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Multidrug resistance and genomic features of Escherichia coli from Sonali chicken meat: a whole-genome sequencing study in Bangladesh

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Abstract

Background:

Antimicrobial resistance in *Escherichia coli* poses a significant public health threat, particularly in low- and middle-income countries where antibiotic use in poultry production is often unregulated. Sonali chickens are widely consumed in Bangladesh but remain underrepresented in antimicrobial resistance surveillance. This study aimed to investigate the prevalence, phenotypic resistance patterns, and genomic characteristics of multidrug-resistant *E. coli* isolated from Sonali chicken meat sold in live bird markets.

Methods:

A cross-sectional study was conducted using 50 Sonali chicken meat samples collected from live bird markets in Chattogram between September and December 2022. Isolation and identification of *E. coli* were performed using standard microbiological and biochemical methods. Antimicrobial susceptibility testing was conducted using the disk diffusion method following Clinical and Laboratory Standards Institute guidelines. Multidrug resistance patterns and Multiple Antibiotic Resistance Index were calculated. Whole-genome sequencing of 26 selected isolates was performed using the Illumina platform. Resistance genes, virulence factors, and plasmid replicons were identified using ResFinder, VirulenceFinder, and PlasmidFinder databases. Descriptive statistics and clustering analyses were applied.

Results:

A total of 74% (37/50) of samples yielded multidrug-resistant *E. coli*. High resistance was observed against tetracyclines, fluoroquinolones, sulfonamides, and β-lactams. Multiple Antibiotic Resistance Index values ranged from 0.18 to 1.00, indicating substantial antibiotic pressure. Genomic analysis identified 29 antimicrobial resistance genes, with frequent detection of *tet(A)*, *tet(M)*, *tet(X4)*, *sul2*, *dfrA* variants, and *blaTEM-1B*. The detection of *tet(X4)* highlights the emergence of resistance to last-resort antimicrobials. A total of 39 virulence genes were identified, mainly associated with adhesion, stress response, and iron acquisition. 23 plasmid replicon types were detected, with p0111, IncFIB(K), and IncX1 commonly associated with resistance genes. Phylogenetic analysis revealed a genetically diverse population with evidence of horizontal gene transfer.

Conclusions:

Sonali chicken meat sold in live bird markets represents a significant reservoir of multidrug-resistant *E. coli* carrying clinically important resistance and virulence determinants. These findings underscore the

urgent need for strengthened antimicrobial stewardship, improved hygiene practices, and integrated genomic surveillance within a One Health framework in Bangladesh.

Trial registration:

Not applicable.

INTRODUCTION

Antimicrobial resistance (AMR) is one of the most critical public health threats of the twenty-first century, undermining decades of medical and agricultural progress. It occurs when microorganisms such as bacteria, viruses, fungi, or parasites develop mechanisms to survive exposure to antimicrobial agents that were previously effective against them. Although AMR is a natural evolutionary process, its current acceleration is largely attributed to the indiscriminate and often inappropriate use of antibiotics in human health, veterinary medicine, and food animal production. According to the World Health Organization, AMR directly caused 1.27 million deaths in 2019, and without intervention, could result in 1.91 million annual deaths by 2050, with 39 million cumulative deaths between 2025 and 2050 (1, 2). Global economic losses are projected to reach cumulative costs of USD 100 trillion by 2050, with annual costs potentially reaching USD 1-3.4 trillion by 2030 (World Bank, 2024). The situation is particularly alarming in low- and middle-income countries (LMICs) such as Bangladesh, where weak regulatory enforcement, limited diagnostic capacity, and unrestricted antibiotic access exacerbate the problem. In these settings, antibiotic misuse in the agricultural sector represents a major driver of resistance, particularly in poultry farming, which depends heavily on antimicrobials for growth promotion, prophylaxis, and disease management, often without veterinary supervision. Studies from Bangladesh have detected multidrug-resistant (MDR) *Escherichia coli* in commercial poultry meat, including isolates resistant to critically important drugs classified by WHO as "Highest Priority Critically Important Antimicrobials" (HPCIA), such as colistin and carbapenems (3). Because resistant bacteria and drug residues can enter soil and water through untreated animal waste, AMR has expanded beyond hospitals and farms to become an environmental and trade-related issue. Addressing this complexity requires a coordinated One Health strategy that links human, animal, and environmental health sectors to promote rational antibiotic use and strengthen integrated surveillance.

Within Bangladesh's rapidly expanding poultry industry, the Sonali chicken—a crossbreed of Rhode Island Red and Fayoumi—has emerged as an economically valuable hybrid whose moderate growth rate, adaptability, and meat quality resembling native breeds have made it popular among smallholders and consumers in districts. Sonali chickens are commonly raised in semi-intensive systems where they scavenge during the day and receive supplemental feed; however, these systems often lack veterinary oversight, record-keeping, or biosecurity measures. Surveys have shown that nearly half of Sonali farms administer antimicrobials frequently without prescription or diagnostic confirmation (4). The routine administration of antibiotics as feed additives or preventive measures imposes strong selective pressure

on gut microbiota, facilitating the development of resistant strains. Recent surveillance has revealed that over 90% of *E. coli* isolates from Sonali and broiler farm environments are MDR, exhibiting high resistance to ciprofloxacin, ampicillin, tetracycline, and trimethoprim–sulfamethoxazole (5). In the absence of effective antibiotic stewardship and regulatory control, Sonali chickens may act as reservoirs and amplifiers of MDR bacteria capable of spreading through the food chain. Investigations have reported that 76% of frozen chicken samples contained *E. coli*, with 86% of those isolates being extended-spectrum β-lactamase (ESBL) producers resistant to β-lactams, tetracyclines, fluoroquinolones, and aminoglycosides, indicating that the poultry value chain is a critical interface for the transmission of resistance genes including bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ variants (6). Human exposure to these bacteria can occur through ingestion of undercooked or contaminated meat, poor food-handling practices, or contact with contaminated environments and even without causing immediate illness, resistant *E. coli* can colonize the human intestine and exchange resistance genes with commensal flora, creating silent reservoirs that may later give rise to difficult-to-treat infections. In urban settings such as Chattogram, high population density, limited awareness of safe food handling, and weak food-safety enforcement amplify these risks, making live bird markets critical interfaces for bacterial exchange and zoonotic transmission.

E. coli is a Gram-negative, facultatively anaerobic bacterium that forms part of the normal gut microbiota of humans and animals but can also cause diseases such as diarrhea, urinary tract infections, and sepsis. Owing to its ubiquity, genetic versatility, and capacity to acquire and disseminate AMR genes through horizontal gene transfer via plasmids, integrons, and transposons, *E. coli* serves as a sentinel organism in AMR surveillance (6). Monitoring resistance trends in *E. coli* provides critical insight into antibiotic use practices and emerging resistance at the animal–human–environment interface. Resistance profiles of *E. coli* isolated from poultry closely mirror antibiotic administration patterns within production systems, making it a valuable bioindicator for antimicrobial misuse in livestock (5). Several studies in Bangladesh have documented widespread MDR among *E. coli* isolates from poultry farms, meat, and environmental samples. A meta-analysis across 17 studies reported an average *E. coli* prevalence of 69.3%, with over 90% of isolates showing MDR phenotypes and harboring clinically significant genes such as bla_{TEM}, $bla_{CTX\text{-}M}$, *tet(A)*, *sul1*, *mcr-1*, and *qnrS*, conferring resistance to β-lactams, tetracyclines, sulfonamides, colistin, and fluoroquinolones (6). This highlight *E. coli* not only as a reliable indicator of antibiotic misuse but also as an active participant in resistance gene transfer. Its presence in the food production environment emphasizes the urgent need for integrated monitoring, responsible antibiotic use, and regulatory control under a One Health framework.

Advances in whole-genome sequencing (WGS) now offer unprecedented capacity to study antimicrobial resistance at a molecular level, providing a comprehensive view of the bacterial resistome and capturing both known and novel genes offering deeper insights than conventional phenotypic or PCR-based methods (7). In Bangladesh, where antimicrobial use in poultry remains largely unregulated, genomic surveillance using WGS has the potential to transform AMR monitoring and policy development. The country's National Action Plan (NAP) for AMR containment (2017–2022, revised 2022–2026)

emphasizes integrated surveillance across human, animal, and environmental sectors under a One Health framework, but currently lacks robust implementation in the poultry sector. Although numerous studies have explored antimicrobial resistance in poultry, most have focused on broiler and layer production systems where antibiotic use is intensive and well documented. In contrast, Sonali chickens represent a major share of Bangladesh's semi-commercial poultry sector but have received limited scientific attention despite their growing contribution to national food security and rural income. Most available studies have relied on phenotypic or PCR-based methods, providing only a partial view of the resistance landscape. The absence of genomic-level data on Sonali-derived *E. coli* represents a critical knowledge gap, especially given the role of live bird markets as hotspots for bacterial exchange and zoonotic transmission. This study therefore applies WGS to *E. coli* isolates obtained from freshly slaughtered Sonali chicken meat collected at live bird markets in Chattogram to generate a comprehensive understanding of their resistance and virulence profiles. The research specifically aims to (1) determine the prevalence and phenotypic resistance patterns of *E. coli* from Sonali chickens, (2) characterize the genetic basis of antimicrobial resistance, virulence, and plasmid content using genome-based analysis, and (3) investigate the genomic epidemiology. By integrating phenotypic and genomic evidence, this work addresses critical data gaps in Bangladesh's AMR landscape and provides baseline genomic insights for this understudied poultry type. The findings will support evidence-based antimicrobial stewardship, strengthen One Health–aligned surveillance frameworks, and inform national policies aimed at reducing the emergence and spread of multidrug-resistant bacteria at the human–animal–environment interface.

METHODOS

Study Site, and Sampling

A cross-sectional study was conducted to investigate multidrug-resistant *Escherichia coli* in Sonali chickens sold at live bird markets (LBMs) in Chattogram City, Bangladesh. A total of 50 meat samples were collected between September and December 2022 from ten major LBMs: Chawkbazar (22.3574°N, 91.8410°E), Sarai Para (22.3519°N, 91.7959°E), Jhautala (22.3578°N, 91.8074°E), Agrabad (22.3331°N, 91.8119°E), Pahartali (22.3582°N, 91.7833°E), 2 No. Gate (22.3671°N, 91.8202°E), Bahaddarhat (22.3689°N, 91.8423°E), Reazuddin Bazar (22.3370°N, 91.8296°E), Oxygen (22.3945°N, 91.8230°E), and Colonel Hat (22.3692°N, 91.7781°E) (Figure 1).

Each meat sample was aseptically collected in sterile Ziplock bags, transported under chilled conditions to the Research Laboratory of the Department of Physiology, Biochemistry, and Pharmacology (DPBP), Chattogram Veterinary and Animal Sciences University (CVASU), and processed immediately upon arrival.

Isolation and Identification of E. coli

Each Sonali chicken meat sample was first rinsed thoroughly with sterile normal saline (0.90% w/v NaCl) to minimize microbial cross-contamination from the external surface. 25gm of meat was aseptically minced using sterile scissors and forceps and were transferred into individually labelled sterile test tubes containing 225 mL Buffered Peptone Water (BPW) (Oxoid, Basingstoke, UK; pH 7.0 ± 0.2). The samples were manually homogenized by vortexing and incubated at 37°C for enrichment. After incubation, aliquots of enriched samples were streaked onto MacConkey agar (Oxoid, UK; pH 7.1 ± 0.2) and incubated at 37°C for 24 hours. Presumptive E. coli (bright pink, dry, indictive of lactose fermentation) were subcultured onto Eosin Methylene Blue (EMB) agar (Oxoid, UK; pH 7.1 ± 0.2) and incubated for an additional 24hours at 37°C. Colonies displaying a moist green metallic sheen on EMB agar were considered presumptive E. coli (8, 9). These were sub-cultured on blood agar to obtain pure cultures. Identification was confirmed by Gram staining and classical biochemical tests. Confirmed E. coli isolates were Gram-negative rods, indole positive, methyl red positive, Voges-Proskauer negative, citrate utilization negative, and capable of fermenting glucose and lactose with acid and gas production. For each sample, one well-isolated colony showing typical E. coli morphology on EMB agar was selected for biochemical confirmation and subsequent analyses. Culture of the 50 Sonali chicken samples yielded 37 (74%) isolates confirmed as E. coli, which were used in subsequent analyses.

Preservation of E. coli Isolates

Pure isolates were inoculated into Brain Heart Infusion (BHI) broth and incubated overnight at 37°C. For long-term storage, 700 µL of culture was mixed with 300 µL of sterile 15% glycerol solution in cryovials, vortexed, and stored at −80 °C.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the *E. coli* isolates was determined using the Kirby–Bauer disk diffusion method (10) on Mueller–Hinton agar (Oxoid, UK) following Clinical and Laboratory Standards Institute (CLSI, 2024) guidelines. A total of 11 antibiotics representing 7 antimicrobial classes were tested, selected based on their importance in human and veterinary medicine and CDC recommendations for *Enterobacteriaceae* infections. Bacterial suspensions were adjusted to a 0.5 McFarland standard (1–2 × 10^8 CFU/mL) in 0.85% sterile saline. Within 15 minutes of preparation, inocula were spread evenly on Mueller–Hinton agar plates, and antibiotic discs (Oxoid Ltd. and Mast Group Ltd., UK) were applied using sterile forceps. Plates were incubated at 37°C for 18 h, after which inhibition zone diameters were measured in millimeters. The results were interpreted as susceptible (S), intermediate (I), or resistant (R) according to CLSI criteria (11). Details of the antibiotics tested, their respective antimicrobial classes, disc potencies, interpretive zone diameters, and manufacturers are summarized in Table S1.

Assessment of Multidrug Resistance (MDR), Multiple Antibiotic Resistance Phenotype (MARP), and Multiple Antibiotic Resistance Index (MARI)

Multidrug resistance (MDR) in *E. coli* isolates was defined as non-susceptibility to at least one agent in three or more distinct antimicrobial classes, following established conventions. Multiple antibiotic resistance phenotypes (MARP) were determined by classifying each isolate according to its unique pattern of resistance across the 7 tested antibiotic classes. Each distinct resistance profile was designated as a specific MAR phenotype, allowing evaluation of resistance diversity and prevalence within the isolate collection.

The multiple antibiotic resistance index (MARI) was calculated for each isolate using the formula MARI = a/b, where "a" is the number of antibiotics to which the isolate was resistant, and "b" is the total number tested (12). Isolates with MARI values >0.2 were considered to have originated from environments with frequent or high antibiotic use, indicative of elevated public and animal health risks. For markets with multiple isolates, a site-specific MARI was determined as the total number of resistance events among all isolates divided by the product of the total number of antibiotics tested and the number of isolates from that site. Site-level MARI values exceeding 0.2 were interpreted as evidence of significant antibiotic pressure at the sampling location.

Whole Genome Sequencing & Genome Assembly

A total of 26 *E. coli* isolates were selected for whole-genome sequencing based on phenotypic diversity and antimicrobial resistance profiles. The bacterial suspension was subjected to DNA extraction utilizing the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) following the protocol of the manufacturer. Whole genome sequencing was performed using Nextera XT DNA Library Preparation Kit followed by 150-bp paired-end sequencing on Illumina Novaseq 6000 sequencing platform. Quality control was performed using Fastp v1.0.1 to assess base quality, GC content, and read length distribution (13). Adapter sequences and low-quality reads (Q < 30) were removed using Trimmomatic v0.39 and BBDuk v38.96 (14, 15).

High-quality reads were assembled de novo using SPAdes v4.2.0 (16). Assembly metrics (number of contigs, genome size, N50) were generated with QUAST v5.2.0 and genome completeness and contamination were assessed using BUSCO v5.3.2 (17, 18). Species identification was confirmed using Gambit v0.5.0 (19) and multilocus sequence typing was conducted with MLST v2.19.0 (20).

The raw sequencing data generated in this study is deposited to the European Nucleotide Archives under BioProject PRJEB108291.

Identification and analysis of AMR genes, virulence genes, and plasmid Inc-types

Antimicrobial resistance (AMR) genes and virulence-associated genes were identified from assembled *Escherichia coli* genomes using **ABRicate** (https://github.com/tseemann/abricate). AMR genes were detected by screening assemblies against the **ResFinder** database (accessed 26 July 2025)-, while virulence genes were identified using the **Virulence Factor Database (VFDB)** (accessed 23 November 2025). Plasmid replicon (Inc) types were identified by comparing assembled sequences against the **PlasmidFinder** database (accessed 10 October 2025). Only hits with **≥95% nucleotide identity and 100% gene coverage** were retained for downstream analyses.

Presence–absence matrices were constructed for AMR genes, virulence genes, and plasmid replicons, with isolates represented as rows and genetic determinants as columns. Gene and plasmid prevalence were calculated across isolates, and distribution patterns were visualized using heatmaps with hierarchical clustering.

AMR genes were further grouped by antimicrobial class and resistance mechanism based on ResFinder annotations, and the relative contribution of each class and mechanism was summarized using bar plots and alluvial (Sankey) diagrams.

Associations between plasmid replicons and AMR genes were explored by constructing plasmid–gene co-occurrence matrices, which were visualized as clustered heatmaps.

The distribution of plasmid replicons across isolates was visualized using heatmaps and hierarchical clustering. Prevalence of each plasmid type was calculated and represented in ranked bar plots. Variation in plasmid carriage among isolates was further examined using principal component analysis (PCA).

Gene prevalence was calculated as the proportion of isolates harboring each virulence gene, and results were visualized in bar plots. Gene distribution across isolates was explored using heatmaps generated with hierarchical clustering (Euclidean distance and complete linkage). PCA was conducted after excluding non-informative genes (zero variance). All data were scaled prior to PCA, and biplots of principal components were generated to visualize isolate clustering based on virulence profiles.

Core-genome SNP analysis and phylogeny

Core-genome single-nucleotide polymorphisms (SNPs) were identified from assembled *Escherichia coli* genomes using **Snippy v3.0**. Core SNP alignments were generated across all isolates, and pairwise SNP distance matrices were calculated. A maximum-likelihood phylogenetic tree was inferred from the core SNP alignment using **FastTree 2.1.** Multilocus sequence typing (MLST) sequence types were mapped onto the phylogeny to evaluate concordance between core-genome relatedness and sequence-type assignment. The resulting phylogenetic tree was visualized and annotated using **iTOL**.

Brief Methodological Overview

Figure 2

RESULTS

MDR E. coli Prevalence in Sonali meat samples

The prevalence of MDR *E. coli* in Sonali chickens presents a serious public health concern, reflecting the widespread and often indiscriminate use of antibiotics in poultry farming. In this study, 74% of the Sonali chicken samples collected from LBMs in the Chattogram district were found to harbor MDR *E. coli*, indicating a high level of resistance to multiple classes of antibiotics (Figure 3).

Distribution of Multidrug-Resistant E. coli Across Live Bird Markets

The prevalence of MDR *E. coli* in Sonali chickens was further examined across various LBMs in Chattogram to identify potential hotspots of resistance. The analysis revealed considerable variation in prevalence among the markets. Notably high rates—around 80%—were recorded in Chawkbazar, Jhautala, Agrabad, 2no Gate, Bahaddarhat, Oxygen, and Colonel Hat. In contrast, relatively lower prevalence rates, approximately 60%, were observed in Sarai Para, Pahartali, and Reazuddin Bazar. These findings suggest that certain LBMs may serve as significant hubs for the dissemination of MDR *E. coli*, emphasizing the need for targeted surveillance and intervention strategies.

Antibiotic Susceptibility Test (AST)

The antibiotic susceptibility profile of *E. coli* isolates from Sonali chicken samples revealed a troubling level of MDR across multiple classes of antibiotics. Antimicrobial susceptibility testing (AST), performed using the disk diffusion method, showed that a majority of the isolates exhibited resistance to commonly used antibiotics (Table 1).

Table 1: Antimicrobial susceptibility patterns of E. coli isolates expressed as percentages of sensitive, intermediate, and resistant responses (n = 37).

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
AMP (10)	2.70%	0.00%	97.30%
FOX (30)	29.73%	13.51%	56.76%
CTX (30)	48.65%	18.92%	32.43%
CAZ (30)	56.76%	16.22%	27.03%
DOX (30)	5.41%	5.41%	89.19%
TE (30)	0.00%	0.00%	100.00%
SXT (1.25/23.75)	8.11%	13.51%	78.38%
GM (10)	32.43%	2.70%	64.86%
CIP (5)	2.70%	2.70%	94.60%
NOR (10)	13.51%	5.41%	81.08%
LEV (5)	10.81%	2.70%	86.49%

*AMP: Ampicillin, FOX: Cefoxitin, CTX: Cefotaxime, CAZ: Ceftazidime, DOX: Doxycycline, TE: Tetracycline, GM: Gentamycin, CIP: Ciprofloxacin, NOR: Norfloxacin, LEV: Levofloxacin, SXT: Trimethoprim/Sulfamethoxazole

MDR Pattern and Multiple Antibiotic Resistance Index (MARI)

The MDR patterns of E. coli isolates from Sonali chickens were evaluated to determine the specific combinations of antibiotics to which resistance was observed. These MDR profiles helped characterize the resistance behavior of the isolates. To further assess the level of antibiotic exposure, the Multiple Antibiotic Resistance Index (MARI) was calculated. Detailed MDR patterns and MARI values for all isolates are shown in **Table S2**. The MARI values among Sonali isolates ranged from 0.27 to 0.94, indicating varying degrees of antibiotic pressure across different live bird markets. A MARI value greater than 0.2 typically suggests that the bacteria originated from environments with high antibiotic usage (21).

The lowest MARI value among *E. coli* isolates from Sonali chickens was 0.18, linked to a resistance pattern of “TE-CIP” from an isolate collected at the Pahartali market. In contrast, the highest MARI value of 1 was recorded at the Sarai Para market. This isolate exhibited resistance to 11 antibiotics, represented by the extensive MDR pattern “AMP-FOX-CTX-CAZ-DOX-TE-GM-CIP-NOR-LEV-SXT.” This indicates a broad resistance spectrum and reflects substantial exposure to antibiotics. The wide range of MARI values among Sonali isolates, from 0.18 to 1, highlights significant variability in antibiotic

pressure across different live bird markets. Particularly, the higher MARI scores point to serious misuse or overuse of antibiotics in poultry farming. These findings underscore the urgent need for stronger antibiotic stewardship and improved management practices to curb the emergence and spread of multidrug-resistant *E. coli*.

Antimicrobial Resistance Gene Landscape in E. coli

A broad spectrum of antimicrobial resistance (AMR) genes was identified among the *Escherichia coli* isolates (Figure 4). Screening against the ResFinder database detected **29 distinct AMR gene subtypes** across the 26 isolates (Table 2). The number of resistance determinants per isolate ranged from 6 to 14 (mean ± SD: 9.3 ± 2.6), reflecting a substantial burden of resistance.

Tetracycline resistance genes were the most prevalent, with *tet(A)* (76.9%), *tet(M)* (69.2%), and *tet(X4)* (61.5%) widely distributed. High frequencies were also observed for sulfonamide and trimethoprim resistance genes, including *sul2* (73.1%), *dfrA12* (65.4%), *dfrA14* (57.7%), and *dfrA17* (53.8%). β-lactam resistance was primarily mediated by *blaTEM* variants, notably *blaTEM-1B* (46.2%), along with *blaLAP-2* (23.1%). Plasmid-mediated quinolone resistance genes were also common, particularly *qnrS1* (61.5%), *qnrS13* (46.2%), and *qnrS4* (42.3%), while efflux-associated genes (*oqxA* and *oqxB*) were detected in 65.4% of isolates.

Table 2: Distribution of antimicrobial resistance genes detected in E. coli isolates (n = 26). *Genes were identified using ResFinder (≥95% identity, ≥100% coverage thresholds). Frequencies represent the proportion of isolates harboring each resistance determinant.*

Gene (Subtype)	No. of Isolates	Frequency (%)	95% CI (%)
blaTEM-1B	12	46.2	27.1–65.3
blaTEM-106	5	19.2	4.1–34.3
blaTEM-135	3	11.5	0–23.8
blaTEM-176	2	7.7	0–17.9
blaTEM-1C	2	7.7	0–17.9
blaLAP-2	6	23.1	6.9–39.3
qnrS1	16	61.5	42.8–80.2
qnrS13	12	46.2	27.1–65.3
qnrS4	11	42.3	23.3–61.3
qnrB19	6	23.1	6.9–39.3
tet(A)	20	76.9	60.7–93.1
tet(M)	18	69.2	51.5–86.9
tet(X4)	16	61.5	42.8–80.2
sul2	19	73.1	56.0–90.2
sul3	4	15.4	1.5–29.3
dfrA12	17	65.4	47.1–83.7
dfrA14	15	57.7	38.7–76.7
dfrA17	14	53.8	34.6–73.0
dfrA15	6	23.1	6.9–39.3
oqxA	17	65.4	47.1–83.7
oqxB	17	65.4	47.1–83.7
aac(3)-IId	9	34.6	16.3–52.9
aadA5	11	42.3	23.3–61.3
aph(3")-Ib	10	38.5	19.8–57.2
aph(6)-Id	10	38.5	19.8–57.2
ant(3")-Ia	7	26.9	9.9–43.9
aph(3')-Ia	8	30.8	13.0–48.6
floR	8	30.8	13.0–48.6

mph(A)	5	19.2	4.1–34.3

Aminoglycoside resistance genes showed moderate prevalence and diversity, including *aadA5* (42.3%), *aph(3")-Ib* (38.5%), *aph(6)-Id* (38.5%), *aph(3')-Ia* (30.8%), and *ant(3")-Ia* (26.9%). In contrast, less frequent determinants included *sul3* (15.4%), *dfrA15* (23.1%), *mph(A)* (19.2%), and *arr-3* (15.4%). Notably, no *blaCTX-M* or *blaOXA* genes were detected.

Co-occurrence of multiple resistance determinants within individual isolates was common, with many isolates harboring genes spanning several antimicrobial classes, indicative of widespread multidrug resistance (Figure S1). Consistently, binary heatmap clustering (Figure 4) revealed distinct groupings of isolates with similar resistance profiles. Isolates with extensive resistance repertoires clustered separately from those with fewer determinants, suggesting potential local dissemination and shared sources of AMR gene circulation.

Spatial distribution of AMR gene burden across live bird markets

Analysis of AMR gene burden across live bird markets revealed marked spatial heterogeneity (Figure 5). Isolates from Agrabad exhibited the highest resistance gene loads (median approximately 10 genes, maximum 15), whereas isolates from Pahartali and 2 No. Gate carried the lowest burdens (median ≤6). Bahaddarhat, Jhautala, and Chawkbazar markets showed intermediate resistance profiles.

When aggregated by antibiotic class, tetracycline, sulfonamide, aminoglycoside, and β-lactam resistance genes predominated across all markets, while fluoroquinolone, phenicol, and macrolide resistance genes were detected less frequently (Figure 6). Resistance genes associated with carbapenems and tigecycline were observed only sporadically.

Plasmid replicon diversity and population structure

Screening with PlasmidFinder identified 23 distinct plasmid replicon types among the 26 *E. coli* isolates, with individual isolates harboring between zero and five plasmids (Figure 4). Hierarchical clustering of plasmid presence–absence profiles revealed distinct distribution patterns across the isolate collection (Figure S2).

The most prevalent replicon was p0111, detected in 50% of isolates, followed by IncX1, ColpVC, IncFIB(K), and IncN, each present in approximately 27–31% of isolates (Figure 4 and Figure 7). Several replicons, including IncR, IncI1, IncHI2, and IncHI2A, were rare and detected in only a single isolate.

Principal Component Analysis (PCA) of plasmid replicon profiles revealed a structured yet moderately heterogeneous distribution of *E. coli* isolates, with PC1 and PC2 explaining 20.1% and 16.4% of the total variance, respectively (Figure 8). The majority of isolates formed a compact cluster, indicating a largely conserved plasmid backbone across the population. In contrast, isolate SM_7 was clearly separated along both axes, highlighting a distinct plasmid composition and suggesting the presence of unique or less prevalent replicon combinations.

Loading score analysis (Figure 9) provided further resolution of the drivers underlying this separation. The primary axis (PC1) was predominantly shaped by pKPC-CAV1321, IncHI2A, IncX1_4, IncR, IncX4_2, and IncHI21, indicating that these plasmids are key determinants of major structural variation within the dataset. The secondary axis (PC2) was strongly influenced by IncHI1A, IncHI1B(R27)_R27, IncFIA(HI1), and Col440I, reflecting an additional layer of plasmid heterogeneity. Several replicons, including ColpVC, IncN, and IncX1, contributed across both components, suggesting overlapping distribution patterns and potential co-occurrence within isolates.

Together, the PCA clustering (Figure 8) and loading patterns (Figure 9) highlight a composite plasmid architecture consisting of a conserved core and a variable accessory component. The strong contribution of IncHI and IncF family plasmids—frequently associated with multidrug resistance and horizontal gene transfer—underscores their central role in driving genomic diversification and shaping the epidemiological and evolutionary trajectories of these isolates.

Virulence Gene Landscape in E. coli from Sonali Chicken Meat

Whole-genome sequencing of 26 *E. coli* isolates identified a diverse repertoire of 39 virulence-associated genes, detected using the VirulenceFinder database (Table 4). Considerable variation in virulence gene distribution was observed among isolates, reflecting heterogeneity in virulence potential within the population.

Table 4: Virulence genes detected in 26 E. coli isolates and their functional categories

Functional Category	Virulence Genes Identified
Adhesion	*fimH, csgA, aslA, iha, hra, lpfa, fdeC, tia, yehA, yehB, yehC, yehD*
Stress Response	*gad, hlyF, anr, terC, nlpl*
Toxin	*hlyE, cib, astA, cma, cea, cvaC*
Capsule Biosynthesis	*kpsMIII, kpsM_K11, kpsE, neuC*
Iron Acquisition	*iutA, iucC, iroN, sitA, fyuA, irp2,*
Serum Resistance	*traT, iss, ompT*
Other/Host Interaction	*shiA, hha, traJ*

Virulence genes were classified into multiple functional categories, including adhesion, stress response, toxin production, capsule biosynthesis, iron acquisition, serum resistance, and other host interaction functions (Table 4). Adhesion-associated genes—such as *fimH, csgA, aslA, iha, hra, lpfA, fdeC, tia,* and *yehA–D*—were widely distributed and detected in the majority of isolates (Figure 10), highlighting their central role in host colonization and biofilm formation. Similarly, stress response–related genes,

including *gad, hlyF,* and *anr*, were frequently identified, suggesting their importance in environmental adaptation and survival under adverse conditions.

In contrast, several virulence determinants associated with specialized pathogenic traits were detected less frequently. These included toxin-associated genes (*hlyE, cib, astA, cma, cea, cvaC*), serum resistance genes (*traT, iss, ompT*), capsule biosynthesis genes (*kpsMIII, kpsM_K11, kpsE, neuC*), and iron acquisition systems (*iutA, iucC, iroN, sitA, fyuA, irp2*), which were present in a subset of isolates. Additionally, genes involved in host interaction and regulatory functions, such as *shiA, hha,* and *traJ*, showed variable distribution. The detection of *traJ* indicates the presence of conjugative elements, highlighting the potential for horizontal transfer of antimicrobial resistance and virulence determinants among isolates. The heterogeneous presence of these genes suggests that while core virulence traits are widely conserved, accessory factors contributing to enhanced pathogenicity and survival may be context-dependent rather than universally required.

Principal component analysis (PCA) of virulence gene profiles demonstrated clear variability in gene distribution (Figure S3). The first two principal components explained a substantial proportion of the total variance (PC1: 67.1%, PC2: 6.7%), indicating that the major differences in virulence gene presence were captured along PC1. The PCA plot revealed distinct clustering of virulence genes into separate groups. A large cluster of genes was observed on the negative side of PC1, suggesting a set of commonly co-occurring virulence determinants. In contrast, several genes were clearly separated along the positive axis of PC1, indicating distinct presence/absence patterns compared to the main group. This separation highlights heterogeneity in virulence gene distribution, suggesting that certain genes contribute disproportionately to the observed variation and may define distinct virulence profiles among the isolates.

Prevalence analysis revealed that adhesion (≈60%) and stress response (≈59%) genes were the most abundant functional categories, highlighting their key role in colonization and environmental adaptation (Figure 11). In contrast, toxin (≈21%) and serum resistance (≈20%) genes showed moderate prevalence, while genes associated with host interaction (≈14%) were less common. Iron acquisition (≈7%) and capsule biosynthesis (≈6%) genes were the least prevalent, indicating that these accessory virulence traits may confer selective advantages only under specific host or environmental conditions.

Concordance between phylogeny, resistome, and virulome patterns

Integration of core-genome phylogeny with AMR gene, virulence gene, and plasmid replicon profiles revealed that closely related isolates often shared similar resistance and virulence determinants (Figures 4 and 10). Nevertheless, notable variation in AMR and virulence gene content was observed within certain phylogenetic clades, indicating heterogeneity among closely related isolates.

Genomic diversity and population structure of *Escherichia coli* isolates

Core-genome SNP–based phylogenetic analysis of 26 *E. coli* isolates revealed substantial genetic diversity within the collection (Figures 4 and 10). The maximum-likelihood phylogeny resolved the isolates into multiple distinct clades, indicating a heterogeneous population structure rather than dominance of a single lineage.

Multilocus sequence typing (MLST) identified several sequence types (STs), with ST48, ST10, ST155, and ST710 among the most frequently observed. Isolates belonging to the same ST generally clustered together within the phylogeny, demonstrating good concordance between MLST assignment and core-genome relatedness. However, some STs were distributed across separate branches of the tree, suggesting genomic diversification within individual sequence types.

DISCUSSION

This study reveals a high prevalence (74%) of multidrug-resistant (MDR) *E. coli* in Sonali chicken meat from live bird markets (LBMs) in Chattogram, reflecting a critical antimicrobial resistance (AMR) threat within Bangladesh's poultry sector. Consistent with earlier work, similar or higher MDR rates have been reported among broiler and Sonali chicken farms in Bangladesh and neighboring countries, reinforcing the regional patterns of unregulated and excessive antibiotic use (7, 22). The detection of elevated MDR rates in specific LBMs highlights their role as significant hubs for the amplification and dissemination of resistant strains, likely due to crowded conditions, environmental contamination, and poor hygiene practices.

The integrated analysis of core genome SNP phylogeny with antimicrobial resistance (AMR), plasmid replicon, and virulence gene profiles revealed a highly diverse *Escherichia coli* population with significant public health implications. The phylogenetic reconstruction demonstrated the presence of multiple sequence types distributed across distinct clades, indicating that the isolates do not originate from a single clonal lineage. However, despite this genetic diversity, overlapping AMR and virulence gene repertoires were frequently observed among phylogenetically unrelated isolates, highlighting the dominant role of horizontal gene transfer in shaping the accessory genome (23, 24).

Critically, the identification of *tet(X4)*—a gene conferring resistance to tigecycline, a last-resort human antimicrobial—marks the first report in Sonali chicken *E. coli* in Bangladesh, emphasizing the need for expanded genomic surveillance and stewardship (7, 25, 26). Mechanistically, drug inactivation through β-lactamases and aminoglycoside-modifying enzymes was dominant, supplemented by target protection and efflux mechanisms. This diversity supports the notion of widespread horizontal gene transfer and adaptive resistance evolution in poultry systems, with cross-resistance and environmental dissemination as ongoing risks. The strong correlation between genotype and phenotype—validated using WGS and ResFinder—confirms that current genomic tools are reliable for AMR surveillance, though discrepancies (possibly due to gene regulation or undetected chromosomal mutations) persist.

The correlation between genotypic and phenotypic resistance patterns was strong for several antibiotic classes, particularly tetracyclines and sulfonamides, validating the utility of WGS in AMR surveillance.

However, some inconsistencies were observed, possibly due to uncharacterized resistance mechanisms, incomplete databases, or differential gene expression. The clustering of isolates based on their AMR gene content indicates localized circulation of specific resistance determinants within LBMs, suggesting potential clonal expansion or frequent horizontal transfer in market environments (7).

Resistance determinants associated with critically important antimicrobials, including β-lactams, fluoroquinolones, tetracyclines, aminoglycosides, and sulfonamides, were widely distributed across the phylogeny. The frequent co-occurrence of these genes with epidemic plasmid families such as IncF, IncI, IncX, and Col-type replicons underscores the role of plasmid-mediated dissemination in the emergence of multidrug-resistant *E. coli* (27, 28). These plasmids are well adapted to *E. coli* and have been repeatedly implicated in the spread of resistance genes between animal, human, and environmental reservoirs, reinforcing their importance in a One Health context (28). This finding aligns with global reports where *IncF* and *IncX* plasmids are the dominant vehicles for multidrug resistance spread in animal and human populations.

Concurrently, Virulence gene profiling of the isolates revealed a diverse repertoire of traits associated with colonization, environmental adaptation, and extraintestinal pathogenicity. Adhesion-associated genes (*fimH, lpfA, yehA–D, aslA, iha, hra, fdeC, tia*) and stress response regulators (*gad, hlyF, anr, terC, nlpI*) were widely distributed, consistent with their central role in host colonization and survival. Iron acquisition systems (*iroN, iucC, iutA, sitA, fyuA, irp2*) and capsule biosynthesis genes (*kpsE, kpsMII, kpsMIII_K11, neuC*), along with toxins (*hlyE, cib, astA, cma, cea, cvaC*) and serum survival factors (*traT, iss, ompT*), were less prevalent, suggesting these accessory virulence determinants may confer selective advantages under specific ecological or host conditions (29). Additional regulatory and host-interaction genes, including *shiA, hha*, and *traJ*, were variably distributed, highlighting the contribution of mobile genetic elements in shaping the genomic landscape of these isolates (30). The heterogeneous distribution of virulence determinants across phylogenetic clusters underscores the modular and mosaic nature of *E. coli* genomes. Certain isolates, such as SM35 and SM16, exhibited distinct virulence patterns, yet similar variability was observed across other strains, indicating that virulence traits are not confined to specific lineages but are dynamically distributed. Collectively, these findings suggest that the observed virulence profiles result from a combination of conserved core functions and accessory traits, reflecting both evolutionary adaptations to host niches and the potential for opportunistic pathogenicity. Notably, several isolates harbored concurrent virulence and multidrug resistance determinants, supporting the circulation of potential high-risk clones with possible zoonotic implications within a One Health framework.

Notably, several isolates harbored combinations of virulence and AMR genes, a convergence that has been increasingly recognized as a major driver of zoonotic risk (31). The co-localization of resistance and virulence traits within poultry-derived *E. coli* is of particular concern, as such strains may act as reservoirs for the transmission of multidrug-resistant and potentially pathogenic bacteria to humans via the food chain, direct contact, or environmental contamination (32). Live bird markets and poultry meat

have previously been identified as critical interfaces for cross-sectoral transmission, facilitating the movement of mobile genetic elements across ecological boundaries (33).

Overall, the integration of core genome phylogeny with resistance, plasmid, and virulence profiling provides a comprehensive framework for assessing zoonotic potential beyond clonal relatedness alone (34, 35).

Integrated analysis of plasmid replicons, antimicrobial resistance genes, and virulence determinants (Figs. 4 and 13) demonstrated substantial genomic heterogeneity among the poultry-associated *E. coli* isolates. The heatmap patterns revealed uneven distribution of mobile genetic elements and resistance determinants, with several isolates (e.g., SM44, SM9, SM16, and SM35) exhibiting comparatively broader repertoires spanning plasmid types, multidrug resistance genes, and virulence-associated factors. Such convergence of resistance and virulence traits is consistent with the “high-risk clone” paradigm described in global genomic surveillance studies, wherein co-selection and plasmid-mediated horizontal gene transfer facilitate persistence, adaptability, and enhanced transmission potential.

The dendrogram-based clustering further indicated that isolates sharing similar plasmid and AMR profiles grouped together, suggesting possible localized dissemination or clonal expansion within live bird market environments. In contrast, other strains displayed relatively limited genetic content, underscoring that the poultry-associated *E. coli* population is heterogeneous in its pathogenic and resistance potential. Collectively, these findings underscore the central role of mobile genetic elements in shaping the genomic architecture of these isolates and highlight the public health importance of monitoring strains that concurrently harbor multidrug resistance and expanded virulence gene repertoires. They further reinforce the necessity of genome-based surveillance strategies within a One Health framework, integrating animal, human, and environmental data to effectively track and mitigate the emergence and dissemination of multidrug-resistant and virulent *E. coli* originating from food-producing animals (36).

CONCLUSIONS

This study provides the first comprehensive genomic characterization of multidrug-resistant (MDR) *Escherichia coli* isolated from Sonali chicken meat sold at live bird markets in Chattogram, Bangladesh. A high prevalence of MDR *E. coli* was observed, with isolates exhibiting extensive resistance to commonly used antimicrobials including tetracyclines, β-lactams, sulfonamides, and fluoroquinolones. The elevated Multiple Antibiotic Resistance Index (MARI) values across isolates further indicate substantial antibiotic selection pressure within the poultry production and marketing chain. Whole-genome sequencing revealed a diverse resistome comprising 43 antimicrobial resistance genes, with dominant determinants including *tet(A)*, *sul1*, *sul2*, and *blaTEM-1B*. Notably, the detection of the tigecycline resistance gene *tet(X4)* represents an important finding and suggests the emergence of resistance to last-resort antimicrobials in poultry-associated *E. coli* in Bangladesh. The presence of multiple plasmid replicons—particularly p0111, IncFIB(K), and IncX1—alongside AMR genes highlights the critical role of mobile

genetic elements in facilitating horizontal gene transfer and dissemination of resistance within the poultry environment. Virulence profiling indicated that many isolates harbor genes associated with adhesion, iron acquisition, and stress response, suggesting the potential for these strains to persist in diverse hosts and environments. Core-genome SNP phylogeny and comparative genomic analyses further demonstrated genetic diversity among isolates while maintaining a conserved genomic backbone, reflecting ongoing evolutionary adaptation under antimicrobial pressure. Collectively, these findings demonstrate that Sonali chicken meat sold at live bird markets may serve as a reservoir of MDR *E. coli* carrying clinically relevant resistance and virulence determinants. Given the close interaction between poultry, humans, and market environments, these strains pose a potential zoonotic and food-safety risk. Strengthening antimicrobial stewardship in poultry production, improving hygiene practices in live bird markets, and implementing integrated genomic surveillance under a One Health framework are essential to mitigate the emergence and spread of antimicrobial resistance in Bangladesh.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with institutional and national guidelines for research involving animal-derived food samples. Ethical approval was obtained from the Institutional Animal Ethics Committee of Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh. No live animals were directly used in this study, and samples were collected from retail live bird markets.

Consent for publication

Not applicable.

Availability of data and materials

The whole-genome sequencing data generated in this study have been deposited in the European Nucleotide Archive under BioProject accession number PRJEB108291. Additional datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MMH: Conceptualization, methodology, data curation, formal analysis, bioinformatics analysis, writing—original draft.
SAR and RBD: Laboratory investigation, data collection, validation.
RR and MM: Laboratory investigation and Data analysis.
MNI, SA, and SC: Supervision and laboratory support.
GK, SM, MA, GS, and AS: Whole-genome sequencing and bioinformatics analysis.
KB: writing—review and editing and data interpretation.
MRP: Co-supervision, writing—review and editing.

SAK: Conceptualization, Collaboration with the Hungarian team, Study design, supervision, writing—review and editing.
All authors read and approved the final manuscript.

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Declaration of generative AI and AI-assisted technologies in the writing process

The authors utilized QuillBot, Grammarly, ChatGPT, and Perplexity exclusively for grammar checking during the manuscript preparation. Following the use of these tools, the text was carefully reviewed and revised as needed. The authors accept full responsibility for the accuracy and integrity of the content presented in this publication.

Data Availability Statement

The data supporting this study can be obtained from the corresponding author upon reasonable request. Public access to the data is restricted due to privacy and ethical considerations.

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Figures

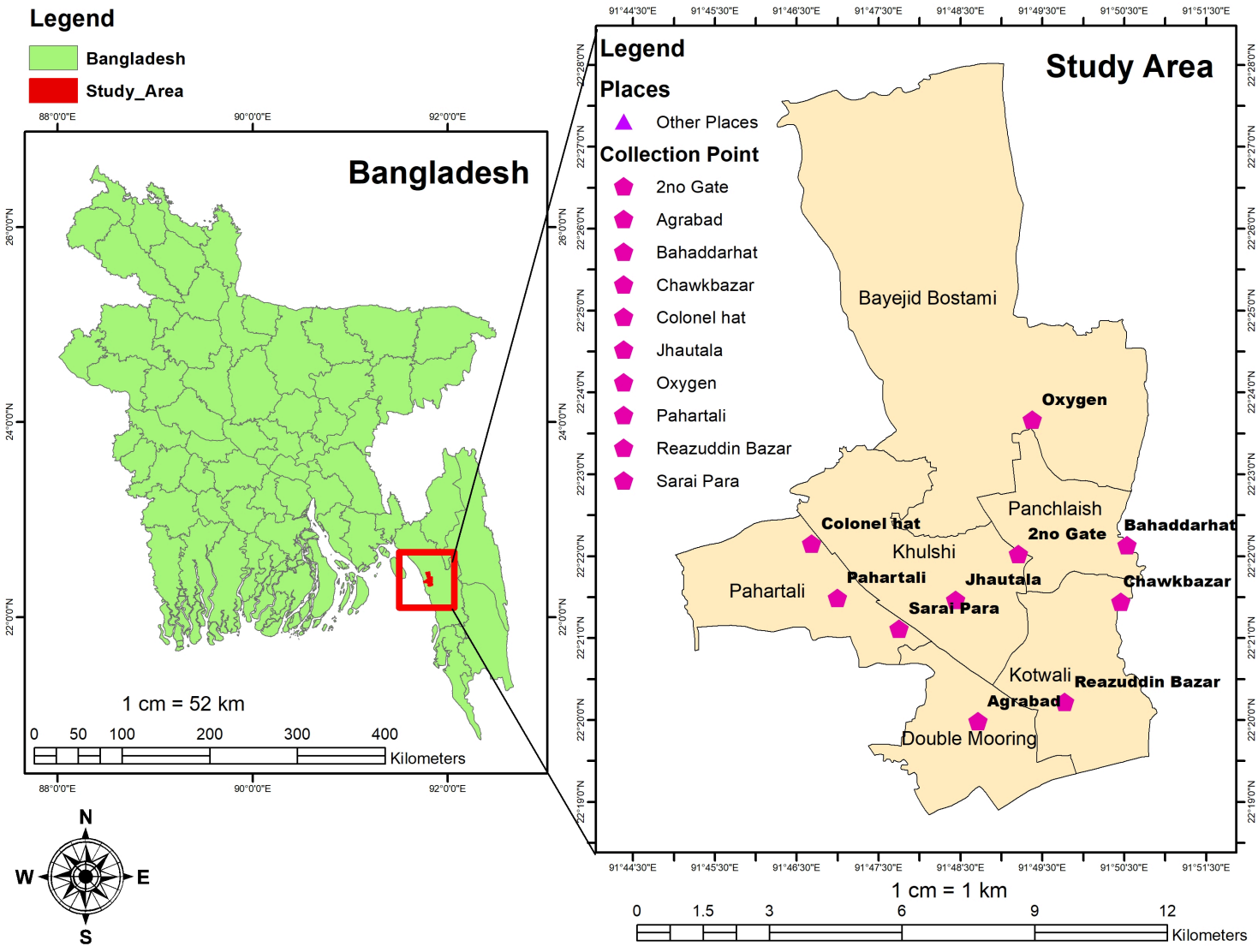


Figure 1

Map of Bangladesh showing the location of the study area (left) and detailed distribution of sampling points across Chattogram City (right).

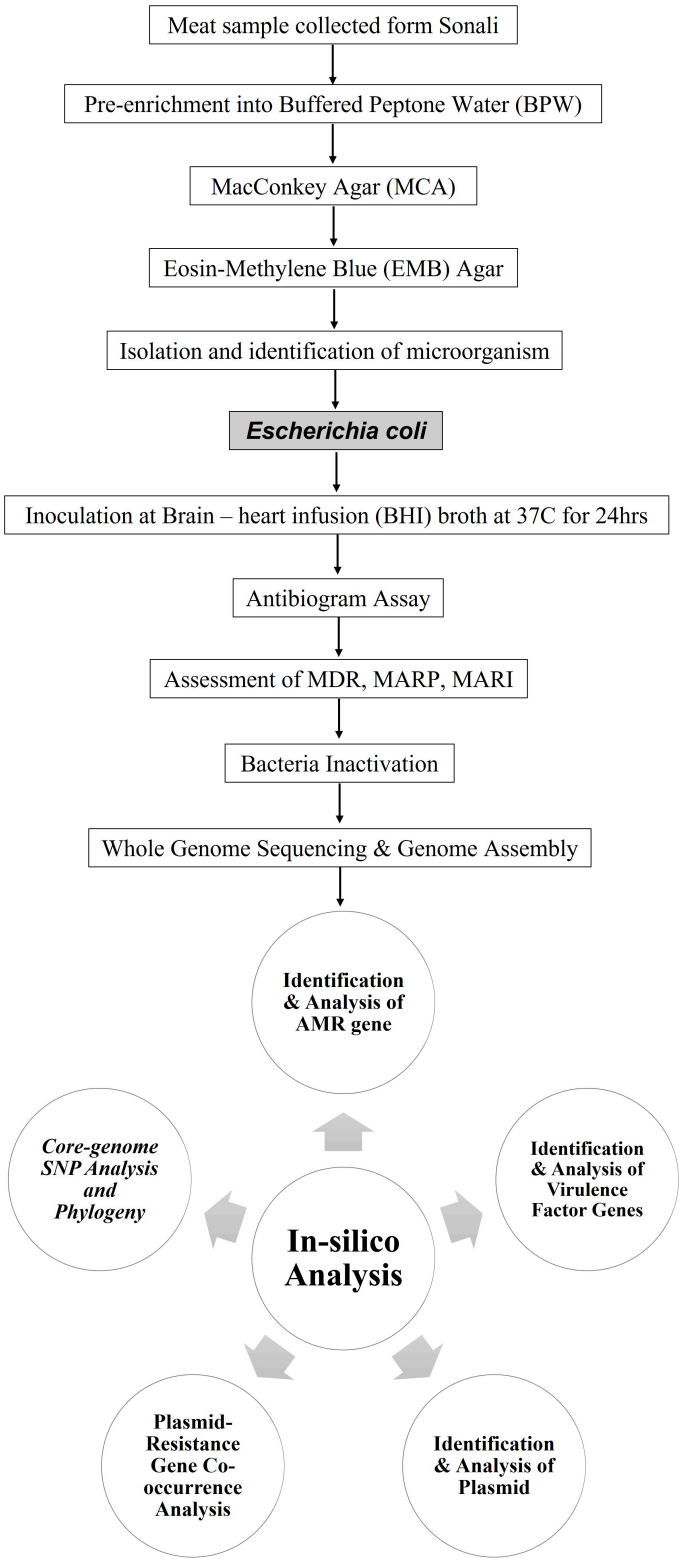


Figure 2

Workflow for isolation, phenotypic characterization, and whole-genome analysis of *Escherichia coli* from Sonali chicken meat.

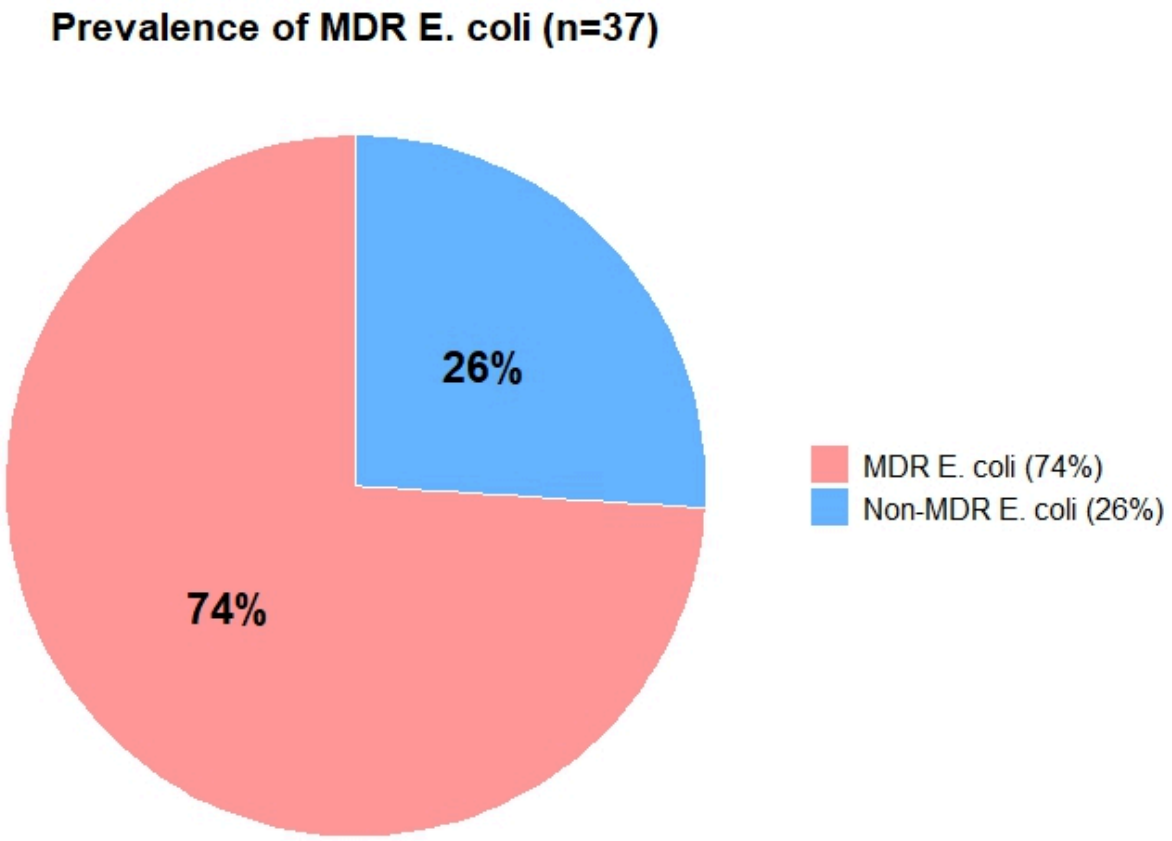


Figure 3

Prevalence of multidrug-resistant (*MDR*) *E. coli* in Sonali chicken samples collected from live bird markets in Chattogram, Bangladesh (n = 37). Out of 50 samples, 37 (74%) were confirmed as MDR *E. coli*, while 13 (26%) were non-MDR.

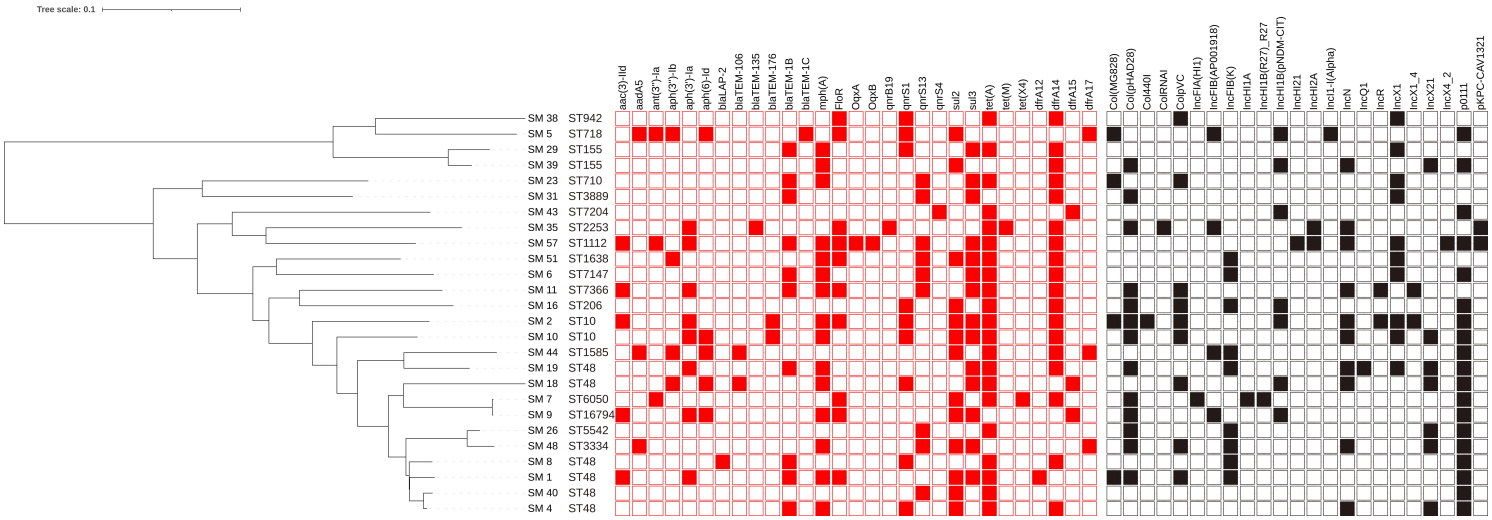


Figure 4

Phylogenetic relationships and distribution of antimicrobial resistance genes and plasmid replicons in Escherichia coli isolates. Core-genome SNP-based phylogenetic tree of Escherichia coli isolates from Sonali chicken meat, annotated with sequence types (STs), antimicrobial resistance (AMR) genes, and plasmid replicons. The phylogenetic tree (left) was constructed based on core-genome single nucleotide polymorphisms, with branch lengths representing genetic distances (scale bar indicated). The heatmap in the middle panel (red squares) shows the presence (filled) or absence (empty) of AMR genes across isolates, including genes conferring resistance to aminoglycosides, β-lactams, tetracyclines, sulfonamides, trimethoprim, phenicols, macrolides, and fluoroquinolones. The right panel (black squares) represents the distribution of plasmid replicon types identified in each isolate. Co-occurrence patterns highlight the role of plasmids in the dissemination of resistance determinants. Isolates are labeled by sample ID and corresponding MLST sequence types.

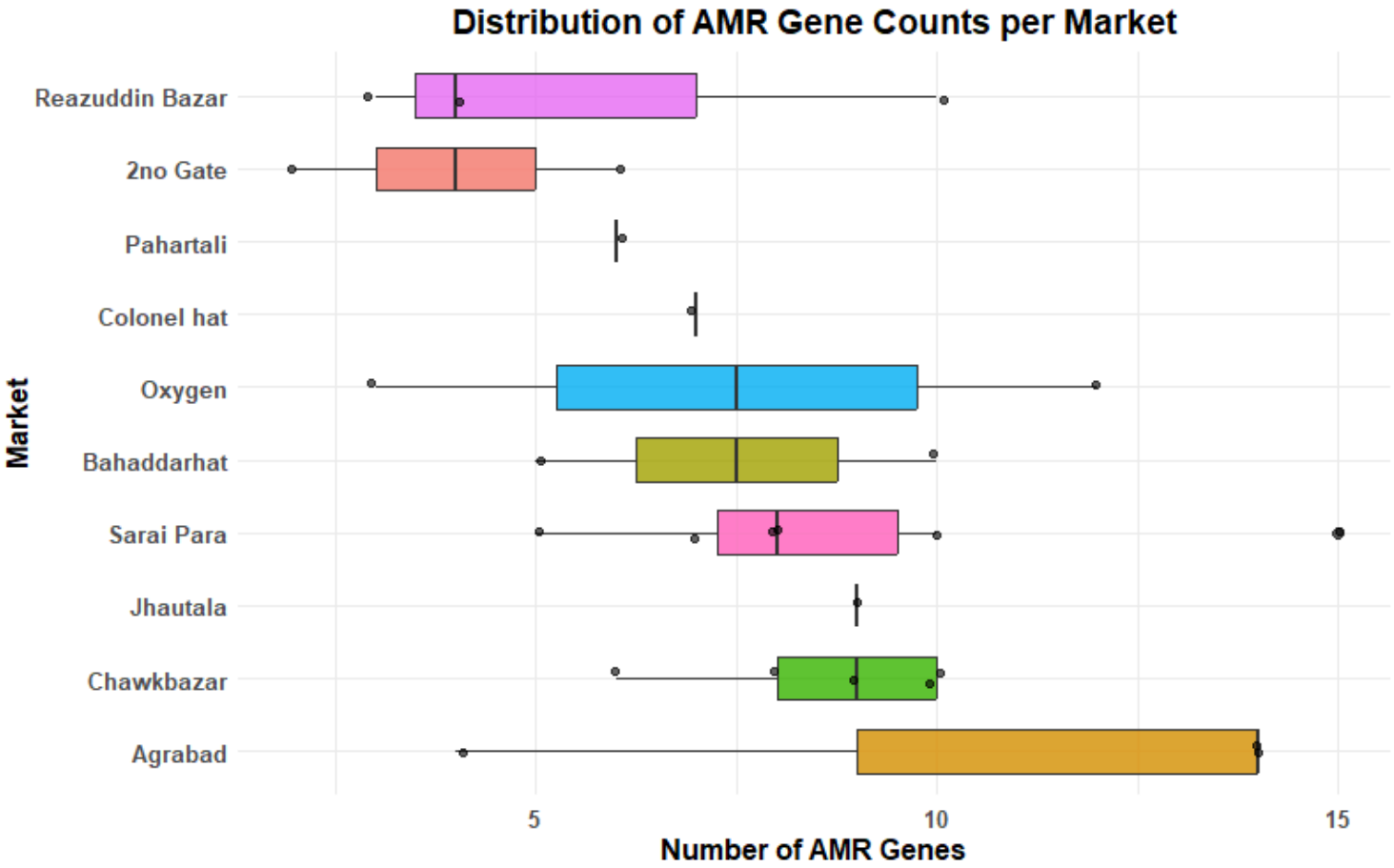


Figure 5

Distribution of AMR gene counts per market. Boxplots show variation in the number of resistance genes per isolate across different live bird markets. Black dots indicate outliers.

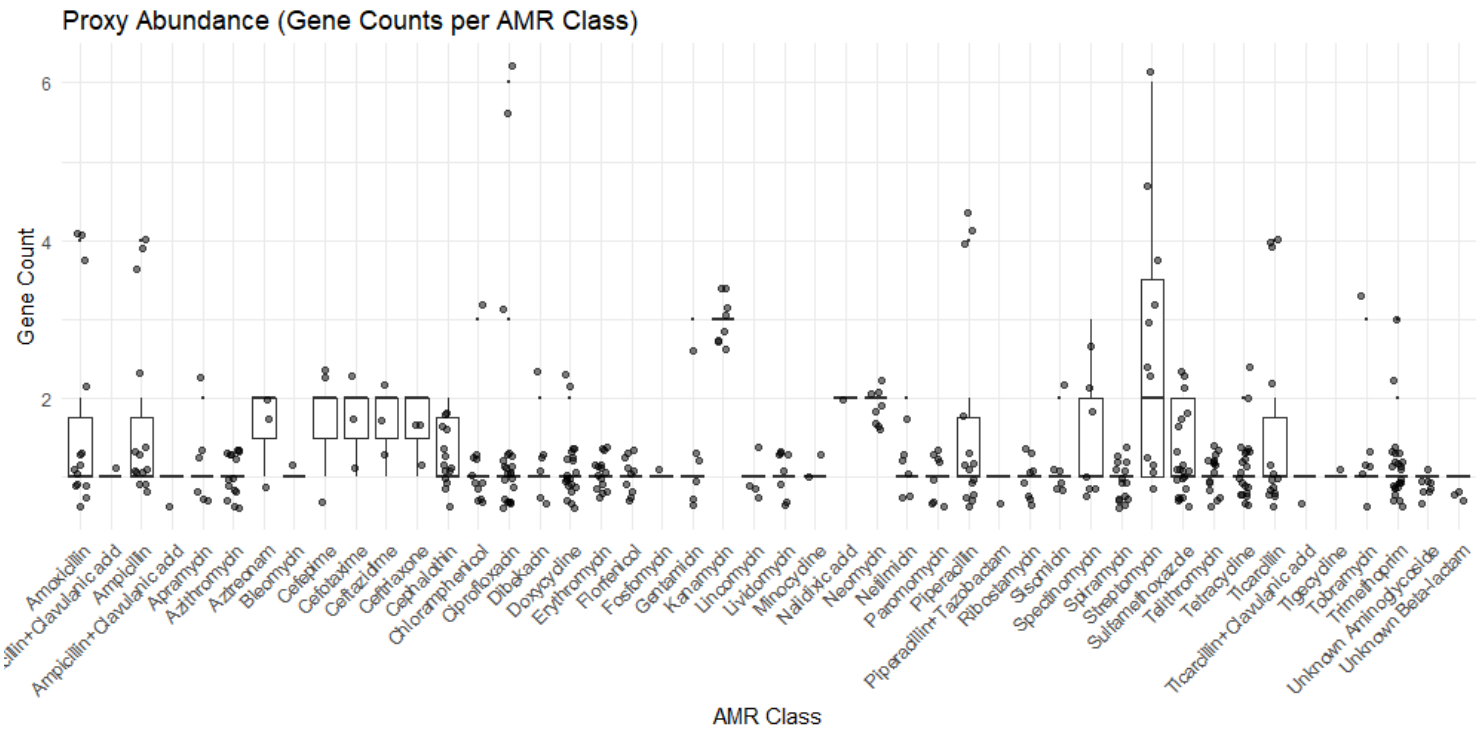


Figure 6

Proxy abundance of AMR classes. Boxplots represent the distribution of gene counts per antimicrobial class across all isolates. Each point corresponds to an individual resistance determinant identified by ResFinder.

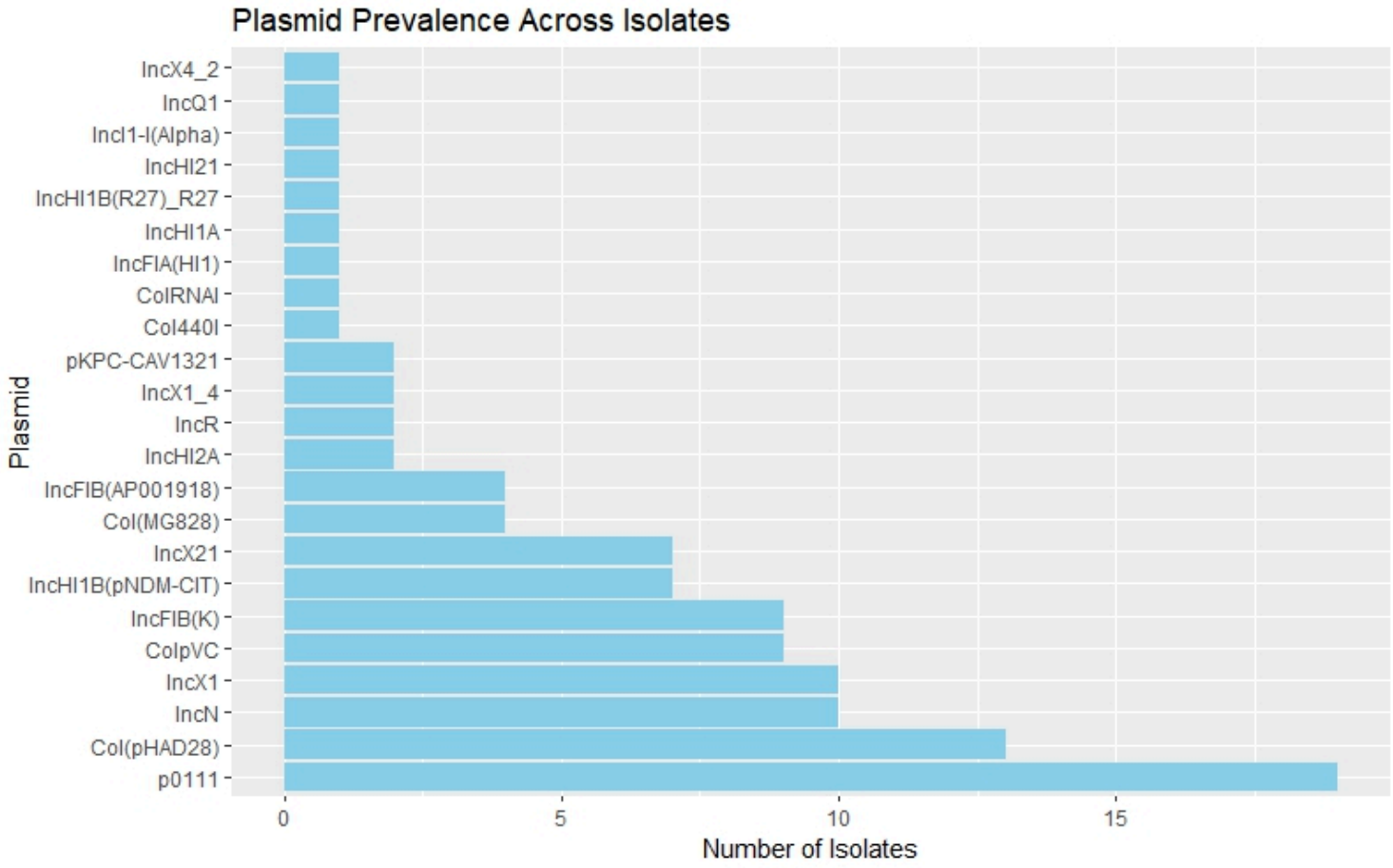


Figure 7

Prevalence of plasmid replicons across 26 E. coli isolates. Bars show the number of isolates carrying each plasmid type. The most prevalent plasmid was p0111, detected in 13 isolates (50%), followed by IncX1, ColpVC, IncFIB(K), and IncN, each found in 7–8 isolates (~27–31%). Several plasmids, including IncR, IncI1, IncHI2, and IncHI2A, were rare, detected in only a single isolate. This pattern indicates the coexistence of both widespread and isolate-specific plasmids within the collection.

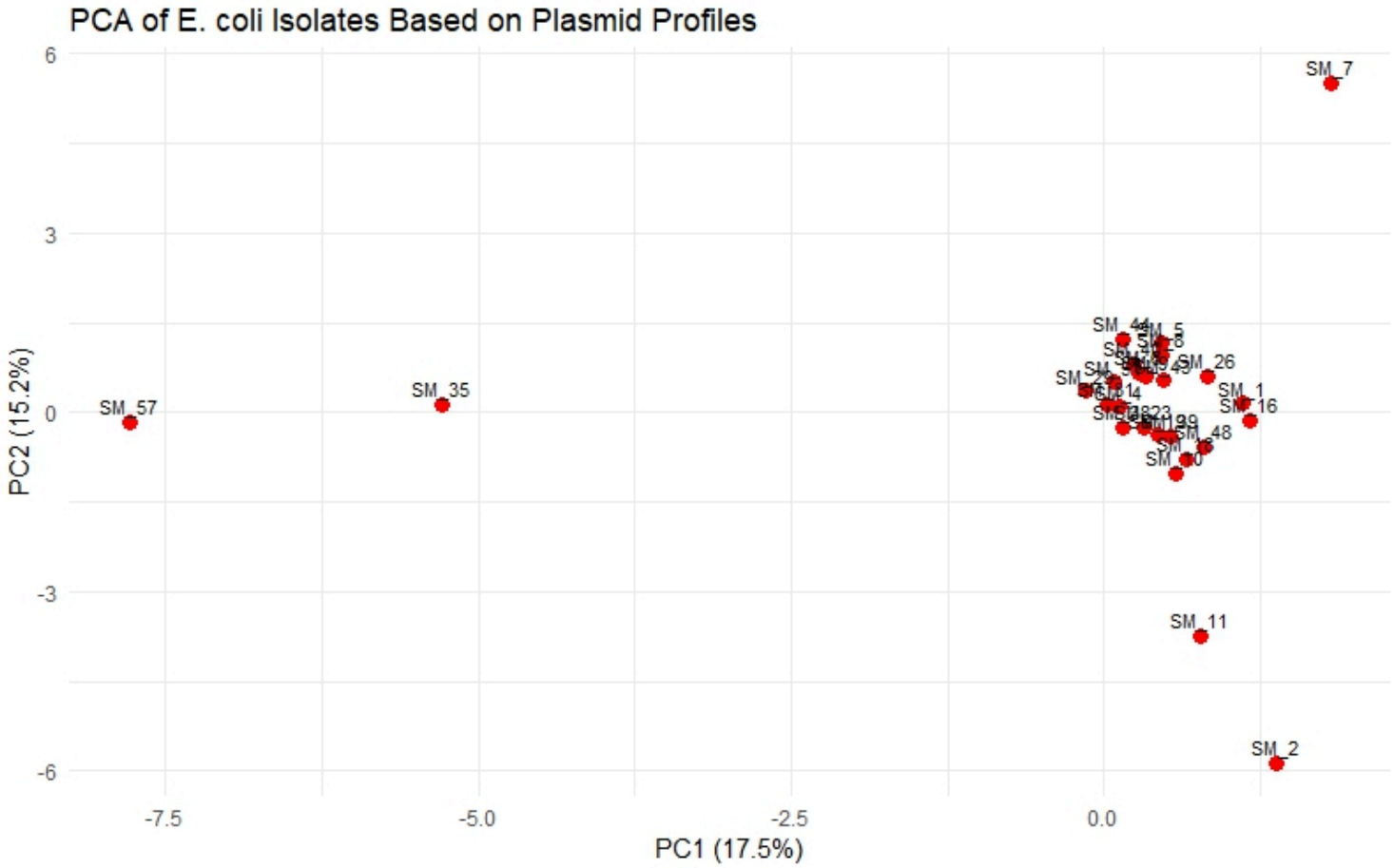


Figure 8

Principal component analysis (PCA) of plasmid carriage profiles in 26 E. coli isolates. Each point represents an isolate positioned according to its plasmid profile. The first two principal components explain 20.1% (PC1) and 16.4% (PC2) of the total variance. Most isolates cluster closely together, indicating similar plasmid content, whereas SM_7 is separated on the negative PC1 axis, reflecting its unique plasmid profile compared with the rest of the collection.

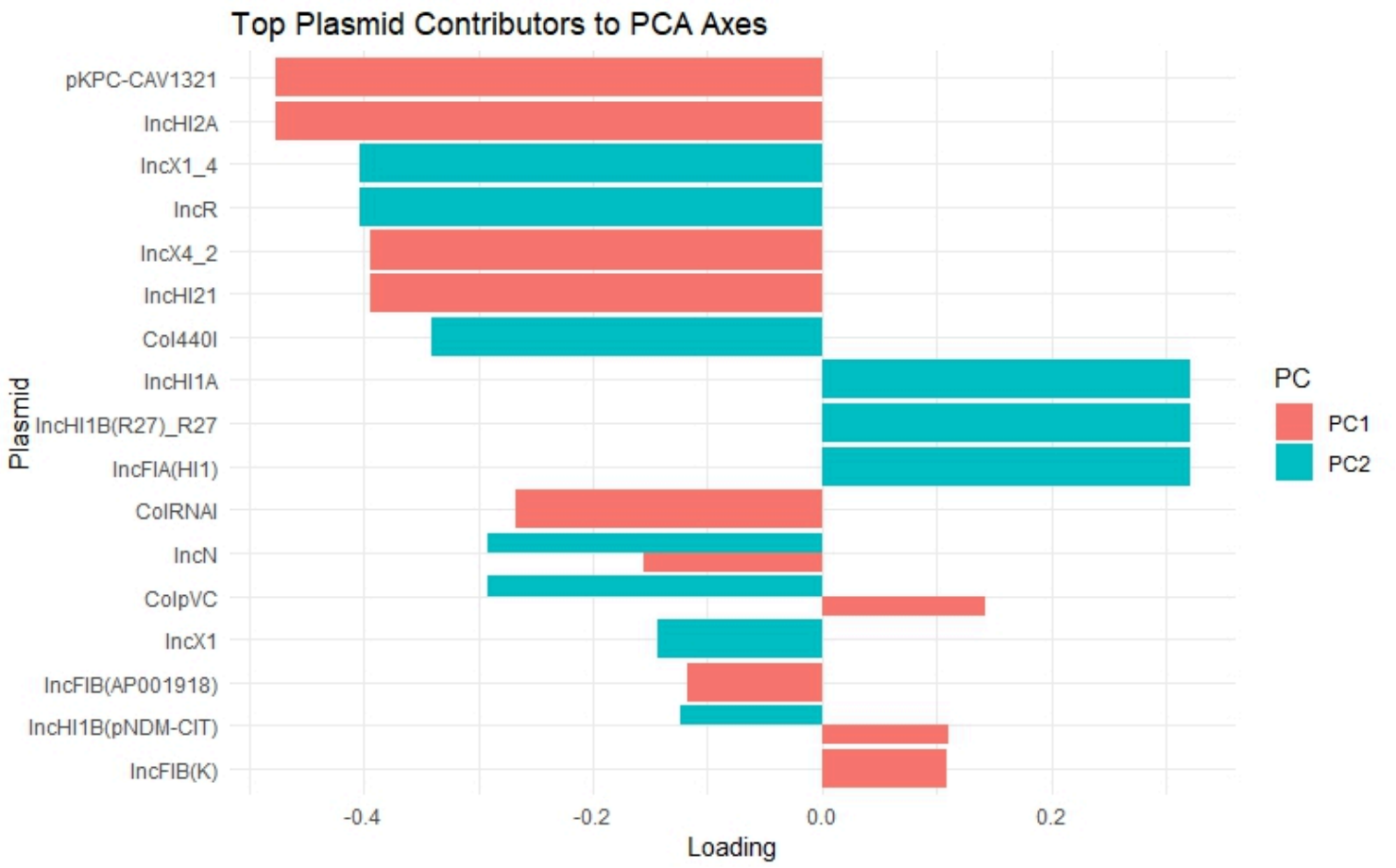


Figure 9

Top plasmid contributors to principal component axes (PC1 and PC2). The bar plot shows loading values derived from PCA of plasmid presence/absence data across 26 E. coli isolates. Each bar represents the contribution of a plasmid replicon to variation along PC1 (red) or PC2 (blue). Positive and negative loading values indicate the direction of influence on isolate separation in PCA space, while larger absolute values indicate stronger contributions. Plasmids with the highest loadings were identified as the main drivers of differentiation among isolates.

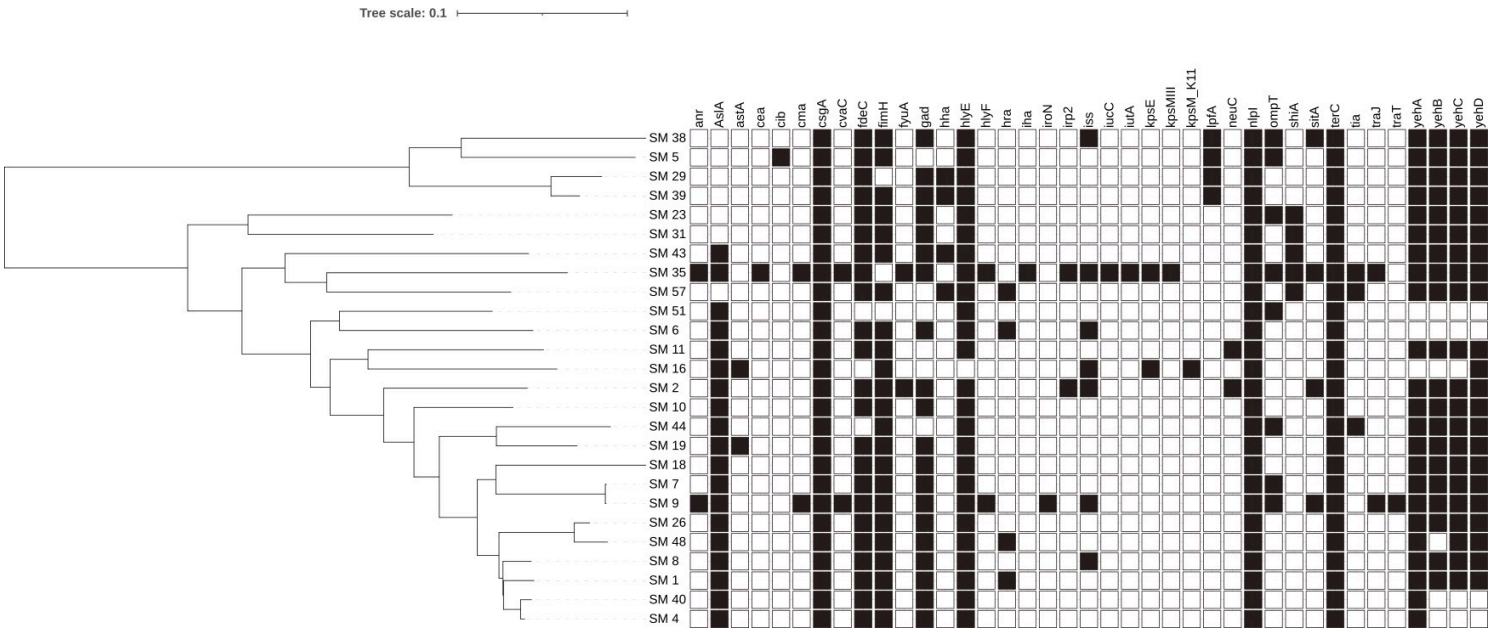


Figure 10

Phylogenetic relationships and distribution of virulence-associated genes in *Escherichia coli* isolates. Core-genome SNP-based phylogenetic tree of *Escherichia coli* isolates annotated with virulence-associated gene profiles. The phylogenetic tree (left) represents genetic relatedness among isolates, with branch lengths proportional to SNP differences. The heatmap (right; black squares) indicates the presence (filled) or absence (empty) of virulence genes associated with adhesion (e.g., *fim*, *pap*, *sfa*), iron acquisition (e.g., *iro*, *iuc*), serum survival, toxin production, and stress response. Conserved and variable virulence gene patterns across different sequence types suggest heterogeneity in pathogenic potential. The combined visualization highlights the coexistence of phylogenetic diversity and virulence gene distribution among poultry-associated *E. coli* isolates.

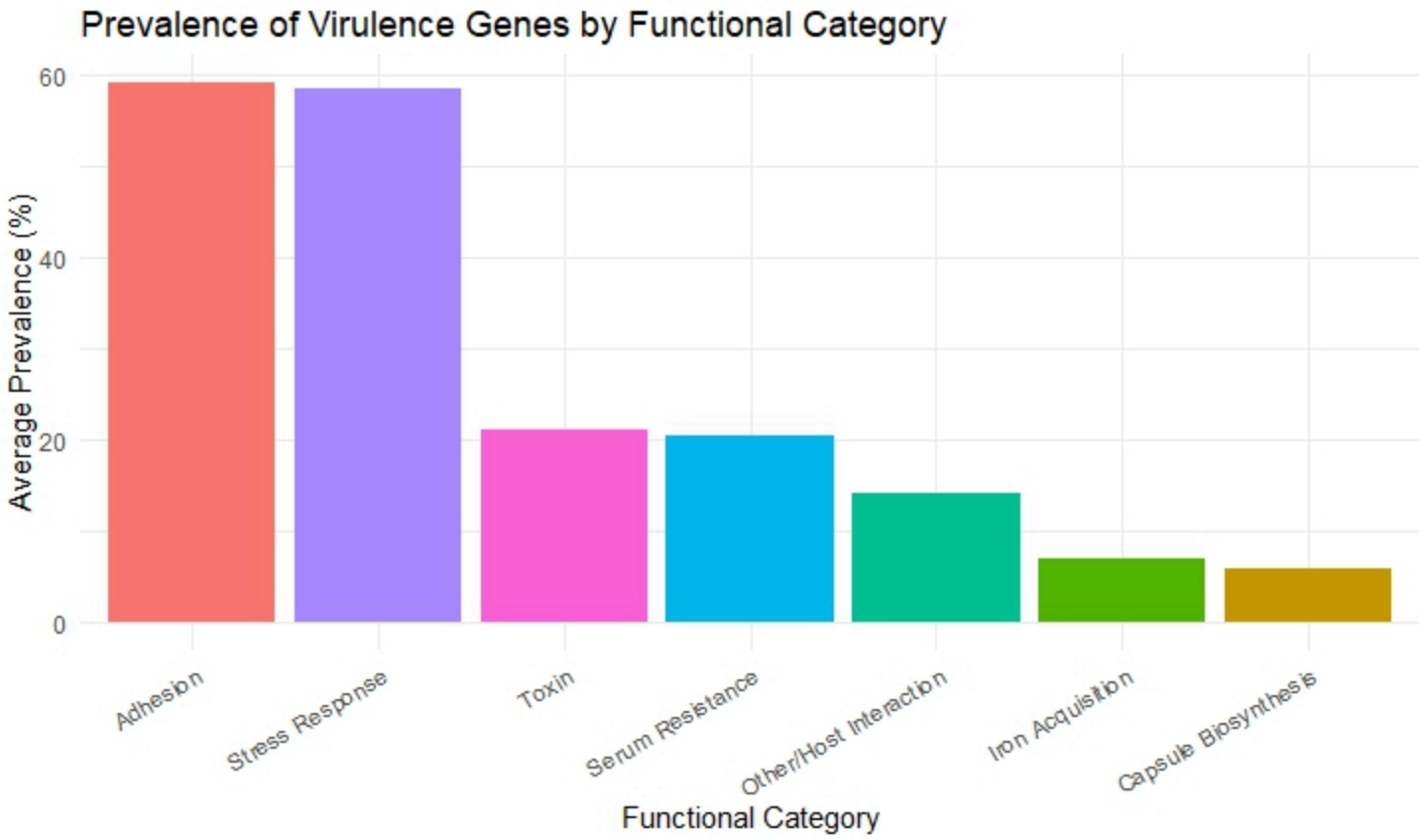


Figure 11

Prevalence of virulence genes by functional category. Bar plot showing the average prevalence (%) of virulence genes among isolates, grouped by functional categories. Stress response genes exhibited the highest prevalence (above 90%), followed by adhesion-related genes (around 85%) and toxin genes (approximately 50%). Capsule biosynthesis, iron acquisition, and serum resistance genes were detected at much lower prevalence levels (<10%). This distribution highlights that stress response and adhesion functions are the most dominant virulence mechanisms among the isolates analyzed.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS3PCAbasedonVirulencegenes.jpeg
- FigureS2PlasmidPresenceAbsence.jpeg
- FigureS1Frequencynew.png
- SupplementaryFiles.docx