UKR Journal of Agriculture and Veterinary Sciences (UKRJAVS)
Homepage: https://ukrpublisher.com/ukrjavs/
Email: submit.ukrpublisher@gmail.com
Volume 2, Issue 5 (September-October) 2026 ISSN: 3107-6173 (Online)

Genomic Landscape and Zoonotic Transmission Risk of Shiga Toxin-Producing *Escherichia coli* in Raw Bovine Milk from Khyber Pakhtunkhwa, Pakistan

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DOI: *https://doi.org/10.5281/zenodo.24051323*

Article History
Original Research Article
Received: 02 September 2026
Accepted: 10 October 2026
Published: 03 November 2026
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Citation: Safir Ullah, Saeed Ul Hassan Khan, Itizaz Ehsan, Hamid Irshad, Sagar M. Goyal, Sher Bahader Khan, Simon G. Patching, & Irshad Ahmad. (2026). Genomic landscape and zoonotic transmission risk of Shiga toxin-producing Escherichia coli in raw bovine milk from Khyber Pakhtunkhwa, Pakistan. UKR Journal of Agriculture and Veterinary Sciences, 2(5), 33-46.

Abstract

Shiga toxin-producing Escherichia coli (STEC) are major foodborne pathogens, with raw milk serving as a key transmission risk. This study aimed to investigate the prevalence, genomic characteristics, virulence determinants, and antimicrobial resistance profiles of STEC isolated from raw bovine milk in Khyber Pakhtunkhwa, Pakistan. A total of 800 raw bovine milk samples were collected across sixteen districts of Khyber Pakhtunkhwa. Samples were processed for E. coli isolation, with STEC confirmed by virulence gene detection. Phenotypic antimicrobial susceptibility testing was performed against twelve antibiotics. Whole-genome sequencing (WGS) of twelve representative STEC isolates enabled comprehensive analysis of virulence determinants, resistance genes, plasmid replicons, and phylogenetic relationships. E. coli prevalence was 40.1% (321/800), with STEC detected in 5.0% (40/800) and Extended-Spectrum Beta-Lactamase (ESBL)-producers in 1.4% (11/800). The Peshawar district showed the highest STEC contamination (13.3%). Antimicrobial resistance was alarmingly high for amoxicillin (100%), ceftriaxone (55.6%), and cefotaxime-clavulanic acid (55.6%). WGS identified resistance mechanisms including efflux pumps (acrAB-tolC, emrAB), ESBL genes (blaCTX-M-15), and plasmid-mediated quinolone resistance (qnrS1). Virulence genes included Shiga toxins (stx1, stx2), type III secretion effectors, and colonization factors. Multiple plasmid replicon types (IncA/C2, IncX1, IncI1, IncFII) and sequence types (ST10, ST540, ST1141, ST1201, ST1210, ST8149, ST8191) confirmed polyclonal contamination. Raw bovine milk in Khyber Pakhtunkhwa harbors genetically diverse, multidrug-resistant STEC strains carrying broad virulence and resistance genes. The combination of Shiga toxin genes, ESBL genes, and mobile resistance elements in foodborne pathogens poses a serious public health threat, highlighting the need for improved surveillance, pasteurization, and implementation of Pak One Health Alliance (POHA) interventions.

Keywords: *STEC, milk, whole-genome sequencing, virulence, resistance genes, MDR.*

1. Introduction

Escherichia coli (E. coli) is a Gram-negative, facultatively anaerobic bacterium belonging to the family *Enterobacteriaceae*. It is a ubiquitous inhabitant of the gastrointestinal tracts of humans and other warm-blooded animals, where it typically exists as a harmless commensal, playing a beneficial role in nutrient

metabolism and colonization resistance. However, specific lineages have evolved pathogenic mechanisms, enabling them to cause a spectrum of intestinal and extraintestinal diseases (Kaper *et al.,* 2004; Naidoo *et al*., 2025). The ecological versatility and genetic plasticity of *E. coli* underpin its success as both a commensal and a pathogen. Pathogenic *E. coli* strains are categorized into distinct pathotypes based on their virulence determinants and clinical manifestations (Geurtsen *et al*., 2022; Pokharel *et al*., 2023). A pathotype of significant global concern is Shiga toxin-producing *E. coli* (STEC), also known as verocytotoxin-producing *E. coli* (VTEC) (Alhadlaq *et al*., 2024). STEC infections are a leading cause of foodborne illness, with the potential to progress to severe complications such as hemorrhagic colitis and life-threatening hemolytic uremic syndrome (HUS), which is characterized by acute kidney failure, hemolytic anemia, and thrombocytopenia (Karmali *et al.,* 1983; Freedman *et al*., 2023). The principal virulence factor of STEC is the production of Shiga toxins (Stx), potent cytotoxins that inhibit protein synthesis in eukaryotic cells. These toxins are encoded by bacteriophages integrated into the bacterial genome and are classified into two major types, Stx1 and Stx2, with numerous subtypes (e.g. Stx1a, Stx1c, Stx2a, Stx2c) that vary in toxicity and epidemiological association (Scheutz *et al.,* 2012; Wang *et al*., 2024). Another critical virulence determinant is intimin, an outer membrane adhesin encoded by the *eae* gene, which is found on the locus of enterocyte effacement (LEE) pathogenicity island. Intimin facilitates the formation of intimate attaching and effacing (A/E) lesions on intestinal epithelial cells, a hallmark of STEC and enteropathogenic *E. coli* (EPEC) infections (Nataro & Kaper, 1998; Yang *et al*., 2020). Additional virulence factors include enterohemolysin (EhxA), which contributes to cellular damage and immune modulation (Paton & Paton, 1998; Schwidder *et al*., 2019), and various plasmid-encoded factors that enhance colonization and survival. Ruminants, particularly cattle, are the primary asymptomatic reservoirs of STEC, shedding the bacteria in their feces. Consequently, the primary transmission route to humans is through the consumption of contaminated foods, including undercooked ground beef, raw leafy vegetables, and critically unpasteurized (raw) milk and dairy products (Oliver & Murinda, 2012). Direct contact with farm environments or infected animals also poses a risk. A compounding public health challenge is the escalating prevalence of antimicrobial resistance (AMR) in *E. coli*. The selective pressure exerted by the widespread use of antibiotics in human medicine and livestock production has fueled the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains (Alkofide *et al*., 2020; Almutairy, 2024). Of particular concern is the global dissemination of strains producing extended-spectrum β-lactamases (ESBLs), enzymes that hydrolyze and confer resistance to penicillins, cephalosporins, and aztreonam (Jacoby & Munoz-Price, 2005). Resistance to fluoroquinolones and aminoglycosides is also increasingly common, severely limiting therapeutic options (World Health Organization, 2020). Contaminated food chains, including dairy products, are significant vectors for transmitting these resistant pathogens to humans (Dehkordi *et al.,* 2014). Indeed, raw milk is a key reservoir of MDR bacteria (Ahmad *et al*., 2021; Khan *et al*., 2026).

Modern molecular and genomic tools are indispensable for tracking and understanding these pathogens. Multilocus sequence typing (MLST) provides a standardized method for assessing the population structure and relatedness of isolates (Ibarz Pavón & Maiden, 2009; Zueter *et al*., 2024). Whole-genome sequencing (WGS) offers unparalleled resolution, enabling high-resolution outbreak investigation, comprehensive detection of virulence and resistance genes, and insights into phylogenetic evolution and transmission dynamics (Kwong *et al.,* 2015; Blankenship *et al*., 2023).

In regions like Khyber Pakhtunkhwa, Pakistan, where dairy farming is a foundation of the rural economy and consumption of raw milk is culturally prevalent, the risk of STEC exposure is heightened. Common practices such as pooling milk from multiple animals and inadequate cold-chain management can make contamination levels worse. The aim of this study was to investigate the prevalence, virulence profiles, and antimicrobial resistance patterns of STEC isolated from the raw milk supply chain in Khyber Pakhtunkhwa. By addressing critical knowledge gaps, the findings are expected to inform risk assessments, guide targeted interventions to improve dairy hygiene, and ultimately contribute to enhanced food safety and public health protection both locally and globally.

2. Materials and Methods

2.1. Sample Collection and Microbiological Characterization

This study was conducted from December 2021 to June 2023. A total of 800 raw bovine milk samples were randomly collected across sixteen districts of Khyber Pakhtunkhwa, Pakistan, from diverse sources including milk shops, dairy farms, milk vendors, and collection centers. Samples were transported under a cold chain to the Veterinary Research Institute, Peshawar, with supplementary analyses performed at collaborating laboratories in Islamabad and in the United States of America. The sample size was designed to ensure statistical reliability for detecting pathogenic STEC. Isolates were recovered by culture on Eosin-Methylene Blue (EMB) agar, MacConkey agar, and selective sorbitol-MacConkey

agar (SMAC/CT-SMAC). Presumptive *E. coli* colonies were confirmed through Gram staining, motility testing, and a series of biochemical assays (IMViC tests). Isolates positive for indole, methyl red, and triple sugar iron tests, but negative for Voges-Proskauer and citrate utilization, were identified as *E. coli*. STEC screening included sorbitol fermentation profiling and latex agglutination for the O157 serotype. For molecular characterization, DNA was extracted using the boiling method. A multiplex PCR protocol was employed to detect key STEC virulence genes (*stx1*, *stx2*, *eae*, *hlyA*, *rfbO157*, *fliC H7*) and prevalent ESBL resistance genes (*bla SHV*, *bla TEM*, *bla CTX-M*). Amplified PCR products were separated by electrophoresis on a 2% agarose gel, visualized under UV light, and documented. These integrated phenotypic and genotypic procedures ensured molecular profiling of all isolates.

2.2. Antimicrobial Susceptibility and Multidrug-Resistance Testing

The antibiotic resistance profiles of ESBL-positive STEC isolates were determined using the Kirby-Bauer disk diffusion method. Following Clinical and Laboratory Standards Institute (CLSI) (2020) guidelines, bacterial suspensions were standardized to a 0.5 McFarland turbidity standard and swabbed onto Mueller-Hinton agar plates. A panel of twelve antibiotic discs was applied, representing multiple antimicrobial classes: β-lactams penicillin, amoxicillin, ceftriaxone, aminoglycosides gentamicin, streptomycin, tetracyclines oxytetracycline, quinolones enrofloxacin, norfloxacin, florfenicol. Plates were incubated at 37 °C for 24 hours. Inhibition zone diameters were measured and interpreted as susceptible, intermediate, or resistant according to CLSI (2020) breakpoints. Isolates demonstrating resistance to at least one agent in three or more distinct antimicrobial classes were classified as MDR.

2.3. Whole Genome Sequencing and Bioinformatics Analysis

Twelve STEC isolates, selected based on their virulence profiles and antimicrobial resistance phenotypes, were subjected to WGS. Genomic DNA was extracted and sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) at the University of Minnesota Genomics Center. Raw sequencing reads were assessed for quality using FastQC (Andrews, 2010) and subsequently assembled *de novo* with the PATRIC assembler (Wattam *et al*., 2017). Genome annotation was performed using Prokka (Seemann, 2014). Downstream bioinformatic analyses were conducted primarily with web-based tools hosted by the Center for Genomic Epidemiology (CGE). Serotyping was performed using Serotype Finder 2.0 (Joensen *et al*., 2015), with thresholds set at ≥85% identity and ≥60% coverage of target genes. Virulence gene profiling was carried out with Virulence Finder 2.0 (Malberg Tetzschner *et al*., 2020), applying thresholds of ≥90% identity and ≥60% coverage. Core genome MLST (cg-MLST) was conducted using cg-MLST Finder 1.1 (Larsen *et al*., 2012), which utilizes Prokka-based annotation to examine allelic variation across the *E. coli* core genome. It was also employed to generate pangenome statistics and to identify a refined set of highly discriminatory loci through a random forest algorithm integrated with a binary tree-traversal method (Kaas *et al*. 2014). AMR gene detection was performed using a comprehensive approach. Annotated protein sequences were screened against the Comprehensive Antibiotic Resistance Database (CARD) version 3.2.9 (Alcock *et al*., 2023) using the Resistance Gene Identifier (RGI) tool. Screening was performed with three stringency levels.

3. Results

3.1. Overall Prevalence of *E. coli*, STEC, and ESBL Isolates

This study was conducted to determine the prevalence and genomic characteristics of STEC in the raw milk supply of Khyber Pakhtunkhwa, Pakistan. An analysis of 800 raw bovine milk samples collected from diverse sources including milk shops, dairy farms, vendors, and collection centers revealed an overall *E. coli* prevalence of 40.1% (321/800). Within this, 5.0% (40/800) of samples were confirmed as STEC through the detection of key virulence genes (*stx1*, *stx2*, *eae*, *hlyA*), with ESBL-producing isolates identified in 1.4% (11/800) of the total. The O157:H7 serotype was not detected in any of the isolates. Selected STEC isolates underwent comprehensive phenotypic screening for antimicrobial resistance and subsequent WGS. Genomic analysis was employed to identify virulence and resistance genes, determine sequence types (STs) via MLST, and clarify phylogenetic relationships, providing critical insights into the lineage, spread, and public health risk posed by these MDR pathogens in the regional dairy chain. Table 1 presents the distribution of *E. coli*, STEC, and ESBL strains across milk sources. Out of 200 samples from dairy farms and individual farmers, 73 tested positive for *E. coli*, with eight positive for STEC and two for ESBL. Milk collection centers had a low incidence, with one STEC-positive sample and no ESBL detection. Milk shops had the highest contamination rates, with 207 *E. coli* positives, 30 STEC, and eight ESBL-positive samples. Milk street vendors showed lower contamination, with 28 *E. coli* positives, one STEC, and one ESBL. Statistical analysis revealed no significant association between milk sources and pathogen prevalence.

Table 1. *District-wise prevalence levels of E. coli, STEC, and ESBL-producing isolates from raw milk in Khyber Pakhtunkhwa, Pakistan (n = 800)*

District	*E. coli* n (%)	STEC n (%)	ESBL n (%)	Total samples
Abbottabad	7 (28.0)	0 (0)	0 (0)	25
Bannu	10 (50.0)	1 (5.0)	0 (0)	20
Charsadda	21 (52.5)	2 (5.0)	2 (5.0)	40
Chitral	9 (45.0)	0 (0)	0 (0)	20
Dera Ismail Khan	32 (37.6)	6 (7.1)	3 (3.5)	85
Dir (Upper & Lower)	15 (37.5)	0 (0)	0 (0)	40
Haripur	12 (30.0)	0 (0)	0 (0)	40
Karak	8 (40.0)	0 (0)	0 (0)	20
Kohat	21 (46.7)	4 (8.9)	1 (2.2)	45
Lakki Marwat	12 (40.0)	1 (3.3)	0 (0)	30
Mansehra	7 (20.0)	0 (0)	0 (0)	35
Mardan	11 (27.5)	2 (5.0)	0 (0)	40
Nowshera	12 (40.0)	2 (6.7)	0 (0)	30
Peshawar	78 (52.0)	20 (13.3)	5 (3.3)	150
Swabi	19 (47.5)	2 (5.0)	0 (0)	40
Swat	47 (33.6)	0 (0)	0 (0)	140
Total	**321 (40.1)**	**40 (5.0)**	**11 (1.4)**	**800**

3.2. Antimicrobial Susceptibility Profile of STEC Isolates

The disk diffusion assay that we used offers several advantages over other antimicrobial susceptibility methods, including simplicity, low cost, the ability to test large numbers of microorganisms and antimicrobials, and ease of result interpretation (CLSI, 2020). Norfloxacin and florfenicol exhibited the highest sensitivity among the tested antibiotics, with 24 (66.1%) and 20 (55.6%) isolates sensitive, respectively, and only six (16.7%) isolates showing resistance to each. Enrofloxacin demonstrated a good sensitivity rate, with 22 (61.1%) sensitive isolates and six (16.7%) resistant. Sensitivity to gentamicin and sulphamethoxazole was demonstrated by fourteen (38.9%) and twelve (33.3%) isolates, respectively. Conversely, ceftriaxone and cefotaxime-clavulanic acid displayed the higher resistance rates at 20 (55.6%) each, followed by amoxicillin with 22 (61.1%) resistant isolates. Penicillin and streptomycin both demonstrated resistance in 16 (44.4%) of the isolates. Oxytetracycline resistance was observed in twelve (33.3%) isolates. The Chi-square value of 37.01 ($p = 0.022$) indicates a statistically significant distinction in the resistance patterns among the various antibiotics tested in Table 2.

Table 2. *Antimicrobial susceptibility testing of STEC isolated from raw milk in Khyber Pakhtunkhwa, Pakistan*

Antibiotic	Disc	Resistant n (%)	Intermediate n (%)	Sensitive n (%)
Penicillin	P-10	16 (44.4)	12 (33.3)	8 (22.2)
Amoxicillin	AMC	22 (61.1)	6 (16.7)	8 (22.2)
Augmentin	Ag	14 (38.9)	10 (27.8)	12 (33.3)
Sulphamethoxazole	SXT	10 (27.8)	14 (38.9)	12 (33.3)
Gentamicin	CN-10	12 (33.3)	10 (27.8)	14 (38.9)
Streptomycin	STM	16 (44.4)	6 (16.7)	10 (27.8)
Oxytetracycline	TET	12 (33.3)	16 (44.4)	8 (22.2)
Ceftriaxone	CTX	20 (55.6)	12 (33.3)	4 (11.1)
Norfloxacin	NRF	6 (16.7)	6 (16.7)	24 (66.1)
Enrofloxacin	ENR	6 (16.7)	8 (22.2)	22 (61.1)
Florfenicol	FRN	6 (16.7)	10 (27.8)	20 (55.6)
Cefotaxime-Clavulanic	CTXCL	20 (55.6)	10 (27.8)	6 (16.7)
Chi-square = 37.01, p = 0.022 (R × C Method using EPI-info software)				

3.3. Core Genome Multilocus Sequence Typing (cg-MLST) Analysis

cg-MLST was performed to elucidate the genomic diversity and relatedness of the *E. coli* isolates. Analysis was conducted using cg-MLST Finder 1.1, which screened for 2,513 predefined loci. Among the eighteen sequenced isolates, 2,163 to 2,439 loci (86.1-97.1%) were identified, with allele match percentages ranging from 76.0% to 96.0%. A pangenome analysis of the eighteen isolates, comprising a total of 9,231 genes, categorized the gene repertoire as follows: the core genome (genes present in all isolates) consisted of 3,267 genes (35.4%); the accessory genome (genes present in two or more isolates) contained 3,030 genes (32.8%); and the unique genes (strain-specific) accounted for 2,934 genes (31.8%). As expected, the proportion of shared core genes increased when analyzing subsets of strains, reflecting reduced genetic diversity and higher similarity within smaller, more closely related groups. Phylogenetic relationships were inferred from the cg-MLST data (Table 3).

Table 3. *Allelic profiles (housekeeping gene allele numbers) and sequence types (ST) of twelve STEC isolates as determined by MLST*

Adk	fumC	gyrB	icd	Mdh	purA	recA	ST
64	11	5	8	693	6	335	8149
10	11	183	8	8	8	2	1141
10	11	183	8	8	8	2	1141
64	11	3	8	693	8	335	8191
10	4	4	654	773	8	6	–
10	11	4	8	8	18	2	10
10	11	5	8	7	8	2	1210
6	7	57	1	8	8	2	540
10	11	4	8	8	8	2	10
10	11	5	8	7	8	2	1201

3.4. Virulence Gene Spectrum of STEC Isolates

WGS revealed a comprehensive arsenal of virulence genes among the twelve STEC isolates. Table 4 details the repertoire of virulence genes identified, with a focus on their clinical roles. The isolates possess a complete enterobactin system for iron acquisition, including biosynthesis genes (*entA*, *entB*, *entC*, *entD*, *entE*, *entF*, *entS*) and uptake genes (*fepA*, *fepB*, *fepC*, *fepD*, *fepG*), which is crucial for survival in the iron-limited host environment. Adherence and colonization are facilitated by Type 1 fimbriae genes (*fimA*, *fimB*, *fimC*, *fimD*, *fimE*, *fimF*), enabling initial attachment to host cells. Critically, the presence of *stx1* and *stx2* genes confirms their potential to cause severe human illness, as these encode Shiga toxins responsible for hemorrhagic colitis and HUS. Additional accessory factors, such as the type III secretion system effectors (*espX1*, *espX4*, *espX5*) and the mucin-degrading enzyme *aslA*, suggest enhanced pathogenic potential and adaptation to mucosal niches, highlighting the public health risk posed by these isolates.

Table 4. *WGS-based virulence genes and their clinical significance in STEC isolates (n = 12) from raw bovine milk in Khyber Pakhtunkhwa, Pakistan*

Virulence gene	**Role in pathogenesis**	**Novelty in context**
Enterobactin system (iron acquisition)		
entA, entB, entC, entD, entE, entF, entS	Biosynthesis and secretion of the siderophore enterobactin	Core virulence: Essential for survival in the iron-limited host environment; common in *E. coli*
fepA, fepB, fepC, fepD, fepG	Transport and uptake of ferric-enterobactin complex	Core virulence: Completes the iron acquisition pathway; necessary for systemic infection
Adherence and colonization		
fimA, fimB, fimC, fimD, fimE, fimF	Structural subunits and assembly of Type 1 fimbriae	Primary adhesins: Fundamental for initial attachment to host epithelial cells; ubiquitous

Toxin and effector delivery		
espX1, espX4, espX5	Putative type III secretion system (T3SS) effectors	Associated with virulence in some STEC/EPEC strains; suggests a specific sub-lineage
stx1, stx2	Shiga toxin production	Primary determinants for hemorrhagic colitis and HUS
hlyC	Enterohemolysin production	Contributes to cellular damage and immune modulation
yagV/ecpE	Putative fimbrial assembly protein	May enhance colonization potential
Miscellaneous		
aslA	Arylsulfatase-like enzyme; role in mucin degradation	Potential niche factor: May facilitate mucosal colonization; less commonly highlighted in standard STEC profiling

3.5. Antimicrobial Resistance Gene Spectrum of STEC Isolates

Table 5 profiles the AMR gene repertoire of the twelve STEC isolates, highlighting both intrinsic resistance mechanisms and acquired determinants. The isolates are equipped with robust multidrug efflux systems, primarily the resistance-nodulation-division (RND) family pump AcrAB-TolC (encoded by *acrA*, *acrB*) and major facilitator superfamily (MFS) pumps (*emrA*, *emrB*, *mdfA*), which provide broad-spectrum defense against antibiotics including fluoroquinolones, tetracyclines, and disinfectants (Saidijam *et al*., 2006; Venter *et al*., 2015; Ahmad *et al*., 2025). Notably, the high frequency of chromosomal β-lactamase genes (*ampC*, *ampH*) in eleven isolates indicates significant resistance to penicillins and cephalosporins. The presence of *eptA* is also clinically relevant, as it can confer baseline resistance to last-resort polymyxins (colistin) through lipid A modification (Xu *et al*., 2018). Furthermore, regulatory two-component systems (*baeR*, *cpxA*) and acid resistance regulators (*gadW*, *gadX*) enhance the bacteria's ability to survive antibiotic stress and host stomach acidity, respectively, thereby promoting overall fitness and persistence.

Table 5. *WGS-based antimicrobial resistance genes and their clinical significance in STEC isolates (n = 12) from raw bovine milk in Khyber Pakhtunkhwa, Pakistan*

AMR gene / system	**Primary function / Mechanism**	**Novelty in context**
Multidrug efflux pumps (RND family)		
acrA, acrB, acrD, acrE, acrF, acrS	Components and regulators of the AcrAB-TolC efflux system	Core intrinsic resistance: The primary multidrug efflux machinery in *E. coli*; ubiquitous presence (12 isolates)
Multidrug efflux pumps (MFS)		
emrA, emrB, emrK, emrR	Components of EmrAB and EmrKY efflux systems	Broad-spectrum resistance: Contribute to resistance against fluoroquinolones, tetracyclines, and biocides
mdfA	Multidrug and disinfectant resistance efflux pump	Confers resistance to chloramphenicol, fluoroquinolones, and disinfectants; associated with environmental adaptation
β-Lactam resistance		
ampC, ampC1, ampH	Chromosomal AmpC β-lactamase and regulators	Mediates resistance to penicillins, cephalosporins (except cefepime), and β-lactam/β-lactamase inhibitor combinations; detected in 11 isolates

Peptide antibiotic resistance		
bacA	Confers resistance to bacitracin	Common in gut microbiota; indicates adaptation to peptide antibiotics
eptA	Modifies Lipid A, providing resistance to polymyxins (colistin)	Chromosomal *eptA* can confer baseline resistance; detection warrants attention
Regulatory systems		
baeR, baeS, cpxA	Two-component systems regulating stress response and efflux pump expression	Upregulate efflux and envelope stress responses upon antibiotic exposure, promoting survival
gadW, gadX	Regulators of the acid resistance system	Critical for stomach acid survival; indirectly promotes antibiotic tolerance by enhancing general fitness

3.6. Plasmid Replicon Analysis

Plasmid replicons were identified by screening whole genome sequences against the PlasmidFinder database, using a minimum coverage and identity threshold of 90% (Carattoli *et al*., 2014). Table 6 illustrates the diversity of plasmid replicon types identified among the STEC isolates, highlighting their potential to carry and disseminate resistance and virulence determinants. The profiles range from simple, low-replicon combinations, such as the co-occurrence of IncFIB(AP001918) and IncFIB(K)-Kpn3, to highly complex multi-replicon arsenals carrying up to five distinct plasmid types. Several isolates carry plasmids of significant epidemiological concern, including broad-host-range IncA/C2 and epidemic IncX1 types, which are frequently associated with the spread of multidrug resistance. The presence of IncI1-Alpha and IncFII(pCoo) plasmids is notable, as these are often linked to mobile resistance elements and enterotoxin genes, respectively. Furthermore, the detection of the p0111 plasmid in one isolate represents an uncommon finding in this specific context, suggesting the potential acquisition of unique genetic traits. Collectively, this plasmid diversity underscores the substantial capacity for horizontal gene transfer among these isolates.

Table 6. *WGS-based plasmid replicon types and their clinical significance in STEC isolates (n = 12) from raw bovine milk in Khyber Pakhtunkhwa, Pakistan*

Plasmid types detected	**Notable feature / importance**
IncFII(pSE11), IncA/C2, Col440I, IncFIB(AP001918)	Common multi-replicon profile; includes broad-host-range IncA/C2
IncFII(pSE11), Col440I, IncA/C2, IncFIB(K)-Kpn3, IncX1	Diverse and notable: Carries five distinct replicons, including epidemic plasmid types (IncA/C2, IncX1)
Col440I, IncFII (pCoo), IncFIB(AP001918), IncY	Features the enterotoxin-associated IncFII(pCoo) plasmid
IncA/C2, IncFIB(K)-Kpn3, IncX1, IncI1-Alpha, IncFII(pHN7A8)	High importance: Highly diverse arsenal (5 types) including key resistance/virulence plasmids (IncA/C2, IncI1, IncX1)
IncFII(pSE11), IncFIB(K)-Kpn3, IncI1-Alpha	Carries the mobile genetic element-associated IncI1 plasmid
Col440I, IncFIB(K)-Kpn3, IncY	Minimal but notable profile with IncY
IncA/C2, IncFIB(K)-Kpn3, IncFII, p0111	Notable: Unique presence of the p0111 plasmid, uncommon in this context
IncFIB(K)-Kpn3, IncFIB(AP001918)	Basic, low-replicon count profile
IncFIB(AP001918), IncFIB(K)-Kpn3	Basic, low-replicon count profile; shared with SF359

3.7. Outline of Key Resistance Determinants

Table 7 categorizes the specific AMR determinants identified in the isolates according to their functional mechanism. The resistance arsenal is dominated by RND family efflux pumps (Venter *et al*., 2015; Ahmad *et al*., 2025), with the core AcrAB-TolC complex providing intrinsic multidrug resistance, while the presence of MdtABC-TolC offers a specific defense against aminocoumarins. MFS pumps (Saidijam *et al*., 2006; Ahmad *et al*., 2025) expand this profile significantly; the detection of *mdtM* is noted as a novel finding in STEC from food sources, while *mdfA* and *tet(A)* provide resistance to disinfectants and tetracyclines, respectively. Of critical clinical importance are the enzymatic inactivation mechanisms, highlighted by the presence of the CTX-M-15 ESBL gene, which confers resistance to penicillins and cephalosporins and represents a major public health concern (Moghaddam *et al*., 2017). Additionally, the detection of the plasmid-mediated quinolone resistance (PMQR) gene *qnrS1* threatens a key class of therapeutic antibiotics. Table 7 also notes rare determinants like the *Ugd* target alteration gene and global regulators such as *marA*, which orchestrate broader resistance responses.

Table 7. *WGS-based efflux pumps and key resistance determinants/notable features of STEC isolates (n = 12)*

Mechanism category	**Key gene/ determinant**	**Primary resistance profile**	**Notable importance**
RND efflux pumps	*AcrAB-TolC*	Multidrug	Core multidrug efflux complex; common finding.
	MdtEF-TolC	Multidrug	Associated with bile salt and drug resistance.
	MdtABC-TolC	Aminocoumarin	Less common; specific to aminocoumarins.
MFS efflux pumps	*mdfA*	Disinfectants, Tetracycline	Broad substrate specificity; notable for biocide resistance.
	mdtM	Multidrug	Novel finding: Uncommon in STEC from food sources.
	tet(A)	Tetracycline	Classic tetracycline-specific efflux pump.
Target alteration	*Ugd*	Peptide antibiotics	Rare: Very low prevalence; unusual in this context.
Target protection	*qnrS1*	Fluoroquinolone	Plasmid-mediated quinolone resistance (PMQR); clinically critical.
Enzymatic inactivation	*CTX-M-15*	Penams, Cephalosporins	High importance: ESBL gene; major public health concern.
	aph(3)-Ib / aph(6)-Id	Aminoglycosides	Co-occurring streptomycin resistance genes.
Regulators	*marA*	Multidrug	Global regulator of multidrug resistance.

4. Discussion

The present study provides a comprehensive genomic analysis of STEC isolated from raw bovine milk in Khyber Pakhtunkhwa, Pakistan, revealing concerning prevalence rates and complex resistance mechanisms that pose significant public health risks. The overall *E. coli* prevalence of 40.1% (321/800) aligns with findings from a parallel study in the same region (Ullah *et al*., 2024). This consistency validates the representativeness of our sampling across the sixteen districts and diverse milk supply sources. The detection of STEC in 5.0% (40/800) of samples is particularly significant, as these strains carry the potential to cause severe human illnesses including hemorrhagic colitis and HUS. This prevalence rate agrees with the STEC positivity reported in a recent investigation from Khyber Pakhtunkhwa (Ullah *et al*., 2024), suggesting stable circulation of these pathogens in the regional dairy chain. This finding aligns with studies from similar dairy-intensive regions globally and within South Asia (Ramya *et al*., 2018; Kamaruzzaman *et al*., 2020).

Absence of the O157:H7 serotype in our isolates is consistent with other studies from Pakistan that have documented the predominance of non-O157 serotypes in animal reservoirs (Irshad *et al*., 2020; Ismail *et al*., 2021; Ullah *et al*., 2025). This, coupled with the detection of other STEC strains, underscores the epidemiological shift towards non-O157 serogroups as predominant contaminants in dairy products, a trend increasingly reported in both developed and developing countries (Mathusa *et al*., 2010; Luna-Gierke *et al*., 2014;

Babolhavaeji *et al*., 2021; Bahgat *et al*., 2024; King *et al*., 2025; Nemati *et al*., 2025; Howard *et al*., 2026). This finding has important diagnostic implications, as clinical laboratories frequently target only the O157 serotype, potentially missing non-O157 infections that may be equally severe.

Geographically, the Peshawar district demonstrated the highest STEC prevalence at 13.3%, followed by Kohat (8.9%) and Dera Ismail Khan (7.1%). This distribution likely reflects the higher density of dairy operations and milk marketing networks in these urban and peri-urban areas. The statistically significant variation in resistance patterns ($\chi^2 = 37.01$, $p = 0.022$) across antibiotics underscores the heterogeneous selection pressures operating in different localities, possibly related to variable antimicrobial usage practices in veterinary medicine. The antimicrobial susceptibility profile revealed alarmingly high resistance to penicillins (amoxicillin 61.1%, penicillin 44.4%) and cephalosporins (ceftriaxone 55.6%, cefotaxime-clavulanic acid 55.6%). These findings corroborate recent reports documenting high amoxicillin and ceftriaxone resistance among STEC isolates from identical sources in Pakistan.

The detection of ESBL-producing isolates in 1.4% of total samples, confirmed genotypically by the presence of *bla*CTX-M genes, represents a critical public health concern as these enzymes confer resistance to critically important cephalosporins, limiting therapeutic options for severe infections. Conversely, the preserved susceptibility to norfloxacin (66.1% sensitive), enrofloxacin (61.1%) and florfenicol (55.6%) suggests these agents may remain effective options, although the presence of plasmid-mediated quinolone resistance genes (*qnrS1*) in our genomic analysis warrants caution against over-reliance on any single antibiotic class. The 44.4% streptomycin resistance observed aligns with the co-occurrence of APH(3)-Ib and *APH(6)-Id genes identified in our WGS data, representing a classic resistance mechanism commonly linked to mobile genetic elements. The virulence gene repertoire identified through WGS reveals the pathogenic potential of these isolates. The comprehensive enterobactin system for iron acquisition (*entA-F*, *entS*, *fepA-G*) is essential for survival in the iron-limited host environment, representing core virulence machinery that enables systemic infection. The Type 1 fimbriae genes (*fimA-F*) facilitate initial attachment to host epithelial cells, a prerequisite for colonization and subsequent toxin delivery. Critically, the presence of *stx1* and *stx2* genes confirms the potential to cause severe human illness, as these encode Shiga toxins responsible for hemorrhagic colitis and HUS. The detection of type III secretion system effectors (*espX1*, *espX4*, *espX5*) suggests a specific sub-lineage with enhanced pathogenic potential, while the mucin-degrading enzyme *aslA* may facilitate mucosal colonization, a factor less commonly highlighted in standard STEC profiling (Oliver *et al*., 2005).

WGS provided unprecedented resolution of the genetic determinants underlying the phenotypic resistance profiles. The ubiquitous presence of RND family efflux pumps (Venter *et al*., 2015; Ahmad *et al*., 2025) (*acrA*, *acrB*, *acrD*, *acrE*, *acrF*, *acrS*) across all twelve sequenced isolates confirms the role of AcrAB-TolC as the primary multidrug efflux machinery in *E. coli*, providing intrinsic resistance that serves as a first-line defense against diverse antimicrobial classes. The detection of MFS pumps (Saidijam *et al*., 2006; Ahmad *et al*., 2025) (*emrA*, *emrB*, *emrK*, *emrR*, *mdfA*) expands the resistance repertoire, with *mdfA* specifically conferring resistance to chloramphenicol, fluoroquinolones, and disinfectants - the latter having particular significance for environmental adaptation and persistence in dairy processing environments (Du *et al*., 2018). Of notable clinical importance was the identification of *mdtM* as a novel finding in STEC from food sources, representing an uncommon multidrug efflux pump that extends the resistance spectrum (Law & Alegre, 2018). The detection of *eptA*, conferring baseline resistance to last-resort polymyxins (colistin) through lipid A modification, warrants attention despite plasmid-mediated *mcr* genes being more clinically critical. This finding aligns with the chromosomal *eptA* variants increasingly recognized in *E. coli* from animal sources (Huang *et al*., 2018). The ESBL burden was confirmed through detection of CTX-M-15, the globally dominant extended-spectrum β-lactamase, which together with chromosomal AmpC β-lactamases (*ampC*, *ampC1*, *ampH*) in eleven isolates explains the high-level cephalosporin resistance observed phenotypically. This combination of enzymatic inactivation mechanisms represents a major public health concern, as it effectively compromises entire classes of β-lactam antibiotics. The co-occurrence of aminoglycoside resistance genes *aph(3)-Ib* and *aph(6)-Id* explains the streptomycin resistance profile and reflects the common association of these determinants on mobile genetic elements. The two-component regulatory systems (*baeR*, *baeS*, *cpxA*) identified in our isolates play crucial roles in upregulating efflux pump expression and envelope stress responses upon antibiotic exposure, promoting bacterial survival. Additionally, the acid resistance regulators (*gadW*, *gadX*) enhance fitness by enabling stomach acid survival, indirectly promoting antibiotic tolerance and facilitating transmission through the oral route (Manges *et al*., 2019). The global regulator *marA* orchestrates broader multidrug resistance responses, integrating multiple resistance

mechanisms into a coordinated defense strategy (Ruiz *et al*., 2008).

Plasmid replicon typing revealed extraordinary diversity, with profiles ranging from simple low-replicon combinations to complex multi-replicon arsenals carrying up to five distinct plasmid types. The detection of broad-host-range IncA/C2 and epidemic IncX1 plasmids is of significant epidemiological concern, as these are frequently associated with dissemination of multidrug resistance across bacterial populations and geographical boundaries (Carattoli, 2013). The presence of IncI1-Alpha and IncFII(pCoo) plasmids is notable for their association with mobile resistance elements and enterotoxin genes, respectively. Furthermore, detection of the p0111 plasmid in one isolate represents an uncommon finding in this context, suggesting acquisition of unique genetic traits potentially linked to niche adaptation.

Cg-MLST analysis revealed substantial genetic diversity among isolates, with the core genome comprising 3,267 (35.4%) genes, the accessory genome 3,030 (32.8%) genes, including 2,934 (31.8%) unique genes. This distribution indicates an open pangenome characteristic of *E. coli* populations from diverse sources, enabling adaptation to varied environmental niches. The identification of multiple sequence types (ST10, ST540, ST1141, ST1201, ST1210, ST8149, ST8191) through MLST confirms the polyclonal nature of STEC contamination in the regional dairy chain, suggesting multiple sources of introduction rather than a single outbreak strain. The detection of MDR STEC in milk shops (30 STEC-positive, 8 ESBL-positive) compared to dairy farms (8 STEC-positive, 2 ESBL-positive) implicates post-farm gate handling and marketing practices as critical control points. Milk shops, where pooling of milk from multiple producers occurs, represent amplification points for contamination and resistance dissemination. The lower contamination in collection centers (one STEC-positive) suggests that organized sector operations with better hygiene infrastructure may mitigate risks, although the absence of statistical association between source and prevalence indicates that contamination is widespread across the supply chain. These findings align with growing international recognition of raw milk as a vehicle for transmitting multidrug-resistant STEC, including observations from Brazil (Oliveira *et al.,* 2018; Lopes *et al*., 2025), India (Singh *et al.,* 2019; Nair *et al.,* 2020), United Kingdom (Treacy *et al*., 2019) and the United States of America (Thomas *et al*., 2022).

Our observations have emphasized how developing countries such as Pakistan face challenges from STEC contamination due to inadequate testing facilities, limited public awareness, and widespread raw milk consumption. Genetically diverse STEC strains with zoonotic potential in dairy animals confirm their role as carriers of clinically significant pathogens, raising concerns about transmission to humans through the food chain. The public health implications are therefore substantial. STEC infections, particularly those mediated by Stx2 as identified in our isolates, carry a high risk for progression to HUS, a life-threatening complication primarily affecting young children and the elderly. Multidrug resistance profiles complicate clinical management when treatment becomes necessary, as antibiotic therapy in STEC infections remains controversial due to risk of toxin release but becomes unavoidable in systemic infections. The presence of ESBL determinants further compromises therapeutic options, potentially necessitating use of last-resort antibiotics with increased toxicity or cost.

5. Conclusion

This study discovered that raw milk in Khyber Pakhtunkhwa, Pakistan, serves as a reservoir for genetically diverse, multidrug-resistant STEC strains. The complex combination of Shiga toxin genes, ESBL determinants, and mobile resistance elements in foodborne pathogens poses a critical public health threat. These findings highlight the urgent need for enhanced surveillance programs incorporating WGS-based characterization. Implementation of hazard control systems in dairy value chains and promotion of pasteurization are essential interventions. A One Health approach integrating human, animal, and environmental health sectors is vital to mitigate STEC-associated disease risks in Pakistan.

Acknowledgments

The authors gratefully acknowledge the support and resources provided by the Veterinary Research Institute, Peshawar; the Animal Sciences Institute, National Agricultural Research Centre (NARC), Islamabad; and the University of Minnesota, USA.

Funding

This work was supported by the Higher Education Commission (HEC) of Pakistan under the International Research Support Initiative Program (IRSIP).

Conflict of Interest

The authors have no conflicts of interest to declare.

Data Availability

Data are presented in the manuscript, and further data are available from the corresponding author upon reasonable request.

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