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Genomic insights into plasmid mediated AMR genes, virulence factors and mobile genetic elements in raw milk *Escherichia coli* from Gujarat, India

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Antimicrobial resistance in raw milk is a critical challenge for public health. This study aimed to investigate the in-vitro and in-silico analysis of antimicrobial resistance, mutations, virulence factors (VFs), mobile genetic elements, and diversity of *Escherichia coli* in cattle raw milk of Gujarat, India during May 2023- April 2024. 56 isolates were recovered from 50 raw milk samples. Among them, 26 were identified as *E. coli* and subjected to antimicrobial susceptibility testing. The extensively drug-resistant 10 isolates were further confirmed by *16s rRNA* identification. Whole-genome Nanopore sequencing was performed on 10 representative strains, and data were further used in bioinformatics analysis. The results revealed that wide array of ARGs and mutations were detected. Plasmid-mediated ARGs were detected in three isolates that include bla_{TEM-1}, *sul2*, *dfrA14*, *aph*^{6}*-Id*, *aph(3")-Ib*, and *tet(B)*, responsible for resistance against antibiotic classes such as beta-lactam, folate-pathway, aminoglycosides, and tetracyclines, respectively. Additionally, the conserved VFs in isolates facilitated serum survival and toxin production. The unit transposons Tn2 and Tn10 were detected in two isolates. The isolates exhibited considerable diversity, belonging to different serotypes, sequence types (ST), and phylogroups. This research provides crucial insights for developing effective monitoring, mitigation, and educational strategies to prevent the spread of pathogenic *Escherichia coli* through the food chain.

Keywords *Escherichia coli*, Raw milk, ARGs, WGS, Mobile genetic elements, Multi- locus sequence type

Antimicrobial resistance (AMR) has been termed the "silent pandemic," transcending geographical and human-animal borders. AMR represents a critical global health crisis, projected to cause 10 million deaths and a $100 trillion economic loss by 2050, if no multi-sectoral action is taken[1]. In 2017, the Indian government established the National Action Plan for Antimicrobial Resistance (NAP-AMR) to control AMR[2]. After that, in 2022 Gujarat government declared 'Network Program on AMR, Superbugs and One health.

Multi-drug resistance (MDR) and extensively drug resistance (XDR) refer to the ability of bacteria to with stand the effects of at least three and five classes of clinically relevant drugs, respectively[3]. Before the corona virus 2019 (COVID-19) pandemic began, AMR was already top priorities for global health[4].

Milk is an ideal medium for bacterial growth, including some pathogenic types[5]. In Gujarat, India, raw milk is an affordable and readily available protein source, widely produced and consumed in both urban and rural areas. Gujarat uses both traditional and intensive milk production methods. Mastitis, a common disease impacting milk hygiene and quality in dairy cattle, is prevalent worldwide. *Escherichia coli* (*E. coli*) normally found in human and animal intestines can become pathogenic when transmitted through contaminated milk and dairy products[6]. *E. coli* is also present in meat and dairy cattle manure, posing a potential risk of infection to humans[7].

In recent years, various acquired resistance mechanisms, such as plasmids, transposons, integrons, and bacteriophages, have been identified in the spread of resistance genes in bacteria[8,9]. The significant rise in antibiotic resistance, particularly due to the presence of extended-spectrum beta-lactamase (ESBL) genes is alarming[10]. Additionally, continuous accumulation of mutations can lead to the emergence of genomes resistant

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to clinically relevant antibiotics. Various virulence genes also play critical roles in pathogenicity and disease manifestation[11].

Understanding the mechanisms and determinants of AMR in bacteria at the molecular level is crucial for controlling and comprehending the changing dynamics of resistance[12]. Whole genome sequencing (WGS) has become a powerful approach for screening antibiotic resistance, understanding pathogen diversity, and evaluating the number and functions of mutations. WGS also holds great promise in developing new antibiotics, enhancing diagnostic capabilities, and controlling AMR across human, animal, and environmental sectors[13].

This study analyzes raw milk *E. coli* using phenotypic AST and WGS data. We screened for antibiotic resistance in classes such as beta-lactams, beta-lactam inhibitors, quinolones, phenicols, aminoglycosides, tetracyclines, and folate pathway antagonists. To understand molecular changes, we examined virulence factors, mobile genetic elements (including plasmids, insertion sequences, transposons and phagosomes), multilocus sequence typing (MLST), serotyping and phylogroups analysis. Comparative genome analysis of the isolated *E. coli* was performed using a reference strain.

Results

Prevalence of *E. coli* in raw milk

A high prevalence of bacterial isolates was found in cattle raw milk samples from Gujarat, India. From 50 raw milk samples, 56 bacteria were isolated, with 26 identified as *E. coli* based on morphological and biochemical characteristics. *E. coli* isolates displayed bright pink colonies on MacConkey agar and a metallic green sheen on Eosin Methylene Blue agar. Microscopically, the isolates appeared as Gram-negative, moderately sized, motile rods. They tested positive for catalase, methyl red, and indole tests, but negative for the Voges-Proskauer test in biochemical assays.

Antibiotic susceptibility testing of *E. coli*

Antimicrobial susceptibility testing revealed the highest resistance rates within the beta-lactam class of antibiotics, with 96.15% ($n=25$) of isolates resistant to ampicillin and 92.30% ($n=24$) to cefepime (Table 1). Additional resistance rates were observed against various antibiotics: amoxyclav (88.46%) ($n=23$), imipenem (69.23%) ($n=18$), cephalothin (84%) ($n=22$), ticarcillin (80.76%) ($n=21$), cefotaxime (69.23%) ($n=18$), ceftazidime (76.92%) ($n=20$), ceftriaxone (69.23%) ($n=18$), aztreonam (65.38%) ($n=17$), piperacillin/tazobactam (88.46%) ($n=23$), and norfloxacin (69.23%) ($n=18$). Resistance rates ranging from 23 to 50% were observed against ciprofloxacin, chloramphenicol, streptomycin, tetracycline, doxycycline, sulphamethizole, and trimethoprim. Among the 26 isolates, 10 were identified as extensively drug-resistant (XDR), 9 as multi-drug-resistant (MDR), and 7 as sensitive. Multi-drug resistance (MDR) and extensively drug resistance (XDR) refer to the ability of bacteria to with stand the effects of at least three and five classes of clinically relevant drugs, respectively. The zone measurements and breakpoints used in this study adhered to CLSI and EUCAST guidelines.

Class and subclass of antibiotics	Antibiotics with disc content(µg)	Zone size (mm)	No. of *Escherichia coli* (26)	
		*Intermediate Breakpoints	No. of Resistance	% of Resistance
Beta lactam (Penicillins)	Ampicillin (10)	15 to 18	25	96.15
	Amoxyclav (30)	15 to 20	23	88.46
Beta lactam (Carbapenems)	Imipenum (10)	18 to 21	18	69.23
Beta lactam (Cephalosporins)	Cephalothin (30)	13 to 17	22	84.61
	Ticarcillin (75)	17 to 21	21	80.76
	Cefotaxime (5)	16 to 20	18	69.23
	Ceftazidime (30)	15 to 21	20	76.92
	Ceftriaxone (30)	16 to 20	18	69.23
	Cefepime (30)	16 to 20	24	92.30
Beta lactam (Monobactam)	Aztreonam (30)	16 to 22	17	65.38
Beta lactam inhibitor	Piperacillin/tazobactam (75/10)	15 to 20	23	88.46
Quinolones	Ciprofloxacin (5)	20 to 21	13	50
	Norfloxacin (5)	20 to 21	18	69.23
Phenicoles	Cloramphenicol (30)	20 to 22	8	30.76
Aminoglycosides	Streptomycin (300)	12 to 14	9	34.61
Tetracyclins	Tetracycline (30)	16 to 18	12	46.15
	Doxycycline (30)	16 to 18	8	30.76
Folate pathway antagonist	Sulphamethizole (300)	11 to 15	7	26.92
	Trimethoprim (5)	13 to 17	6	23.07

Table 1. Antibiotic susceptibility patterns of *E. coli* isolates.

Amplification of 16 S rRNA gene by PCR

The identification of all 10 XDR isolates was confirmed through the amplification of the *16 S rRNA* gene using PCR with *E. coli* species-specific primers. Purified DNA was extracted from the *E. coli* isolates and used in the PCR assay. The presence of the *16 S rRNA* gene was verified, confirming that all 10 isolates were indeed *E. coli*. These confirmed *E. coli* isolates were then subjected to whole-genome sequencing (WGS) for further genomic characterization.

Whole genome sequencing and bioinformatics data analysis

Whole-genome sequencing (WGS) was employed to gain an in-depth understanding of the genetic features of 10 XDR *E. coli* isolates. This included the identification of antibiotic resistance genes (ARGs), point mutations, virulence factors, mobile genetic elements, and conducting comparative genome analyses to know their genetic relatedness to *E. coli* from human, animal, and environmental sources. The AMR isolates genomes were submitted to NCBI (Bioproject no: PRJNA1109570) (Table 2).

Antibiotic resistance genes and point mutation in E. coli

Annotated ARGs for the ten sequenced *E. coli* isolates are listed in Table 3. The NCBI AMRFinderPlus and ResFinder tools revealed that, all ten sequenced *E. coli* harbored beta-lactamase genes such as bla_{EC}, as well as efflux resistance genes like *emrD* and *mdtM*. The $bla_{CTX\text{-}M\text{-}15}$ gene was detected in four isolates (AMR1, AMR3, AMR5, and AMR6). Additionally, the $bla_{TEM\text{-}1B}$ gene, a narrow-spectrum beta-lactamase, was found in three isolates (AMR3, AMR4, and AMR7). Notably, one isolate (AMR3) contained three beta-lactamase genes: $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, and bla_{EC}.

Our findings showed that all ten *E. coli* isolates were conferred resistance to several classes of antibiotics (Table 3). The ARGs of the *E. coli* isolates consisted of a plethora of quinolone resistance gene [*qnrS1*], efflux resistance genes [*acrF*, *emrD*, *emrE*and *mdtM*], folate pathway antagonist resistance genes [*sul2* and *dfrA14*], aminoglycosides resistance genes [aph^{6}-Id and *aph*(3")-Ib] and tetracycline resistance gene [*tet*(B)]. The high diversity of pathogenic isolates and the co-carriage of several significant ARGs are worrisome. We identified six isolates harboring quinolone resistance gene.

Figure 1 represents concordance between phenotypic (P) resistance and genotypic (G) prediction (WGS data) of all ten sequenced *E. coli* isolates. Results are presented separately for each isolate and its resistance pattern using different color codes as shown in the figure. Each tile is colored based on whether both the resistant phenotype and genotype agreed (P + G); both phenotype and genotype predicted susceptible (S); phenotype was resistant, but the genotype was sensitive (P); phenotype was sensitive, but the genotype was resistant (G). All sequenced isolates were shown concordance (P + G) to at least three classes of antibiotics.

Several mutations were detected in genomes of *E. coli*. This include mutations in the *marA*, *marR*, *acrR*, *folP*, *soxR*, *soxS*, *nfsA*, *UhpT*, *GlpT*, *rpoB*, *parC*, *parE*, *phoP*, *gyrA*, *murA* and *ompF* genes as depicted in Table 3. The detection of wide array of mutations in chromosomal genes could lead to several resistance patterns.

Virulence factors (VFs) of E. coli

All sequenced *E. coli* isolates contains a wide range of virulence associated genes. The most conserved VFs across ten *E. coli* isolates were: *yehA*, *yehB*, *yehC*, *yehD* (YHD fimbrial cluster, adhesions), *cia* (Colistin i.a.), *cvaC* (Microcin C), *clpK1* (Heat shock survival), *hlyE* (Avian *E. coli* heamolysin), *terC* (Tellurium ion acquisition systems) and *iss* (Increased serum survival) (Table 3). However, other virulence factors such as *traT* (Outer membrane protein complement resistance) and *mchF* (ABC transporter protein) were detected in AMR7 as depicted in Table 3.

Mobile genetic elements of E. coli

The mobile genetic elements of sequenced *E. coli* were diverse as the isolates harbored diverse plasmids, insertion sequences and transposons[14].

Plasmids: As depicted in Table 3, most of the plasmids carried by these *E. coli* isolates belonged to the incompatibility F (IncF) group. These include IncFIB(PB171), IncFIB(AP001918), IncFIC(FII) and IncFII.

Isolate ID	Submission ID (Strain)	Genome Accession No.
AMR 1	ECOCDS01	JBEFLH000000000
AMR 2	ECOCDS02	JBEGCT000000000
AMR 3	ECOCDS03	JBEGCU000000000
AMR 4	ECOCDS04	JBEGCV000000000
AMR 5	ECOCDS05	JBEGCW000000000
AMR 6	ECOCDS06	JBEGCX000000000
AMR 7	ECOCDS07	JBEUPV000000000
AMR 8	ECOCDS08	JBEPUW000000000
AMR 9	ECOCDS09	JBEJGT000000000
AMR 10	ECOCDS10	JBEJGU000000000

Table 2. List of AMR isolates (strain) with genome accession number submitted in NCBI (Bioproject no: PRJNA1109570).

Isolate ID & Status	Class of Antibiotics	ARGs	Point mutation in ARGs	Virulence Factors	Plasmids	Insertion Sequence
AMR1 (MDR)	1.Beta Lactam 2.Quinolone 3.Efflux	1.*bla*CTX-M-15,*blaEC* 2.*qnrS1* 3.*acrF*, *emrD*, *mdtM*	marA, acrR, folP, soxR, nfsA, UhpT, GlpT, rpoB, parC	*Anr*, *capU*, *cia*, *csgA*, *fdeC*, *fimH*, *gad*, *hha*, *hlyE*, *iss*, *nlpI*, *terC*, *yehA*, *yehB*, *yehC*, *yehD*	IncFIB(PB171), IncI1-I(Alpha)	IS30, IS911, ISSfl10, ISEc5, MITEEC1, ISEc1, IS609, IS640
AMR2 (DR)	1.Beta Lactam 2.Efflux	1.*blaEC* 2.*emrE*, *emrD*, *mdtM*, *acrF*	soxS, folP, marR, parC, UhpT, phoP, soxR, parE, gyrA, murA, nfsA, rpoB, ompF, acrR	*capU*, *clpK1*, *csgA*, *fdeC*, *gad*, *hha*, *hlyE*, *iss*, *IpfA*, *nlpI*, *terC*, *yehA*, *yehB*, *yehC*, *yehD*	IncFIB(AP001918), IncR	ISSfl10, MITEEC1, IS911, ISEc38, IS609, ISEc1
AMR3 (MDR)	1.Beta Lactam 2.Quinolone 3.Efflux	1.*bla*CTX M-15, *bla*TEM-1B, *blaEC* 2.*qnrS1* 3.*acrF*, *emrD*, *mdtM*	rpoB, acrR, nfsA, folP, UhpT, soxR, murA, phoP, marR	*Anr*, *capU*, *csgA*, *fdeC*, *hlyE*, *hra*, *iss*, *nlpI*, *terC*, *yehA*, *yehB*, *yehC*, *yehD*	IncFIB(AP001918)	MITEEC1, IS26, ISEc31, ISEc9, ISSfl10, IS911, IS609, ISEc1, IS640, ISEc30
AMR4 (XDR)	1.Beta Lactam 2.Quinolone 3.Efflux 4.Folate pathway antagonist 5.Aminoglycosides	1.*bla*TEM-1B, *blaEC* 2. *qnrS1* 3. *emrD*, *mdtM* 4.*sul2*, *dfrA14* 5.*aph*(6)Id *aph*(3")Ib	ompF, GlpT, marR, murA, parE, rpoB, parC, nfsA, folP	*AslA*, *csgA*, *fimH*, *fyuA*, *hha*, *hlyE*, *irp2*, *iss*, *nlpI*, *terC*, *yehA*, *yehB*, *yehC*, *yehD*	IncX1, IncY	MITEEC1, IS102, IS421, IS609, IS30, ISEc1, ISkpn26
AMR5 (MDR)	1.Beta Lactam 2.Quinolone 3. Efflux	1.*bla*CTX-M-15, *blaEC* 2. *qnrS1* 3. *acrF*, *emrD*, *emrE*, *mdtM*	GlpT, nfsA, soxR, parC, phoP, folP, parE, ompF, rpoB	*Anr*, *csgA*, *fdeC*, *fyuA*, *gad*, *hlyE*, *irp2*, *iss*, *nlpI*, *ompT*, *terC*, *traJ*, *traT*, *yehA*, *yehB*, *yehC*, *yehD*	IncFIB (AP001918), IncFIC (FII)	MITEEC1, ISkpn19, ISEc9, IS609, ISEc5, IS682, IS3, IS30, ISkpn26, IS640, ISkpn8
AMR6 (MDR)	1.Beta Lactam 2.Quinolone 3. Efflux	1.*bla*CTX-M-15 2. *qnrS1* 3. *emrD*, *mdtM*	gyrA, gyrB, parC, pare, pmrA, pmrB, folP, ropB, ampC	*AslA*, *csgA*, *fimH*, *fyuA*, *hha*, *hlyE*, *irp2*, *iss*, *nlpI*, *terC*, *yehA*, *yehB*, *yehC*, *yehD*	IncFIB (AP001918)	MITEEC1, IS640, IS609, ISEc1, ISEc30, IS26
AMR7 (XDR)	1.Beta Lactam 2.Efflux 3. Folate pathway antagonist 4.Aminoglycosides 5.Tetracycline	1.*bla*TEM-1B, blaEC 2. *emrD*, *mdtM* 3.*sul2*, *dfrA14* 4.*aph*(6)Id,*aph*(3")Ib 5.*tet*(B)	murA, nfsA, GlpT, UhpT, ompF, parE, folP, marR, parC, gyrA, rpoB, soxS	*Anr*, *cia*, *csgA*, *cvaC*, *etsC*, *fdeC*, *fimH*, *fyuA*, *hha*, *hlyE*, *hlyF*, *iroN*, *irp2*, *iss*, *iucC*, *iutA*, *IpfA*, *mchF*, *nlpI*, *ompT*, *sitA*, *terC*, *traJ*, *traT*, *yehA*, *yehB*, *yehC*, *yehD*	IncFIB(AP001918), IncFII IncQ1	MITEEC1, IS629, ISEc32, IS609, IS911, ISSfl10, IS100, ISEc38, ISEc31, IS682, IS4, IS3, IS26
AMR8 (DR)	1.Beta Lactam 2.Efflux	1.*blaEC* 2. *mdtM*, *acrF*, *emrD*	folP, parC, gyrA, parE, phoP, acrR, soxR, nfsA, ompF, GlpT, UhpT, soxS, murA, marR	*aalF*, *anr*, *cia*, *csgA*, *faeE*, *faeF*, *fdeC*, *fimH*, *hha*, *hlyE*, *iss*, *IpfA*, *nlpI*, *ompT*, *terC*, *tia*, *traJ*, *yehA*, *yehB*, *yehC*, *yehD*	Col(BS512), IncFIC(FII), IncI1-I(Alpha), IncFIB(AP001918)	MITEEC1, IS640, IS682, ISEc18, IS609, IS629, ISEc1
AMR9 (MDR)	1.Beta Lactam 2.Quinolone 3.Efflux	1.*blaEC* 2.*qnrS1* 3. *mdtM*, *acrF*, *emrD*	soxR, marR, gyrA, acrR, phoP, parE, parC, folP, ampC, GlpT, pmrA, pmrB	*csgA*, *fdeC*, *fimH*, *gad*, *hha*, *hlyE*, *iss*, *IpfA*, *nlpI*, *ompT*, *terC*, *yehA*, *yehB*, *yehC*, *yehD*	Col(BS512), IncFIC(FII)	MITEEC1, ISEc1, IS629, IS609, IS3
AMR10 (DR)	1.Beta Lactam 2.Efflux	1.*blaEC* 2. *mdtM*, *acrF*, *emrD*	marA, folP, phoP, marR, soxS, ompF, UhpT, GlpT, gyrA, rpoB, acrR, soxR, nfsA, parC, parE	*AslA*, *anr*, *clpK1*,*csgA*, *etsC*, *hha*, *hlyE*, *iss*, *mlpI*, *sitA*, *terC*, *traJ*, *traH*, *yehA*, *yehB*, *yehC*, *yehD*	IncFIA, IncFIB(PB171), IncX1	MITEEC1, IS3, ISEc5, ISEc1, ISEc43, ISEc38, IS102, ISEc30, ISkpn8

Table 3. Genomic characteristics of XDR *E. coli* isolated from raw milk.

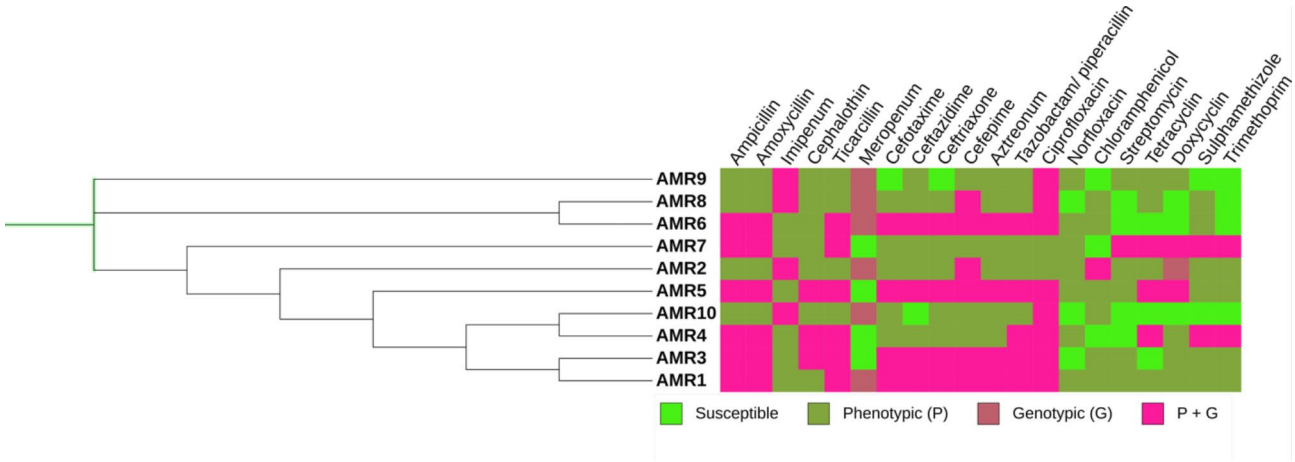

Fig. 1. Phylogenetic relatedness of sequenced *E. coli* and concordance between phenotypic AST result and the genotypic prediction from WGS data visualized in Interactive Tree of Life tool (iTOL). The legend colors shows resistance pattern: neon green (Susceptible), olive green (Phenotypic), rouge (Genotypic) and pink (P+G).

Similarly, smaller plasmids of approximately 2 kb Col(BS512) were also detected in two (AMR8 and AMR9) isolates. Plasmid mediated resistance gene were detected based on their location. The plasmid mediated bla_{TEM-1} gene was detected in two isolates (AMR3 & AMR4) as they were carried IncFIB, IncX1 and IncY plasmids. In AMR4, plasmid mediated AMR genes were detected for different three classes of five antibiotics such as beta lactam (bla_{TEM-1}), folate pathway antagonist (*sul2* and *dfrA14*) and aminoglycosides (*aph*[6]*Id* and *aph*(3")*Ib*). Moreover, AMR 7 isolate contained tetracycline (*tet*(B)) resistance gene located on plasmid.

Insertion sequences (IS) and transposons: Asides from plasmids, different families of autonomous transposable elements (IS) and transposones were detected in sequenced isolates (Table 3). As shown in Fig. 2, different 26 types of IS were detected in 10 sequenced isolates. MITEEC1 (123 bp belonging to the IS630 family) minimum inverted repeat was detected in all 10 isolates. In AMR4, Tn2 unit transposon was detected. Tn10 were detected in AMR7, which is determinant of *tet*(B) tetracycline resistant gene.

Comparative genome analysis

For most sequencing experiments, comparison to other genomes is a crucial step to know which region varies from other genomes. Figure 3 represents, comparative genomic circular map for ten query sequence of *E. coli* using reference strain: *Escherichia coli* strain 25104 plasmid p25104 insertion sequences ISEcp1 transposase (tnpA) gene, extended-spectrum beta-lactamase $bla_{CTX-M-15}$ gene and putative transposase gene (GenBank: KF891471.1). The gap shows mismatch of query sequence with reference sequence. AMR1, AMR3, AMR5 and AMR6 are fully matched with reference strain, which is indicating presence of $bla_{CTX-M-15}$ gene (Table 3).

Phageome analysis of E. coli

Bacteriophage has been identified as involved in the spread of resistance genes in bacteria[15]. The findings shows that 9 of the 10 sequenced *E. coli* harbored at least 1 intake phage and the phage ranged in size from 19.9 kb to 46.5 kb. The coli phages contained open reading frames (ORFs) which could allow for the incorporation of the other mobile genetic elements into the genome of the phages. AMR8, AMR9 & AMR10 harbored 4 intake phages and remaining isolates harbored one or two intake phages as depicted in Table 4. %GC content was

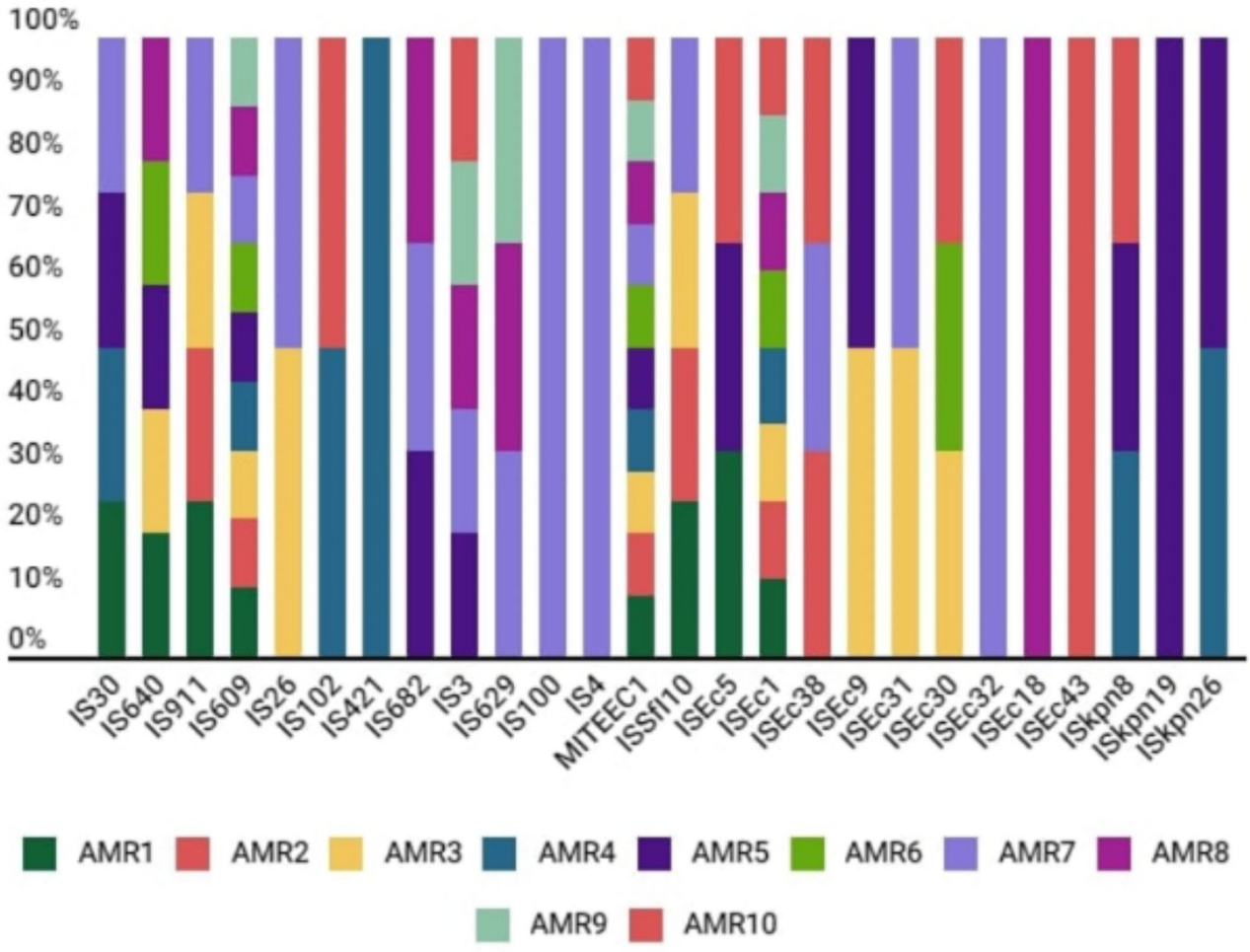

Fig. 2. Stacked chart for prevalence of autonomous transposable elements.

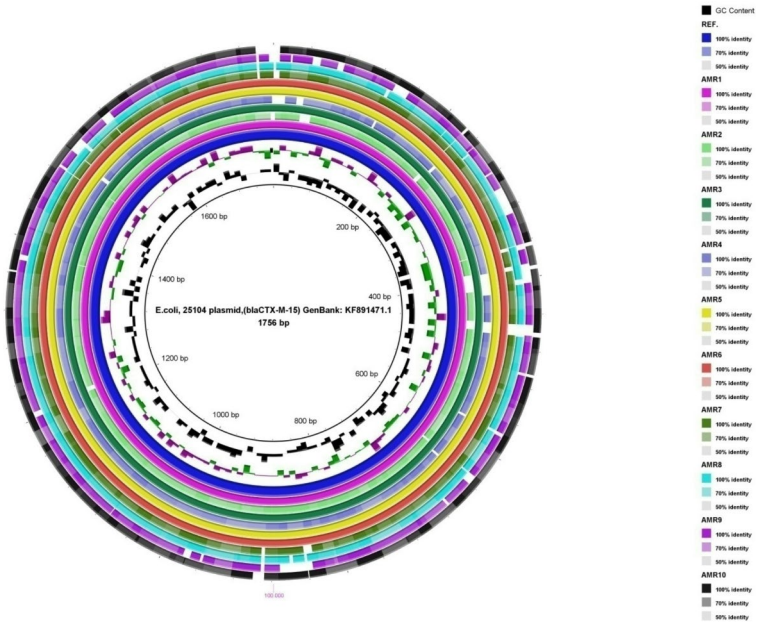

Fig. 3. The comparative genomic circular map was generated for ten sequenced *E. coli* using reference strain: *Escherichiacoli* strain 25,104 plasmid p25104 insertion sequences ISEcp1 transposase (tnpA) gene, extended-spectrum beta-lactamase *bla*CTX-M-15 gene and putative transposase gene (GenBank: KF891471.1). The sequence alignment matches obtained using BLASTn giving a colour-shade gradient from lightest (70% similarity) to darkest (100% similarity). The innermost circle represents guanine-cytosine content and second ring shows GC Skew. The first circular ring (Blue) represents reference genome (1756 bp) and right side color strip shown for each isolate legend colors match with ring color.

Isolate ID	No. of phages	Phage score	No. of ORFs	Region length (Kb)	Region position (Contig No/ position)	Most common phage	GC content (%)
AMR1	1	150	52	11.3	7/87,841–129,946	PHAGE_Entero_BP_4795_NC_004813(9)	50.21
AMR2	1	114	44	34.8	1/216,421–251,316	PHAGE_Klebsi_4LV2017_NC_047818(29)	50.10
AMR3	1	150	51	40.6	14/16,599–57,253	PHAGE_Entero_BP_4795_NC_004813(9)	50.45
AMR4	1	120	25	25.1	13/102,563–127,740	PHAGE_Salmon_118970_sal3_NC_031940(8)	50.40
AMR5	3	150 96 150	44 23 26	36.1 34.5 23	3/137,618–173,735 36/3706–38,283 46/1-23065	PHAGE_Yersin_L_413C_NC_004745(23) PHAGE_Escher_500465_1_NC_049342(13) PHAGE_Entero_mEp460_NC_019716(20)	50.21 50.03 52.11
AMR6	0	0	0	0	-	-	0
AMR7	1	150	49	36.7	40/812-37551	PHAGE_Entero_cdtI_NC_009514(18)	49.54
AMR8	4	150 150 150 110	62 31 51 25	46.5 24.2 38 19.9	7/81,527–128,035 23/2199–26,421 24/1045–39,078 41/1-19970	PHAGE_Phage_Gifsy_1_NC_010392(18) PHAGE_Escher_pro483_NC_028943(20) PHAGE_Entero_SfV_NC_003444(35) PHAGE_Pseudo_phiPSA1_NC_024365(7)	51.03 53.24 49.53 47.37
AMR9	4	150 130 140 103	34 36 55 31	21.9 31 53 32.8	2/309,817–331,795 6/15,377–183,408 20/5885–58,947 25/1075–33,903	PHAGE_Entero_P88_NC_026014(28) PHAGE_Entero_phiP27_NC_003356(9) PHAGE_Entero_DE3_NC_042057(21) PHAGE_Klebsi_4LV2017_NC_047818(20)	53.78 56.47 49.78 49.99
AMR10	4	150 150 106 100	54 31 62 24	40.8 20.7 46.7 19.7	5/128,410–169,300 46/155-20864 29/1473–48,226 47/1315–21,056	PHAGE_Entero_P88_NC_026014(39) PHAGE_Entero_BP_4795_NC_004813(6) PHAGE_Salmon_SW9_NC_049459(32) PHAGE_Pseudo_phiPSA1_NC_024365(7)	52.10 50.42% 52.04% 46.80%

Table 4. The phageome analysis of *E. Coli* using PHASTER.

obtained between 47.37 and 53.24 in *E. coli* isolates. No ARGs were detected on any of the phages from either the RESFINDER or NCBI databases.

Genomic diversity of E. coli

A and B1 phylogroups were detected in *E. coli* isolates. As depicted in Table 4, six isolate having phylogroup A and remaining 4 having phylogroup B1. Multi locus sequence typing (MLST) analysis revealed that, the isolates were genetically diverse as 9 of the 10 sequenced isolates were of diverse sequence types (STs). AMR1 and AMR3

Isolate ID	MLST	Serotype	Phylotype
AMR1	ST650	O9:H30	A
AMR2	ST378	O90:H5	A
AMR3	ST650	O9:H30	A
AMR4	ST486	O170:H9	A
AMR5	ST698	O8:H25	A
AMR6	ST1291	Novel	B1
AMR7	ST24	O8:H25	B1
AMR8	Novel allele	O147:H7	B1
AMR9	ST1292	O168:H7	B1
AMR10	Novel allele	O15:H8	A

Table 5. Genomic diversity of sequenced *E. coli*.

having same ST650. From 10 *E. coli*, 4 novel STs (AMR6, AMR8, AMR9 and AMR10) were identified, from that allelic profile of AMR6 and AMR9 were submitted to Pasteur MLST (database ID- 1612 &1613) and assigned into new sequence type as ST1291 and ST1292 respectively. Pathogenic *E. coli* are serotype based on their O (somatic) and H (flagellar) surface antigen profiles. Several serotypes were identified in the sequenced *E. coli* of raw milk origin such as O9:H30, O90:H5, O170:H9, O8:H25, O147:H7, O168:H7 and O15:H8 including one novel serotype in AMR6 (Table 5).

Discussion

Understanding genetic diversity is key factor to grasping transmission dynamics and predicting spread through the food chain. This study emphasizes the value of WGS and bioinformatics for genomic surveillance of *E. coli* in cattle raw milk from Gujarat, India. This is the first AMR surveillance in this region, revealing a 46% *E. coli* prevalence. In 2013, 41 *E. coli* were found in 50 raw milk samples from Anand, Gujarat, India[16].

Several factors including over use of antibiotics and farm management practices could be responsible for emergence of AMR. Our study found the highest resistance in beta-lactams, with ampicillin (96.15%) and cefepime (92.30%) showing the greatest resistance. This contrasts with 2021 data[17], which reported lower resistance rates for amoxyclav, cefotaxime, cefixime, cefepime, and tetracycline. The higher resistance observed in our study suggests evolving bacterial resistance, likely due to horizontal gene transfer. Resistance rates for amoxyclav, imipenem, cephalothin, ticarcillin, cefotaxime, ceftazidime, ceftriaxone, aztreonam, piperacillin/tazobactam, and norfloxacin ranged between 65% and 90%. Similar patterns were reported by[18] for *E. coli* from buffalo raw milk in Iran. All 10 XDR *E. coli* isolates in our study were confirmed by *16 S rRNA* gene amplification, consistent with[19], which identified 15 of 20 samples as *E. coli* using similar PCR primers.

This study used WGS to analyze beta-lactam resistance genes in five sequenced isolates. In 2023[20], reported that the $bla_{\text{CTX-M-15}}$ gene ($bla_{\text{CTX-M-1}}$ group) was the most common ESBL gene, linked to cephalosporin use in food-producing animals in USA[21]. found that *E. coli* producing $bla_{\text{CTX-M-15}}$, often associated with clonal lineages like ST131, spread extensively through mobile genetic elements, increasing urinary tract and bloodstream infections worldwide. Among the ten sequenced isolates, three harbored the $bla_{\text{TEM-1-B}}$ gene, which confers resistance to third-generation cephalosporins[22]. The concordance between phenotypic AST and genotypic predictions is shown in Fig. 1.

We identified six isolates with quinolone resistance genes (Table 3). Similarly[23], reported similar findings, with qnrS1 being the most common quinolone resistance gene in *E. coli* from human clinical samples in Korea. Additionally, we detected *emrD*, *emrE*, *mdtM*, and *acrF* genes, which encode multidrug efflux pumps. Previously[24] stated that efflux pumps transport various chemicals, leading to broad antibiotic resistance in *E. coli*. Folate pathway antagonist and aminoglycoside resistance genes were also found in two isolates (AMR4 & AMR7). In 2022[25], reported similar resistance genes, including *sul1*, *sul2*, *sul3*, *dfrA1*, *dfrA14*, *dfrA17*, *aadA1*, aph^3-*Ib*, aph^3-*Id*, and aph^6-*Id*, in beef cattle cecal samples from Nigeria.

Bacteria can develop antibiotic resistance through mutations. Mutations in transporter proteins *GlpT* and *UhpT* were found in four *E. coli* isolates (AMR1, AMR7, AMR8, and AMR10) (Table 3)[26]. noted that these transporters are essential for fosfomycin uptake, and mutations or inactivation can lead to fosfomycin resistance. The *folP* gene mutation was present in 10 isolates, increasing the MIC of sulfathiazole fourfold. Mutations in *marA*, *marR*, and *acrR*, detected in all sequenced isolates, reduce susceptibility to ciprofloxacin[27]. Other mutations included *soxR* and *soxS* (important for DNA binding), *nfsA* (involved in reducing quinones and nitroaromatics), and *parC*, *parE*, and *gyrA* responsible for quinolone resistance due to their role in DNA gyrase and topoisomerase IV, essential for bacterial replication[25].

Virulence mechanisms are necessary to overcome host defense systems, and the development of AMR is essential to enable pathogenic bacteria to overcome antimicrobial therapies. In our study, the *E. coli* contained virulence associated genes such as adhesions and iron acquisition systems etc. were detected. Previously, these genes have been implicated in extra intestinal pathogenic *E. coli* as well as in avian coli bacillosis identified in Germany[28]. Hence, these suggest that, *E. coli* from raw milk may be zoonotic pathogens, could be a reservoir of resistance and virulence genes, and has potential to cause human infection[29].

Recent studies have identified various acquired resistance mechanisms, including plasmids, transposons, integrons, and bacteriophages, in the spread of AMR genes. In our study, 8 of 10 *E. coli* isolates harbored

IncF group plasmids (IncFIB(PB171), IncFIB(AP001918), IncFIC(FII), and IncFII), while 2 isolates carried the Col(BS512) plasmid (Table 3). Plasmid-mediated AMR genes were found in 3 *E. coli* isolates, potentially facilitating horizontal gene transfer[30]. Reported similar plasmid-mediated quinolone resistance in ESBL-producing *E. coli* from poultry in Nigeria.

Insertion sequences (IS), part of transposons, can encode antibiotic resistance. We detected 26 types of IS in the isolates, including Tn2 in AMR4 and Tn10 in AMR7, associated with tetracycline resistance (*tet(B)*)[31]. IS26 was found in AMR3 and AMR7 (Fig. 2), and[9] reported IS26 as linked to kanamycin resistance.

Phagosome analysis revealed that phages conferred resistance to chloramphenicol, tetracycline, kanamycin, and ampicillin when transducer into *E. coli* ATCC 13706. Comparative genome analysis visualized genome similarity between reference strains and raw milk AMR *E. coli* strains (Fig. 3). This is the first study to report phage diversity in raw milk *E. coli* in Gujarat, with no previous reports on the phageome (Table 4).

The diversity of ARGs, virulence genes, and mobile genetic elements is evident in the phylogrouping, with two groups (A & B1) detected[32]. found *E. coli* phylogroups B2 and D in raw milk and milk products in Germany. Multilocus sequence typing (MLST), which characterizes bacterial species using sequences from seven housekeeping genes, identifies dominant ST types that may be high-risk food borne pathogens. The pubMLST and Pasteur MLST databases aid in identifying and comparing STs. This study identified four novel STs, including ST1291 and ST1292 (Table 5).

Overall, the study emphasizes the need for genomic surveillance to understand the molecular dynamics of AMR and to develop evidence-based strategies for managing and mitigating the spread of AMR pathogens. A multifaceted, collaborative approach is essential to address the complexities of AMR effectively. The importance of addressing the consumption of raw milk lies in its potential to serve as a source of dangerous MDR bacteria, including *E. coli*, *Klebsiella*, *MRSA*, and *Acinetobacter*[33].

Materials and methods

Sample collection, isolation and identification of *E. coli*

Fifty cattle raw milk samples were aseptically collected in sterile screw-capped glass bottles from dairy farms and bulk milk chilling centers in Gujarat, India during May 2023- April 2024 following standard laboratory protocols (ILRI, 2024). Serially diluted raw milk samples were spread on MacConkey agar and Eosin Methylene Blue agar plates using an automated spiral platter (Interscience Easy Spiral Dilute). Preliminary identification of *E. coli* was performed using Gram staining, followed by confirmation with a biochemical identification kit (HiE.coli, HiMedia Pvt. Ltd., Mumbai, India).

Antibiotic resistance profiling of *E. coli*

Antibiotic susceptibility testing (AST) was performed using Kirby-Bauer disk diffusion method on Mueller-Hinton agar. Nineteen different antibiotics from seven classes were used: beta-lactams (including 3rd and 4th generation cephalosporins), quinolones, phenicols, aminoglycosides, tetracyclines, and folate pathway antagonists. All antibiotic discs were procured from HiMedia Pvt. Ltd., Mumbai, India. Results were interpreted as resistant, intermediate, or susceptible according to the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (2024). Isolates showing resistance to at least three antibiotic classes were defined as multi-drug resistant (MDR), and those resistant to at least five classes were defined as extensively drug-resistant (XDR)[34].

Genomic DNA extraction and amplification of 16 S rRNA gene by PCR

Genomic DNA was extracted from overnight bacterial cultures using the Genomic DNA Extraction Kit (GeNei™) according to the manufacturer's instructions[35]. The concentration and purity of the extracted DNA were determined using a Qubit 4 fluorometer. The confirmation of *Escherichia coli* species-specific primers (F: 5'-GACCTCGGTTTAGTTCACAGA-3' and R: 5'-CACACGCTGACGCTGACCA-3') were used[19]. The amplified PCR products were resolved by electrophoresis on a 2% (w/v) agarose gel at 80 V for 1 h and stained with ethidium bromide. A DNA ladder (GeneRuler 100 bp to 3000 bp DNA Ladder # SM0321, Thermo Scientific) was used as a control[36]. The separated DNA bands were visualized using a gel imager (Invitrogen BioServices Pvt. Ltd., India).

Whole genome sequencing (WGS)

Whole genome sequencing of identified XDR *E. coli* isolates was performed using the MinION nanopore sequencer. The extracted DNA samples were purified using Qiagen Purification Kit according to the manufacturer's instructions. The nanopore sequencing library was prepared using the Ligation Sequencing Kit (SQK-RBK110.96) and loaded on a MinION flow cell (FLO-MIN106D) using the FAK40529 screening protocol. Raw sequencing data were processed and analyzed using NanoForms (https://nanoforms.tech/home/) and assembled using SPAdes3tool[37].

Bio-informatics analysis of whole genome sequence data

WGS assembled contigs were fed into bacterial WGS analysis platforms: AMR Finder plus, Comprehensive Antimicrobial Resistance Database (CARD), Center of Genomic Epidemiology (CGE)[38] and Phage Search Tool Enhanced Release (PHASTER). The sequences of AMR isolates were submitted to NCBI, and the accession number for each sequence is included in Table 2.

In-silico detection of AMR determinants, virulence genes and mobile genetic elements

AMRFinderPlus and CGE based ResFinder 4.3.3 tools used to identify antibiotic resistance genes (ARGs) and chromosomal mutations[39]. ARGs were designated as chromosomal or plasmid-mediated based on their genomic

location using mlplasmids version 2.1.0. Virulence-associated genes were determined using VirulenceFinder 2.0[30]. Mobile genetic elements such as insertion sequences, plasmids, and transposons, were detected using Mobile Element Finder 1.0.3 and PlasmidFinder 2.1[14]. A stacked chart was generated using the web-based Infogram. The CARD database was used to assess genomic relatedness with reference *E. coli* sequences, and comparative genomic circular maps were generated using the BLAST Ring Image Generator (BRIG 0.95)[40].

Detection of phages from WGS assembled sequences
WGS assembled sequences were analyzed using PHASTER (PHAge Search Tool – Enhanced Release) to study the phageome in isolated *E. coli*[15]. This tool identifies and annotates phage sequences in bacterial genomes and plasmids. PHASTER classifies the phageome as intact, questionable, or incomplete based on phage score. This study used only intact phages.

Multi-locus sequence typing (MLST), serotype and phylogroups
The CGE analytical pipeline was used for the identification of the species and its sequence type using MLST 2.0 and replicon sequence typing was manipulated using pMLST 2.0[41,42]. The *E. coli* serotypes (O & H antigen) were determined using Serotype Finder 2.0[43]. The sequenced isolates were classified into different phylogroups using ClermonTyping tool[44].

Conclusion
This study underscores the emergence of a highly diverse array of extensively drug-resistant and extended-spectrum beta-lactamase producing *E. coli* in raw milk, posing a significant health threat to humans. The presence of plasmid-mediated ARGs facilitates the horizontal transfer of these genes, increasing the risk of spreading antibiotic resistance among bacterial populations. WGS data provided deeper insights, revealing a plethora of ARGs, virulence factors, mobile genetic elements, and coliphages, all of which contribute to the spread of antimicrobial resistance and enhance the bacteria's fitness and survival in human hosts. To mitigate the risks posed by these resistant *E. coli* strains and to safeguard public health, urgent actions are required.

Data availability
Sequencing data are available in FASTA format at Bioproject accession PRJNA1109570 in NCBI.

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Author contributions

Devangi Mangroliya: Formal analysis, Investigation, Writing- original draft, Writing- review & editing. Hetvi Adhyaru: Formal analysis, Investigation. Jayesh Kabariya: Investigation, Project administration, Supervision, Writing- review & editing Vimal Ramani: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing- review & editing.

Declarations

Competing interests

The authors declare no competing interests.

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