMérieux) with AST cards, following the manufacturer's instructions and CLSI breakpoints. Isolates resistant to at least one carbapenem were classified as CRE. In addition, colistin MICs were determined using microbroth dilution in Mueller–Hinton broth in 96 microtiter plates. Two-fold serial dilutions (0.25–16µg/mL) were prepared in 96-well microplates and incubated at 37°C for 16–20h. The MIC was defined as the lowest concentration inhibiting visible growth. Colistin resistance was assigned at MIC ≥4µg/mL according to CLSI criteria (Ejaz *et al*., 2021).

Determination of the MAR index: The multiple antibiotic resistance (MAR) index was calculated for each isolate as a measure of cumulative antimicrobial resistance burden. The index was defined as the ratio of the number of antibiotics to which the isolate exhibited resistance to the total number of antibiotics tested. (Woh *et al*., 2023; Qamar *et al*., 2025b)

$$\text{MAR index} = \frac{\text{Number of antibiotics resistant (a)}}{\text{Total antibiotics tested (b)}}$$

MAR index values were calculated separately for isolates from environmental, veterinary, and clinical domains to assess the extent of multidrug resistance. A MAR index ≥0.2 was considered indicative of significant resistance associated with environments of frequent antibiotic use. Fourteen, eleven, and eighteen antibiotics were included in the MAR calculations for environmental, veterinary, and clinical isolates, respectively. The distribution of MAR index categories across the three domains was visualized using stacked plots generated in R (version 4.3.1) with the ggplot2 package.

Phenotypic confirmation of carbapenemase producing bacteria: Carbapenemase production was phenotypically screened using the Modified Hodge Test (MHT) as described in the CLSI recommended phenotypic detection framework and previously published protocols (Qamar *et al*., 2019). Briefly, a suspension of the quality control strain American Type Culture Collection *Escherichia coli* ATCC 25922 was prepared and adjusted to a 0.5 McFarland standard using sterile saline. This suspension was then diluted at a ratio of 1:10 in sterile normal saline and uniformly lawn-inoculated onto Mueller–Hinton agar (MHA) plates to create a confluent bacterial lawn. After allowing the surface to dry for 3–5 minutes at room temperature, a 10 µg meropenem disc was aseptically placed at the center of each MHA plate. Test isolates were then streaked in a straight line from the edge of the meropenem disc to the periphery of the plate using a sterile inoculating loop, ensuring that each isolate was streaked in a single line and adequately spaced when multiple isolates were tested on the same plate. A known carbapenem-susceptible control strain was included in each batch to ensure test validity. All plates were incubated aerobically at 37°C for 18–24 hours under standard laboratory conditions. Following incubation, plates were examined for enhanced growth of the indicator strain along the streak line of the test organism toward the meropenem disc. A positive result was defined by the presence of a characteristic cloverleaf-like indentation, indicating carbapenem hydrolysis by carbapenemase-producing organisms and consequent inactivation of meropenem, which allows growth of the indicator strain in the vicinity of the antibiotic disc.

Molecular detection of carbapenemase and colistin resistant genes:

Bacterial DNA extraction: Fresh colonies of phenotypically confirmed isolates were subjected to DNA extraction using a commercial DNA extraction kit. Briefly, 2–4 fresh bacterial colonies were suspended in 500 µL of nuclease-free distilled water. The suspension was incubated at 95°C for 10 minutes, followed by cooling to room temperature and centrifugation at 10,000 rpm for 10 minutes. Subsequently, 150 µL of the supernatant was collected and stored at −20°C for use as a DNA template in downstream Polymerase chain reaction (PCR) assays.

Molecular detection of antimicrobial resistant genes: Polymerase chain reaction was performed for the detection of resistance genes, including *bla*$_{OXA}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, and *mcr-1*, using a T100™ Thermal Cycler (Bio-Rad, USA). Amplification products were analyzed by agarose gel electrophoresis on 1.5% agarose gels (Bio-Rad, USA). PCR reactions were carried out in a total volume of 25 µL containing PCR buffer, gene-specific primers, 2 mM deoxynucleotide triphosphates (dNTPs), 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase, and 1–2 µL of extracted genomic DNA. Primer sequences used in this study were as follows: *bla*$_{IMP}$ (F: GGAATAGAGTGGCTTAAYTCTC; R: GGTTTAAYAAAACAACCACC), *bla*$_{VIM}$ (F: GATGGTGTTTGGTCGCATA; R: CGAATGCGCAGCACCAG), *bla*$_{NDM}$ (F:

GGTTTGGCGATCTGGTTTTC; R: GGTTTGGCGATCTGGTTTTC), bla_{OXA-23} (F: GATCGGATTGGAGAACCAGA; R: ATTTCTGACCGCATTTCCAT), and *mcr-1* (F: AGTCCGTTTGTTCTTGTGGC; R: AGATCCTTGGTCTCGGCTTG). Thermal cycling conditions consisted of an initial denaturation at 94°C for 10 minutes, followed by 32 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes.

Gel electrophoresis: PCR amplicons were resolved by electrophoresis on 1.5% agarose gels at 120 V for 40 minutes. A 1000 bp DNA ladder (USA) was used as a molecular size marker for fragment size estimation.

Statistical analysis and data visualization: All statistical analyses were performed in R (v4.3.1). Prevalence of Enterobacterales, carbapenem resistance, colistin resistance and co-resistance was calculated as proportions within each domain and species, with exact binomial 95% confidence intervals (CIs). Differences in categorical variables, including resistance frequencies across domains, were assessed using two-sided Fisher's exact tests, with $P<0.05$ considered statistically significant. Data visualization was conducted using ggplot2 and related R packages to generate bar plots, stacked charts, heat maps and domain-level resistance summaries. Standard errors of proportions were calculated for phenotypic resistance estimates.

RESULTS

Characterization of Enterobacterales from human, animal, and environmental reservoirs: Of 200 clinical samples, 82 (41%) were identified as Enterobacterales, comprising 52/100 urine samples (52; 95% CI: 41.8–62.1) and 30/100 blood samples (30; 95% CI: 21.2–40.0). In urine, *E. coli* (24; 46.2%) was the predominant species, followed by *K. pneumoniae* (12; 23.1%), *K. oxytoca* (5; 9.6%), and *P. mirabilis* (5; 9.6%). Blood isolates were primarily *E. coli* (13; 43.3%), *K. pneumoniae* (6; 20%), and *Serratia marcescens* (4; 13.3%). All 200 veterinary samples confirmed *E. coli* (95% CI: 98.2–100.0). From 200 environmental wastewater samples, 240 polymicrobial Gram-negative rods were recovered 98% of HWW (95% CI: 93.0–99.8) and 100% of SWW (95% CI: 96.4–100). Of these, 210 (87.5%) isolates were confirmed as Enterobacterales. In HWW, Enterobacterales comprised *K. pneumoniae* (72; 67.9%), *E. coli* (28; 26.4%) and *P. mirabilis* (6; 5.7%), whereas in SWW, *K. pneumoniae* predominated (96; 92.3%) followed *by E. coli* (8; 7.7%). Overall, was the dominant species in environmental samples, particularly in SWW. Enterobacterales prevalence differed statistically significantly across different sources (Fisher's exact test with Monte Carlo simulation, $P<0.001$), with veterinary and environmental samples exhibiting higher prevalence than clinical samples (Fig. 1).

Antibiogram of Enterobacterales in human animal interface: In this study, the antimicrobial susceptibility of Enterobacterales isolates from clinical, veterinary, and environmental sources was assessed using 18 antibiotics grouped according to the WHO AWaRe classification (11 Watch, 5 Access, 2 Reserve), with testing performed following CLSI M100 guidelines. Intrinsically resistant antibiotic–organism pairs (e.g., ampicillin for *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Proteus vulgaris*; amoxicillin–clavulanate for *Serratia marcescens* and *E. cloacae*; colistin for *Proteus mirabilis*, *P. vulgaris*, and *S. marcescens*) were excluded from comparative analysis, while nitrofurantoin, fosfomycin, and norfloxacin were considered only for urinary isolates. Among clinical isolates, *E. coli* (n=52) exhibited the highest resistance within the Access group, with 94.6% resistant to ampicillin and 86.5% to amoxicillin–clavulanate. Within the Watch group, 86.5% of clinical *E. coli* were resistant to third- and fourth-generation cephalosporins, carbapenem resistance was 32.4%, and fluoroquinolone resistance ranged from 45.9 to 67.6%; Reserve antibiotic resistance (colistin) was 10.8%. Clinical *K. pneumoniae* (n=18) showed 72.2% resistance to amoxicillin–clavulanate (Access), 61.1% to cephalosporins and fluoroquinolones (Watch), 33.3% carbapenem resistance (Watch) and 5.6% resistance to Reserve antibiotics (tigecycline and colistin). Statistically, clinical *E. coli* had significantly higher resistance to ampicillin (94.6% vs. 72.2%, $P<0.05$) and cephalosporins (86.5% vs. 61.1%, $P<0.05$) compared to clinical *K. pneumoniae*, but no significant difference was observed in carbapenem resistance between the two species (32.4% vs. 33.3%, $P>0.05$). All veterinary isolates were *E. coli* (n=200), with Access group resistance highest against ampicillin (97%) and amoxicillin–clavulanate (84%); Watch group cephalosporin resistance ranged from 42 to 50%, carbapenem resistance was 12.5%; Reserve group colistin resistance was 28%. Compared to clinical *E. coli*, veterinary *E. coli* showed significantly higher resistance to ampicillin (97% vs. 94.6%, $P<0.05$) and colistin (28% vs. 10.8%, $P<0.001$), but significantly lower carbapenem resistance (12.5% vs. 32.4%, $P<0.001$). Among environmental Enterobacterales (n=210), *K. pneumoniae* (n=168) exhibited 96% resistance to amoxicillin–clavulanate and piperacillin–tazobactam (Access/Watch), 82.1–96.4% resistance to cephalosporins (Watch) and 29.8% carbapenem resistance (Watch). Environmental *E. coli* (n=36) displayed 100% resistance to ampicillin and 88.9% to amoxicillin–clavulanate and piperacillin–tazobactam (Access), 11.1% carbapenem resistance (Watch), and Reserve group resistance of 33.3% to tigecycline and 22.2% to colistin (as shown in Fig. 2). When comparing environmental *K. pneumoniae* to clinical *K. pneumoniae*, environmental isolates had significantly higher resistance to amoxicillin–clavulanate (96% vs. 72.2%, $P<0.001$) and cephalosporins (range 82.1–96.4% vs. 61.1%, $P<0.001$), but no significant difference in carbapenem resistance (29.8% vs. 33.3%, $P>0.05$). Across all sources, veterinary isolates demonstrated the highest colistin resistance (28%), which was significantly greater than clinical (10.8%, $P<0.001$) and environmental (22.2% for *E. coli*, $P<0.05$) sources, suggesting potential colistin pressure in veterinary settings.

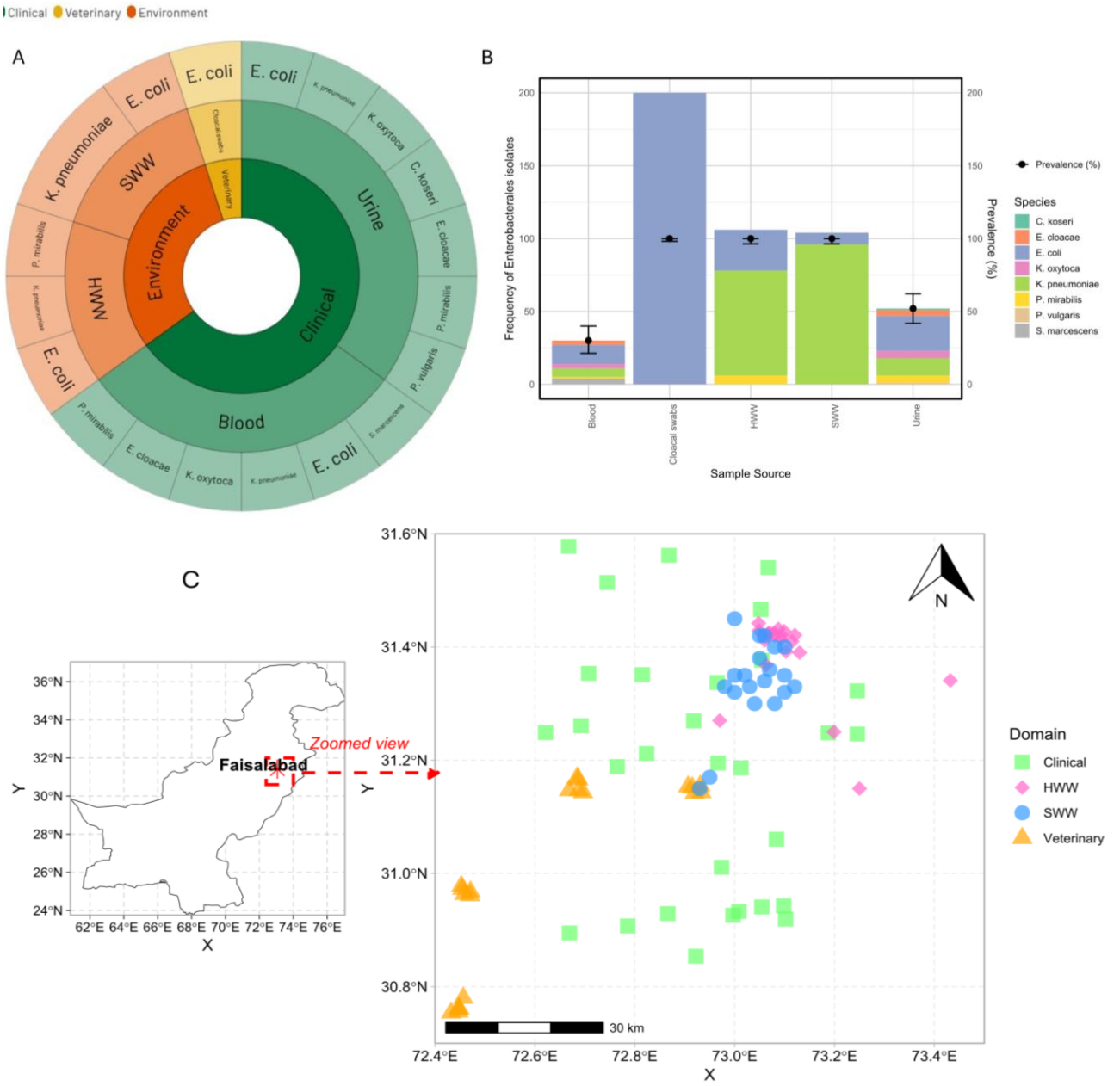

Fig. 1: Distribution and prevalence of Enterobacterales across clinical, veterinary, and environmental sources. (A) Sunburst diagram representing the relative abundance of Enterobacterales species within each domain. The innermost ring corresponds to the sample source (clinical, veterinary, environmental), while the outer ring indicates the proportion of individual bacterial species. (B) Bar chart showing the absolute frequency of each Enterobacterales species across the three domains. Colors correspond to species. (C) Geographic map of Faisalabad depicting sampling locations for clinical facilities, veterinary farms, and hospital and surface wastewater sites. Color markers indicate the specific sites where samples were collected, illustrating the spatial distribution of isolates analyzed in this study.

MIC Distribution of carbapenem and colistin resistant Enterobacterales: Meropenem MICs for CRE ranged from 8 to 1024 µg/mL, whereas colistin MICs ranged from 4 to 32µg/mL across clinical, veterinary, and environmental domains. Among environmental isolates, carbapenem-resistant *E. coli* (n=4) displayed MICs of 64, 128, 256 and 512µg/mL (median 192µg/mL), while *K. pneumoniae* (n=50) showed a bimodal distribution: 8 µg/mL (n=6) and 128µg/mL (n=6), 16µg/mL (n=4) and 32µg/mL (n=4), with the majority (n=18, 36%) at 64µg/mL. The median MIC for environmental *K. pneumoniae* was 64µg/mL, significantly higher than the lower-level resistance cluster at 8–16µg/mL (Mann–Whitney U test, P<0.001). Among veterinary isolates, carbapenem-resistant *E. coli* (n=24) exhibited MICs of 8µg/mL (n=3), 32µg/mL (n=8), 128µg/mL (n=6), 256µg/mL (n=5) and 512µg/mL (n=2), with a median of 128µg/mL. Veterinary CRE had a significantly higher proportion of isolates with MICs ≥128µg/mL (13/24, 54.2%) compared to environmental CRE *E. coli* (3/4, 75%, P>0.05 due to small sample size, but Fisher's exact test P=0.58). In clinical isolates, carbapenem-resistant *E. coli* (n=12) ranged from 16µg/mL (n=1) to 1024µg/mL (n=1), with the majority (10/12, 83.3%) showing high-level resistance at 64–512µg/mL (median 256 µg/mL). Clinical *K. pneumoniae* (n=6) exhibited MICs ranging from 8 to 256µg/mL (median 64µg/mL), while *E. cloacae* (n=3) showed MICs of 128, 256 and 512µg/mL (median 256µg/mL). Comparing across sources, clinical CRE demonstrated significantly higher median meropenem

MICs (256µg/mL for *E. coli* and *E. cloacae*) compared to veterinary (128µg/mL, P<0.05) and environmental *K. pneumoniae* (64µg/mL, P<0.001), indicating that clinical isolates harbored more extreme high-level carbapenem resistance. For colistin resistance, environmental isolates: colistin-resistant *E. coli* (n=8) all had MICs of 8µg/mL (uniform distribution), while environmental *K. pneumoniae* (n=10) ranged from 4–16µg/mL, with most isolates (n=6, 60%) showing elevated MICs at 16µg/mL. The median colistin MIC was significantly higher in environmental *K. pneumoniae* (16µg/mL) than in environmental *E. coli* (8µg/mL, P=0.003, Mann–Whitney U test). Among veterinary isolates, colistin-resistant *E. coli* (n=56) predominantly exhibited elevated MICs: 4 µg/mL (n=10, 17.9%), 8µg/mL (n=28, 50%), 16µg/mL (n=14, 25%) and 32µg/mL (n=4, 7.1%), with a median of 8µg/mL. Veterinary isolates had a significantly broader MIC range and higher proportion of MICs ≥16µg/mL (18/56, 32.1%) compared to environmental *E. coli* (0/8, 0%; P=0.049, Fisher's exact test). In clinical isolates, colistin-resistant *E. coli* MICs were 4µg/mL (n=2) and 8 µg/mL (n=2) (median 6µg/mL), and a single *K. pneumoniae* isolate had an MIC of 4µg/mL. Clinical isolates demonstrated significantly lower colistin MICs (maximum 8µg/mL) compared to veterinary (maximum 32µg/mL, P=0.002) and environmental *K. pneumoniae* (maximum 16µg/mL, P=0.04), suggesting that colistin pressure or resistance mechanisms differ across sources. Notably, the proportion of isolates with colistin MICs ≥8µg/mL was highest in veterinary *E. coli* (46/56, 82.1%), followed by environmental *K. pneumoniae* (6/10, 60%) and lowest in clinical isolates (2/5, 40%; chi-square trend P=0.02). These data (Fig. 3) indicate that veterinary and environmental reservoirs harbor colistin resistance with higher MIC elevations compared to clinical isolates, while carbapenem resistance reaches extreme MIC values (up to 1024µg/mL) predominantly in clinical settings.

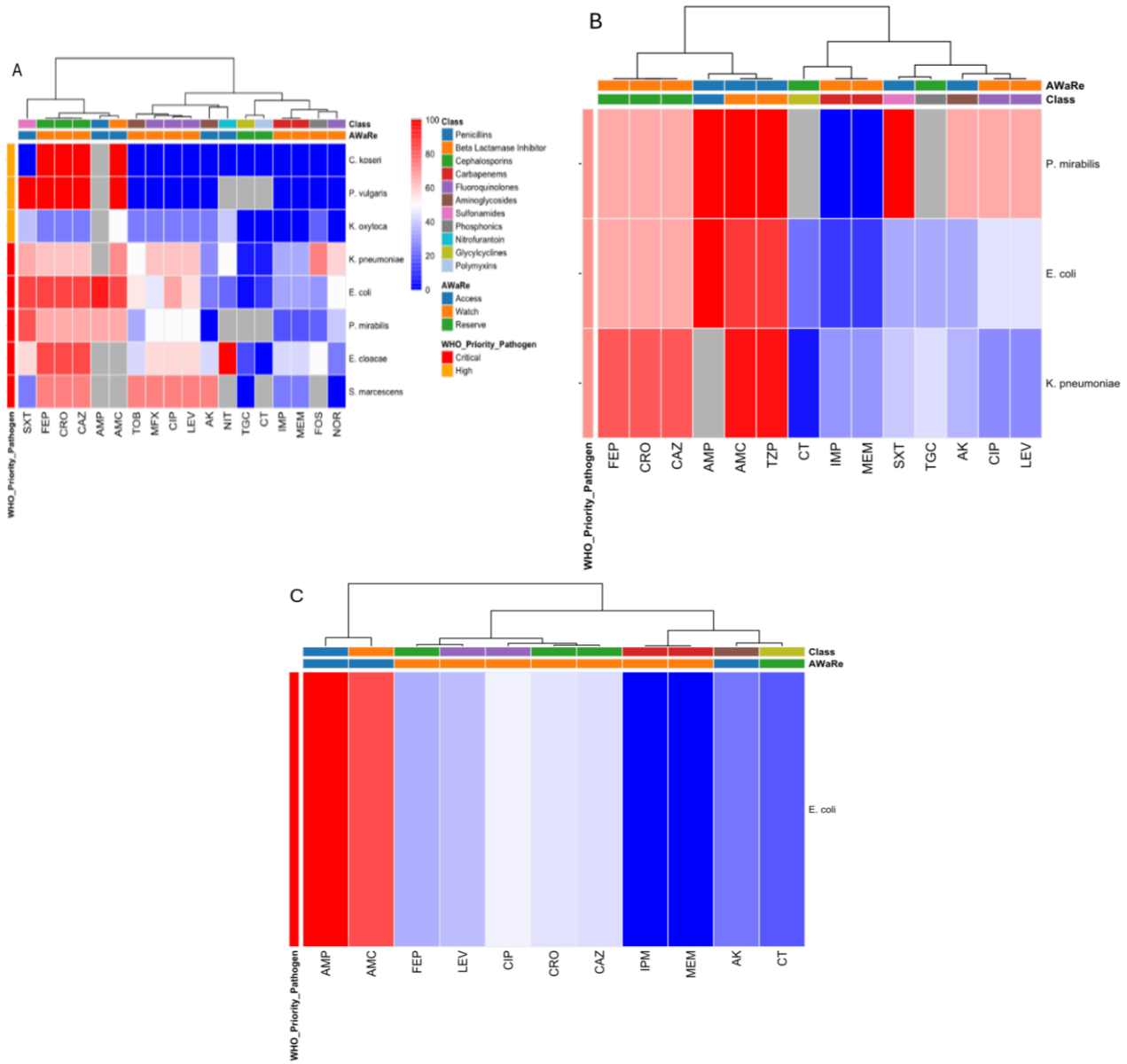

Fig. 2: Antimicrobial resistance profiles of Enterobacterales across domains. (A–C) Heatmaps illustrating the resistance patterns of Enterobacterales isolates from environmental (A), clinical (B), and poultry (C) sources. Rows represent individual bacterial species, and columns represent antibiotics grouped according to the WHO AWaRe classification (Access, Watch, Reserve). The color intensity corresponds to the proportion of resistant isolates for each antibiotic-species combination, highlighting high levels of multidrug resistance across all domains.

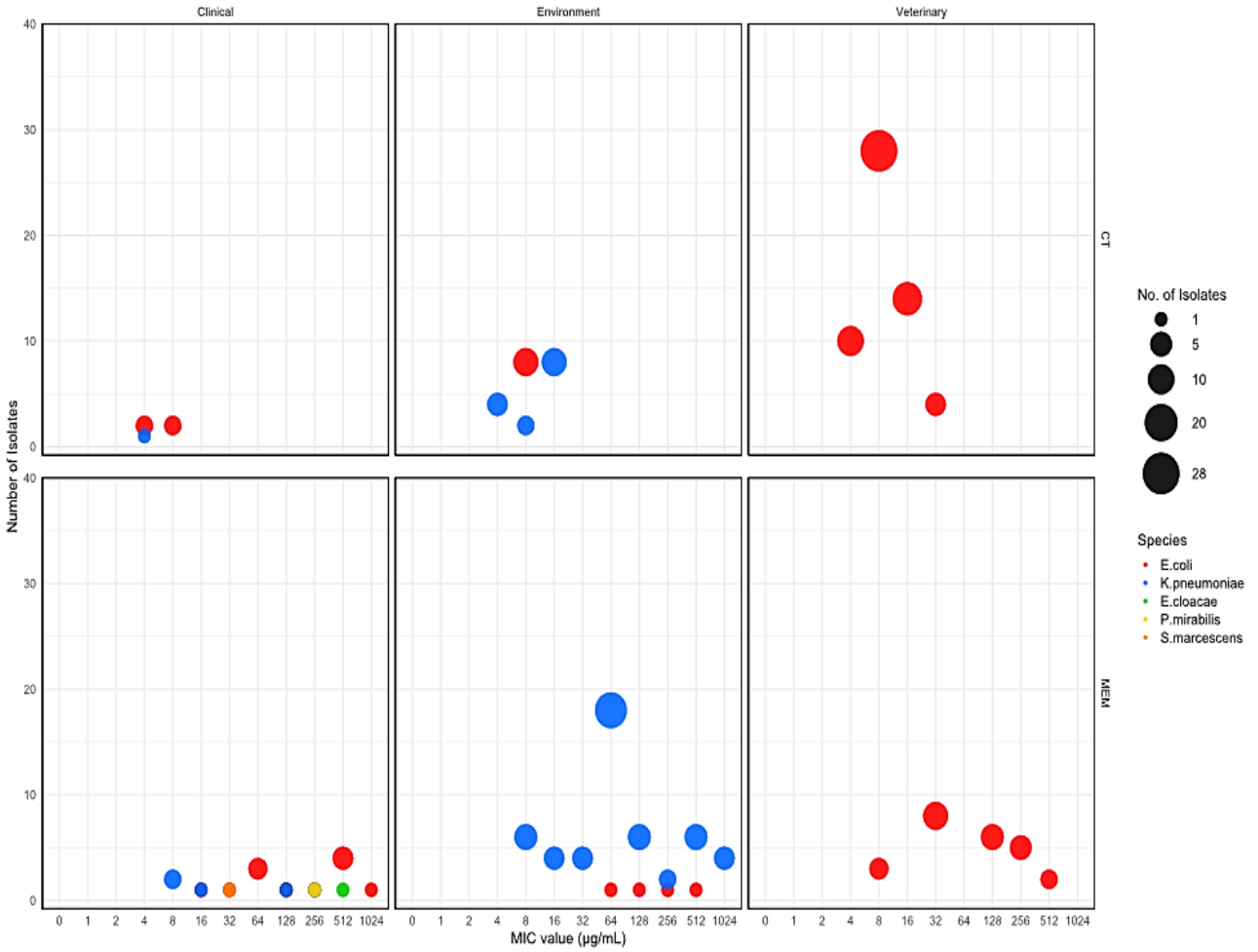

Fig. 3: Minimum inhibitory concentrations of carbapenem- and colistin-resistant Enterobacterales across domains. Bubble diagrams depict the distribution of meropenem and colistin MICs among clinical, veterinary, and environmental isolates. Bubble size corresponds to the number of isolates at each MIC value, while colors indicate bacterial species. Meropenem MICs ranged from 8–1024µg/mL, and colistin MICs ranged from 4–32µg/mL, highlighting the high-level resistance particularly observed in environmental and veterinary isolates.

MAR index analysis: MAR index analysis revealed distinct patterns of multidrug resistance (MDR) across the three domains. A MAR index ≤0.2 indicates low-level resistance, whereas higher values reflect increasing resistance. In the veterinary domain, MAR indices ranged from 0.18 to 1. *E. coli* isolates were predominantly in the >0.2–≤0.5 (n=82; 41%) and >0.5–≤0.8 (n=64; 32%) categories, indicating moderate to high MDR. Fourteen isolates (7%) had MAR indices >0.8–1, exhibiting resistance to nearly all tested antibiotics, while 40 isolates (20%) showed low-level resistance (0–≤0.2). Clinical Enterobacterales displayed MAR indices ranging from 0 to 0.83. *E. coli* most frequently fell within the >0.5–≤0.8 category (n=19; 23.2%), followed by >0.2–≤0.5 (n=15; 18.3%), with only three isolates (3.7%) in the low-resistance range (0–≤0.2). *K. pneumoniae* similarly exhibited MAR indices primarily between 0.2 and 0.8 (n=11; 13.4%), indicating substantial multidrug resistance. Environmental Enterobacterales from HWW and SWW showed MAR indices from 0.14 to 0.86, with *K. pneumoniae* predominating. Most environmental isolates fell within the 0.3–0.5 (n=70; 33.3%) and 0.6–0.8 (n=72; 34.3%) categories, and ten isolates (4.8%) reached MAR indices >0.8–1. *E. coli* also exhibited elevated MAR values, with most isolates between 0.2 and 0.8 (n=34; 17%). Overall, environmental isolates carried the highest multidrug resistance burden, followed by veterinary isolates, while clinical isolates exhibited comparatively lower MAR index values (Fig. 4).

Phenotypic confirmation of carbapenem and colistin resistance: Phenotypic testing confirmed the presence of carbapenem- and colistin-resistant Enterobacterales across veterinary, clinical, and environmental domains. In environmental isolates (n=210), carbapenem resistance was observed in *E. coli* (4/36; 11.1%) and predominated in *K. pneumoniae* (50/168; 29.8%). *E. coli* isolates (n=200) exhibited 12.5% carbapenem resistance, whereas clinical isolates (n=82) showed notable carbapenem resistance in *E. coli* (12/37; 32.4%) and *K. pneumoniae* (5/18; 33.3%). Colistin resistance was detected in all three domains. *E. coli* and *K. pneumoniae* exhibited resistance in 22.2 and 6% of isolates, respectively. *E. coli* showed 28% resistance, while clinical resistance was lower, observed in *E. coli* (10.8%) and *K. pneumoniae* (5.6%). Co-resistance to both carbapenems and colistin was confirmed in all domains: environmental *E. coli* (4/36; 11.1%) and *K. pneumoniae* (2/168; 1.2%), *E. coli* (8/200; 4%) and *E. coli* (1/37; 2.7%). Error bars in Fig. 5 represent the standard error of resistance proportions, reflecting the precision of estimates. Species with smaller sample sizes had larger standard errors, whereas larger groups provided more reliable estimates (Fig. 5).

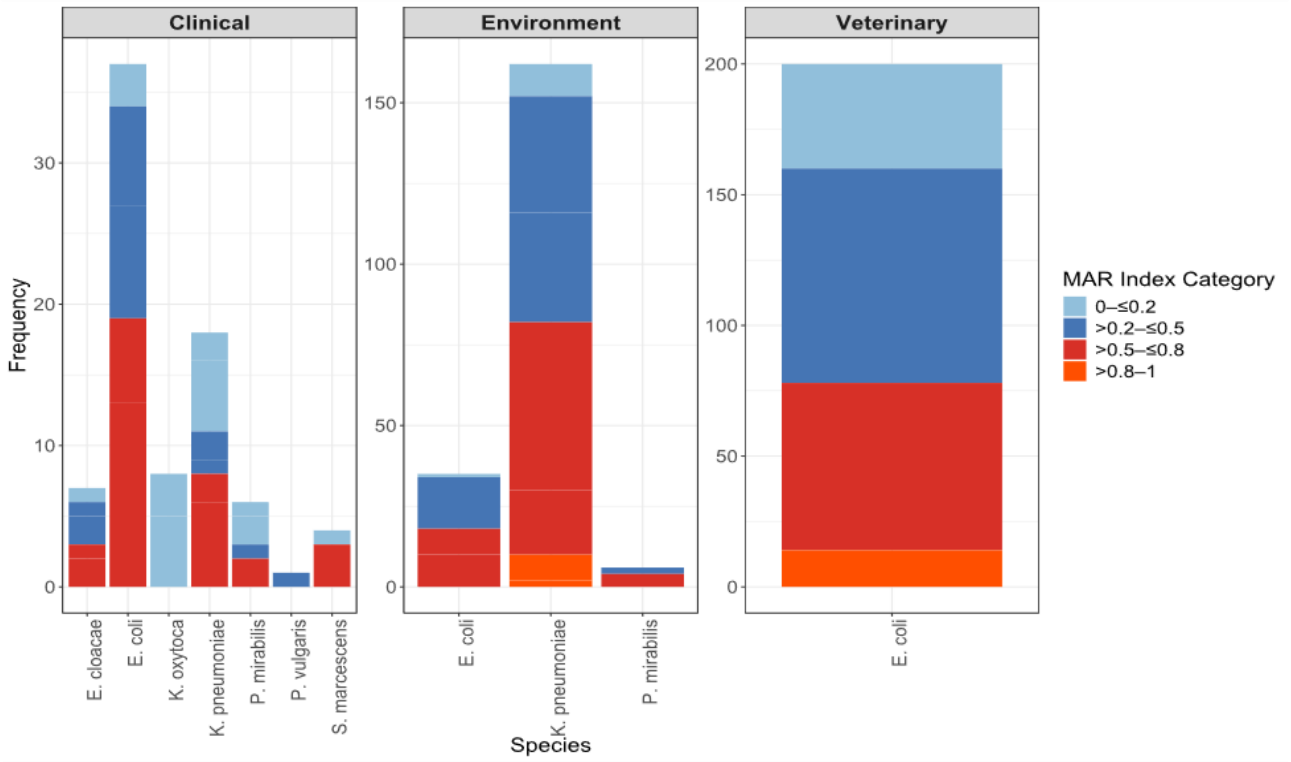

Fig. 4: Distribution of multiple antibiotic resistance indices across domains. Bar diagrams show the MAR index of Enterobacterales isolates from clinical, veterinary, and environmental sources. MAR index categories are indicated along the x-axis (0–≤0.2, >0.2–≤0.5, >0.5–≤0.8, >0.8–1), and the *y*-axis represents the number of isolates in each category. Color shading corresponds to bacterial species, highlighting the higher multidrug resistance burden in environmental and veterinary isolates compared with clinical isolates.

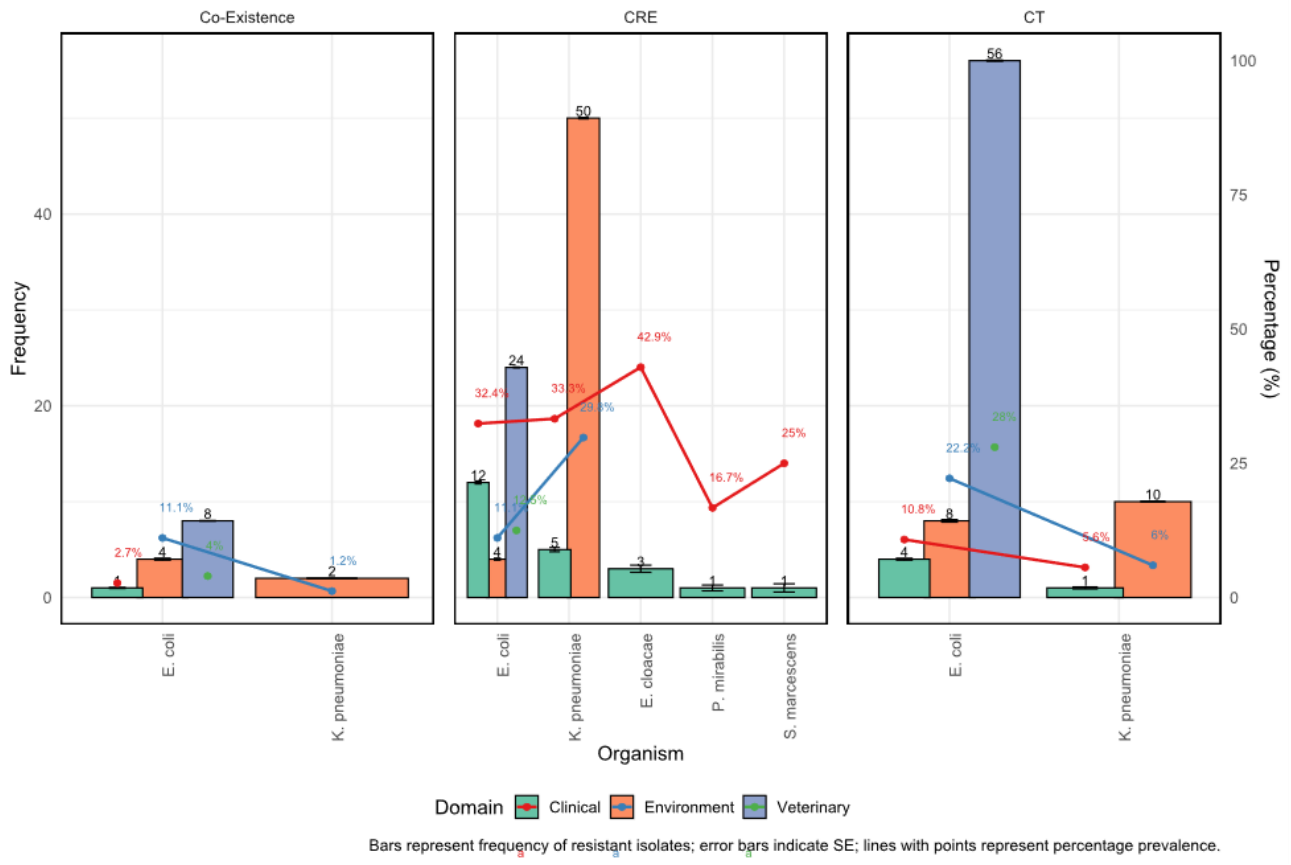

Fig. 5: Prevalence of carbapenem- and colistin-resistant Enterobacterales and co-resistance across domains. Bar charts show the proportion of Enterobacterales isolates resistant to carbapenems, colistin, or both (co-resistance) in clinical, veterinary, and environmental samples. Data are presented for *E. coli* and *K. pneumoniae* isolates, highlighting the relative burden of single and combined resistance in each domain. Error bars represent the standard error of the resistance proportion, indicating the precision of the estimates.

Genotypic Confirmation of CRE and Col-R: Genotypic analysis confirmed the presence of carbapenemase genes in CRE, while the mcr-1 gene was detected in all phenotypically colistin-resistant isolates across domains. In the veterinary domain, all CRE *E. coli* isolates (24/24; 100%) carried carbapenemase genes, with bla_{VIM} (41.7%), bla_{IMP} (37.5%) and bla_{NDM} (25%); one isolate co-harbored bla_{IMP} and bla_{NDM}. Colistin resistance was ubiquitous in *E. coli* (56/56; 100%), indicating widespread plasmid-mediated mcr-1. Among clinical isolates, CRE *E. coli* (12/22; 54.5%) frequently carried bla_{NDM} (66.7%) and bla_{VIM} (58.3%), with lower prevalence of bla_{IMP}, and bla_{OXA} (33.3% each). Multiple co-occurrences were observed: bla_{IMP} + bla_{NDM} (n=4), bla_{IMP} + bla_{VIM}(n=3), bla_{VIM}+ bla_{NDM} (n=3), bla_{NDM} + bla_{OXA} (n=1) and bla_{VIM} + bla_{OXA} (n=3). *K. pneumoniae* (5/22; 2.7%) harbored bla_{NDM}, blaVIM, and bla_{OXA} (40% each), with two isolates co-harboring bla_{NDM} + bla_{OXA}. CRE *E. cloacae* (3/22; 13.6%) carried bla_{IMP} (66.7%) and other carbapenemases at 33.3%; one isolate co-harbored bla_{IMP} + bla_{NDM}. All clinical *E. coli* and *K. pneumoniae* exhibited mcr-1-mediated colistin resistance. In environmental isolates, carbapenem-resistant *E. coli* (4/55; 7.3%) carried bla_{IMP} (50%), bla_{NDM} (50%) and blaVIM (25%), including one isolate co-harboring bla_{IMP} + bla_{NDM}. *K. pneumoniae* (51/55; 92.7%) predominantly carried bla_{NDM} (54.9%), followed by bla_{VIM} (41.2%), bla_{IMP} (29.4%) and bla_{OXA} (15.7%), with multiple co-occurrences. Colistin resistance mediated by mcr-1 was detected in all environmental isolates tested (18/18; 100%) (Fig. 6).

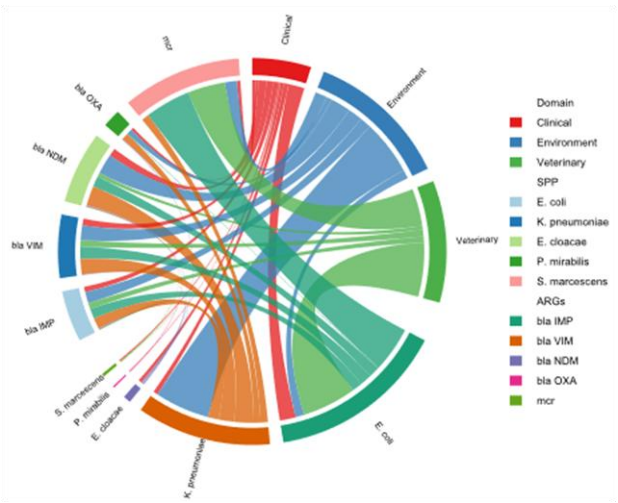

Fig. 6: Chord diagram illustrating the linkage analysis of cross-sectoral sharing of antimicrobial resistance genes in isolates**.** The diagram depicts the distribution of key carbapenemase and colistin resistance genes among Enterobacterales isolated from clinical, veterinary, and environmental domains. Segment size reflects the relative frequency of each gene within a domain, while chord thickness represents the number of isolates linking genes to specific domains or co-harboring multiple resistance determinants. The extensive interconnections emphasize environmental reservoirs as major hubs facilitating the dissemination of last-resort resistance genes across the human–animal interface.

DISCUSSION

The prevalence of Enterobacterales across clinical, veterinary, and environmental sources in the present study reflects their ecological versatility and supports the One Health concept of interconnected human, animal and environmental microbiomes (Destoumieux-Garzón *et al*., 2018). In the present study, *E. coli* and *K. pneumoniae* were the predominant *Enterobacterales* species across all three domains. The results of the study are in accordance with those of a study conducted in Nigeria, where 47.8% of *E. coli* isolates were recovered from the environment and clinical samples (Kasanga *et al*., 2024). Similarly, a study conducted in Pakistan, where 65% *E. coli* and 25% *K. pneumoniae* were detected in clinical samples (Abrar *et al*., 2019). Across the study conducted in Germany, *E. coli* was detected from the process waters and wastewater of poultry and pig slaughterhouses (Savin *et al*., 2021). The carbapenem resistance is particularly alarming because carbapenems are usually considered last-resort antibiotics. AMR is a global One Health issue because the same resistant Enterobacterales that cause urinary tract infections and bloodstream infections in hospitals can also persist in animal gut flora and then be disseminated via wastewater into rivers or canals (Delpy *et al*., 2024). In our study, employing a comprehensive One Health framework, we assessed AMR in Enterobacterales isolates, revealing high levels of resistance to β-lactams and colistin antibiotics. These results are in accordance with the study conducted in Ethiopia analyzing 5-year trends in *E. coli* and *K. pneumoniae* from clinical isolates, among the *E. coli* isolates, the highest rates of resistance were recorded against ampicillin (88%), piperacillin (84%) and tetracycline (80%) and imipenem (5%) (Kitaba *et al*., 2024). Similarly, a study conducted in Russia on antimicrobial resistance among clinical isolates of *K. pneumoniae* and *E. coli* in Russian hospitals reported >80% cephalosporin resistance in *K. pneumoniae* and 60% *in E. coli* (Kitaba *et al*., 2024). Recently, a Chinese study on clinical *E. coli* and *K. pneumoniae* reported that the percentages of resistance of *E. coli* and *K. pneumoniae* to second- and third-generation cephalosporins ranged from 19.7 to 55.2%. The highest carbapenem resistance rates were 1.9 and 16.7% for *E. coli* and *K. pneumoniae*, respectively (Liu *et al*., 2025). A study conducted in Pakistan detected colistin-resistant *E. coli* in commercial poultry cloacal swabs. The *E. coli* isolates from poultry exhibited 100% resistance to amoxicillin/clavulanate, 98.9% to ampicillin, and 93.8% to tetracycline. The *E. coli* from clinical isolates exhibited 90% resistance to ciprofloxacin, 88% to ampicillin, and 85% to ceftriaxone. Among these, MDR *E. coli* isolates from poultry and humans showed colistin resistance in 78.6 and 48.1%, respectively (Huda *et al*., 2025). In present study, CRE detected 29.8% in environmental *K. pneumoniae*, 12.5% in veterinary *E. coli*, and 33.3% in clinical *K. pneumoniae*. Col-R was observed in 22.2% *E. coli* from environment. In another study conducted in Pakistan, they found that among clinical sources, 64.5% were *Klebsiella pneumoniae* CRE, 17.4% *E. coli* CRE, and 5.81% Col-R among *Enterobacterales* (Furqan *et al*., 2022). The results of the study were consistent with those of a study conducted in Pakistan, which reported carbapenem-resistant Enterobacteriaceae in clinical settings at levels below 30% (Saeed *et al*., 2021). A Pakistani meta-analysis confirmed a rate of CRE (pooled) across clinical and veterinary sources and reported 24% *K. pneumoniae* and 9% *E. coli* (Umair *et al*., 2024). The results were in accordance with the study conducted in India, where they report the 26.5%

CRE and 18.7% colistin resistance from tertiary care hospitals (Hait *et al*., 2024). Carbapenemase genes confer resistance in Enterobacterales, while plasmid-mediated *mcr-1* enables resistance by modifying lipid A in the bacterial outer membrane, facilitating horizontal gene transfer across clinical, veterinary, and environmental reservoirs (Liu *et al*., 2016). In the current study, genotypic analysis confirmed the presence of bla_{IMP} (33-50%), bla_{VIM} (25-58%), bla_{NDM} (25-66.7%), in CRE isolates, with 100% prevalence of mcr-1 in phenotypically confirmed colistin-resistant strains across domains. The results of the study agree with the study conducted in Malaysia on carbapenem- and colistin-resistant *E. coli* from broiler chickens, which reported bla_{NDM} and bla_{OXA-48} in 37.5% of *E. coli* isolates (Aklilu *et al*., 2022). Similarly, a study conducted in South Africa identified carbapenemase genes $bla_{OXA-48-like}$ (76.8%), bla_{NDM} (21.1%) and bla_{VIM} (1.3%) in carbapenem-resistant Enterobacteriaceae and detected no *mcr-1* genes (Lowe *et al*., 2022). Overall, our findings demonstrate that carbapenem- and colistin-resistant Enterobacterales are widely disseminated across clinical, veterinary, and environmental reservoirs, reinforcing the interconnected nature of AMR within a One Health framework. The convergence of high-level phenotypic resistance, elevated MDR indices, and the widespread presence of carbapenemase genes and *mcr-1* highlights the efficiency of cross-sector transmission and horizontal gene transfer. The substantial resistance burden observed in environmental and animal sources indicates that these compartments act as active reservoirs and amplification points for last-resort antibiotic resistance. These results underscore the urgent need for integrated One Health surveillance, strengthened antimicrobial stewardship, and improved environmental management to curb the further spread of high-risk resistant Enterobacterales.

Conclusions: This study highlights the widespread dissemination of carbapenem- and colistin-resistant Enterobacterales across human, animal, and environmental sectors in Pakistan. The detection of bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA} and *mcr-1* genes, along with high multidrug resistance rates, underscores a serious public health threat and the urgent need for integrated One Health surveillance and antimicrobial stewardship.

Authors contribution: Aatika: Writing –original draft, Investigation, Data curation; MUQ: Conceptualization, Funding acquisition, Supervisor, Project administration, Review & editing; SM: Methodology, Investigation, review & editing; MHR: Resources, Methodology, Supervision, Review & editing.

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