



RESEARCH ARTICLE

Phylogenetic and Molecular Insights into Antimicrobial Resistance and Virulence Determinants of Ovine *Escherichia coli* in Ningxia, China

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ABSTRACT

Diarrhea is a common cause posing significant economic losses in sheep farming, primarily caused by various infectious pathogens, among which *Escherichia coli* is one of the key etiological agents. However, limited data exist on the antimicrobial resistance profiles and virulence-associated factors of *E. coli* isolated from diarrheic sheep in Ningxia, China. In this study, a total of 32 fecal samples were collected from diarrheic sheep at farms in Ningxia to investigate the resistance phenotypes and virulence gene profiles of *E. coli*. The 16S rRNA gene amplification and phylogenetic analysis were used to identify bacterial isolates. *E. coli* recovered from 59.4% (19/32) of the samples. Antimicrobial susceptibility was determined by the disk diffusion method, antibiotics included β -lactams, quinolones, aminoglycosides, tetracyclines, polymyxins, and sulfonamides. The *eaeH* gene was detected in all isolates (19/19); several strains also harbored virulence genes associated with enteroaggregative (*EAEC*), enteropathogenic (*EPEC*), and enterohemorrhagic (*EHEC*) *E. coli*. Antimicrobial susceptibility revealed that all isolates were resistant to ampicillin and trimethoprim-sulfamethoxazole. In conclusion, these findings indicate a high prevalence of virulence traits and multidrug resistance among the ovine *E. coli* isolates from Ningxia, underscoring the urgent need to strengthen antimicrobial stewardship and pathogen surveillance in the region.

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INTRODUCTION

Escherichia coli (*E. coli*) is a facultative, typically commensal Gram-negative bacterium commonly found in the intestines of animals and humans. In humans and other warm-blooded animals, *E. coli* typically lives in the digestive tract and is expelled along with stool (Li *et al.*, 2023). Intestinal bacteria produce a variety of metabolites that affect animal health, especially in the intestines (Rychlik, 2025). Under normal circumstances, it contributes to the intestinal microbiota and helps maintain microbial homeostasis. Based on their pathogenic traits, *E. coli* can be classified as either enteropathogenic or extra-intestinal pathogenic. Extra-intestinal pathogenic *E. coli* (ExPEC) has evolved the capacity to colonize extra-intestinal areas through adaptive modifications. It results in infections in humans and pets, as well as disorders in

multiple organs (Smith *et al.*, 2007). However, certain pathogenic strains of *E. coli* harbor various virulence factors, such as adhesins (*eae*), Shiga toxins (*stx1*, *stx2*), and enterotoxins (*est*, *elt*), which can disrupt the intestinal epithelial barrier and induce clinical symptoms such as diarrhea (Kaur and Dudeja, 2023; Ramatla *et al.*, 2024). These pathogenic strains are important causes of disease in livestock production. Bacteria, viruses, parasites, and fungi are the most common microbiological pathogens causing diarrheal diseases (Fidan *et al.*, 2009; Nawaz *et al.*, 2021; Zafar *et al.*, 2024; Xu *et al.*, 2025). Furthermore, the spread of antibiotic-resistant *E. coli* and their resistance genes is frequently associated with infections in farm animals. In recent years, antibiotics have been extensively used in livestock and poultry production to stimulate growth, prevent disease, and provide treatment (Osterberg *et al.*, 2016). Nevertheless, widespread and indiscriminate use of

these drugs has contributed to the emergence of antimicrobial resistance (AMR) among pathogenic bacteria and a consequent decrease in antibiotic effectiveness (Zaib *et al.*, 2019). Antibiotic usage is considered a major risk factor for the development and spread of AMR (de Man *et al.*, 2000). It exacerbates the potential for zoonotic transmission of resistant strains to humans and poses a significant global public health threat (O'Brien, 2002). Studies have shown that animal feces serve as an important reservoir for the spread of pathogenic bacteria. As a major component of the gut microbiota, *E. coli* isolated from feces is often used as an indicator organism in AMR surveillance, facilitating assessment of regional antimicrobial resistance risks. The factors influencing *E. coli* resistance patterns vary and may include age, prior antimicrobial exposure, geographical location, and other environmental and management factors (Cao *et al.*, 2019; Enne *et al.*, 2008). Understanding the genetic makeup of resistant strains is essential for evaluating region-specific AMR risks.

Ningxia, located in northwest China, is one of the country's key regions for herbivorous livestock production, with the sheep industry playing a central role in its agricultural economy (Zhang *et al.*, 2024). The veterinary medicinal products used in food-producing animals for various purposes and their residues remain in their bodies (Żmudzki *et al.*, 2012). In sheep husbandry, gastrointestinal tract (GIT) abnormalities are perhaps the most significant contributors to inadequate productivity, but they frequently coexist with other issues. Converting primary forage, herbage, or cereal crops into a commercial product is the main motivation for raising sheep for farming. Numerous bacterial, viral, and parasite illnesses cause morbidity and mortality as well as a reduction in total output. Johne's disease, Salmonellosis, *Escherichia coli* infection, enterotoxemia from *Clostridium perfringens* Types B, C, and D, plague in small ruminants, parasitism, campylobacteriosis, and other diarrheal illnesses are the most prevalent illnesses linked to GIT lesions (Ali and Alsayeqh, 2022; Yaseen *et al.*, 2025). Helminth parasitism, in particular, damages health by resulting in anemia, diarrhea, inappetence, and, in extreme cases, death (Kumar *et al.*, 2015). In young lambs, *E. coli* is a common infection, particularly in unhygienic settings. It spreads quickly throughout a flock or herd and causes severe, watery diarrhea. The bacteria can be fatal if left untreated and are frequently linked to scours in newborns. As a common cause of lamb diarrhea, *E. coli* warrants close monitoring of its resistance and virulence characteristics, which have practical significance for sustainable local animal husbandry. The study aimed to find the prevalence of virulence and resistance genes among ovine *E. coli* isolates from Ningxia. The isolates were tested for antimicrobial susceptibility, and resistance genes were detected by PCR. Insights into detection of virulence and AMR genes in ovine *E. coli* in this region, offering evidence-based support for clinical antimicrobial therapy, zoonotic disease control, and the development of antibiotic stewardship strategies within a One Health framework.

MATERIALS AND METHODS

Sample collection: From August to September 2024, a total of n=150 fresh fecal samples were aseptically collected from sheep at a farm in Ningxia, China. The n=32 diarrheic sheep

were diagnosed out of 150 samples by fecal scoring, and these samples were used for further processing. Sterile disposable sampling tubes were used during collection, and each sample was individually labeled. Samples were transported on dry ice to the Clinical Laboratory of Nanjing Agricultural University for further processing.

***Escherichia coli* isolation and purification:** Cultures were transferred to fresh LB broth (Thermo Fisher Scientific) and incubated for 12 hours under the same conditions. Following enrichment, cultures were streaked aseptically onto MacConkey agar (Thermo Fisher Scientific) and incubated at 37°C for 18–24 hours. Colonies with a characteristic metallic green sheen on EMB agar (Thermo Fisher Scientific) were considered presumptive *E. coli*. These colonies were inoculated into LB broth and incubated at 37°C overnight to obtain pure cultures for further analysis (Ema *et al.*, 2022).

Bacterial identification: The purified bacterial cultures were first examined by Gram staining under a light microscope. For molecular identification, a single colony was inoculated into 5mL LB broth and incubated at 36 ± 1°C with shaking at 200rpm for 24 hours. A 1mL aliquot was centrifuged at 8,000rpm for 5 minutes, and the pellet was used for genomic DNA extraction. The 16S rRNA gene was amplified with universal primers 27F (5'-AGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Li *et al.*, 2022). The expected amplicon is ~1,466 bp. 25µL: 12µL of 2× Taq MasterMix (Dye plus), 1µL each of forward and reverse primers (10µmol/L), 2µL of DNA template, and 9µL of ddH₂O. Cycling conditions were: initial denaturation at 95°C for 10 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes; and a final extension at 72°C for 5 minutes. Agarose gel electrophoresis was carried out, and sequencing was conducted at Shanghai Sangon Biotech Co., Ltd China.

Phylogenetic analysis of 16S rRNA genes of fecal *E. coli* isolates: *E. coli* obtained in the current research and reference strains of various geographic isolates were aligned in the multiple sequence alignment using ClustalW algorithm with the help of MEGA version 11.0 (Molecular Evolutionary Genetics Analysis). The neighbor-joining (NJ) method was applied to a phylogeny tree, and genetic distances were estimated along the Kimura 2-parameter model. Bootstrap analysis was done with some replicates (1,000) to assess the strength of the phylogenetic connection. The subsequent phylogenetic tree was applied to evaluate the genetic-relatedness of the isolates of Ningxia with reference strains of other regions.

Antimicrobial susceptibility testing and resistance gene detection: Antimicrobial susceptibility was determined by the disk diffusion method. Antibiotic disks included β-lactams (ampicillin, cefotaxime, ceftazidime), quinolones (ciprofloxacin), aminoglycosides (streptomycin, gentamicin, amikacin), tetracyclines (tetracycline), polymyxins (polymyxin B), and sulfonamides (trimethoprim-sulfamethoxazole). The bacteria were multidrug resistant, and MDR was defined as resistance to at least one antibiotic in three or more

antimicrobial classes (Pinheiro *et al.*, 2020). Disks were purchased from Hangzhou Microbial Reagent Co., Ltd, and tests were performed in triplicate. Results were interpreted using CLSI M100-S33 breakpoints; the breakpoints were listed in Table 1. PCR was employed to detect 20 resistance genes across five antibiotic classes in the 19 *E. coli* isolates: β -lactam resistance genes (*blaTEM*, *blaSHV*, *blaCTX-M*, *blaOXA*), aminoglycoside resistance genes (*aac(3)-II*, *aac(6)-Ib*, *aadA*, *strA*, *strB*, *aph(3)-VI*), quinolone resistance genes (*qnrA*, *qnrB*, *qnrS*), sulfonamide resistance genes (*sul1*, *sul2*, *sul3*), and tetracycline resistance genes (*tetA*, *tetB*, *tetC*, *tetD*) (Table 2). The 25 μ L PCR reaction mixture consisted of 12.5 μ L of 2 \times Taq Master Mix, 1 μ L each of forward and reverse primers, 1 μ L of DNA template, and 9.5 μ L of ddH₂O. PCR products were analyzed on a 1% agarose gel, and the band patterns were visualized and recorded using a gel documentation system.

Table 1: Criteria for the determination of drug content and sensitivity of drug-sensitive paper

Antibiotics	Abbreviation	Inhibition zone diameter/mm		
		R	I	S
Amikacin	AMK	≥ 14	15-16	≥ 17
Cefotaxime	CTX	≥ 16	23-25	≥ 22
Ampicillin	AMP	≥ 11	12-14	≥ 15
Gentamicin	GEN	≥ 12	13-14	≥ 15
Streptomycin	STR	≥ 11	12-14	≥ 15
Ciprofloxacin	CIP	≥ 15	16-20	≥ 21
Trimethoprim-sulfamethoxazole	SXT	≥ 10	11-15	≥ 16
Ceftazidime	CAZ	≥ 17	18-20	≥ 21
Polymyxin B	PB	≥ 8	9-11	≥ 12
Tetracycline	TET	≥ 14	15-18	≥ 19

Table 2: Primer information of resistance genes

Antibiotics	Genes	Sequences (5'→3')		Size/bp	Annealing temperature/°C
β -lactams	<i>blaTEM</i>	F:TCGCCGCATACACTATTCTCAGAATGA	R:ACGCTCACCGGCTCCAGATTTAT	445	61
	<i>blaSHV</i>	F:ATGCGTATATTCGCTGTG	R:CCTCATTCAGTCCCGTTTCC	502	54
	<i>blaCTX-M</i>	F:CGCGCTACAGTACAGCGATA	R:CCTTAGGTTGAGGCTGGGTG	434	57
	<i>aac(3)-II</i>	F:ACTCTGATCGGATACCCGTC	R:CTCCGTCAGCGTTTCAGCTA	237	57
	<i>aac(6)-Ib</i>	F:CATGACCTTGCGATGCTCTA	R:GCTCGAATGCCTGGCGTGT	470	57
Aminoglycosides	<i>aadA</i>	F:TTTGCTGGTTACGGTGAC	R:GCTCCATTGCCAGTCG	499	54
	<i>strA</i>	F:TGCTCCTCTTCTCCATCC	R:CGCCGCAATATGTTCTA	197	54
	<i>strB</i>	F:ATGATGCAGATCGCCATGTA	R:CGGTCGTAGAGGCAATCTG	326	54
	<i>aph(3)-VI</i>	F:ATACAGAGACCACCATACAGT	R:GACAATCAATAATAGCAAT	234	48
	<i>blaOXA</i>	F:TTTTCTGTTGTTGGGTTTC	R:TTTCTTGGCTTTTATGCTTG	447	48
Quinolones	<i>qnrA</i>	F:AGAGGATTTCTCACGCGAGG	R:TGCCAGGCACAGATCTTGAC	580	57
	<i>qnrB</i>	F:GGCATCGAAATTCGCCACTG	R:TTTGCCGCCCCGCCAGTCGAA	264	61
	<i>qnrS</i>	F:GCAAGTTCAATGAACAGGGT	R:TCTAAACCGTCGAGTTCGGCCG	428	54
Sulfonamide	<i>sul1</i>	F:TGGTGACGGTGTTCGGCATTG	R:GCGAGGGTTTCCGAGAAGGTG	789	61
	<i>sul2</i>	F:CGGCATCGTCAACATAACC	R:GTGTGCGGATGAAGTCAG	722	54
	<i>sul3</i>	F:CATTCTAGAAAACAGTCGTAGTTCCG	R:CATCTGCAGCTAACCTAGGGCTTTGGA	990	57
Tetracyclines	<i>tetA</i>	F:GCTACATCTGCTTGCCTTC	R:CATAGATCGCCGTGAAGAGG	210	57
	<i>tetB</i>	F:TTGGTTAGGGGCAAGTTTTG	R:GTAATGGGCCAATAACACCG	659	54
	<i>tetC</i>	F:CTTGAGAGCCTTCAACCCAG	R:ATGGTCTCATCTACCTGCC	418	57
	<i>tetD</i>	F:AAACCATTACGGCATTCTGC	R:GACCGGATACCCATCCATC	787	54

Table 3: Primer information of virulence genes

Genes	Sequences (5'→3')		Size/bp	Annealing temperature/°C
<i>aatA</i>	F:CATAGGCGTTTCTCTTCCGAT	R:CCTGTGCTTCATACAGATTCGTT	1226	54
<i>Let</i>	F:GGCGTTACTATCCTCTCTAT	R:TGGTCTCGGTCCAGATATGT	272	54
<i>stx1</i>	F:ACACTGGATGATCTCAGTGG	R:CTGAATCCCCCTCCATTATG	614	54
<i>aggR</i>	F:ACGCAGAGTTGCCTGATAAAG	R:AATACAGAATCGTCAGCATCAGC	400	54
<i>aatB</i>	F:GAATGCAGAGCAAGCGTTCAAT	R:GCCGACTTTAGCCCTCCATA	40	57
<i>stx2</i>	F:CAGTCGTCACCTCACTGGTTTCATCA	R:GGATATTCTCCCACTCTGACACC	283	57
<i>ipaH</i>	F:TGAGAGCTGTGAGGACCGT	R:CATGCCATGGTCCCGAGAGG	383	61
<i>eaeH</i>	F:TGTTCCGGAAGACACTAATGGT	R:CGCCGTAGAGCCGAGAGTAA	400	57
<i>bfpA</i>	F:GATAAACTGATACTGGGCGAGC	R:AGTGAAGTTCGGGAAGCAC	826	54
<i>eaeA</i>	F:GGAACGGCAGAGGTTAATCTGCAG	R:GGCGCTCATCATAGTCTTTC	346	57
<i>estA</i>	F:CAACTGAATCACTTGACTCTT	R:TAAATAACATCCAGCACAGG	158	49

Virulence genes detection: PCR-based detection of virulence genes was performed on 19 *E. coli* isolates. Eleven virulence genes associated with five *E. coli* pathotypes: *EHEC* (enterohemorrhagic *E. coli*), *EIEC* (enteroinvasive *E. coli*), *EPEC* (enteropathogenic *E. coli*), *EAEC* (enteroaggregative *E. coli*), and *ETEC* (enterotoxigenic *E. coli*), were screened. The corresponding virulence genes for each pathotype were as follows: *eaeA*, *eaeH*, and *bfpA* (*EPEC*); *stx1*, *stx2*, *eaeA*, and *eaeH* (*EHEC*); *aggR*, *aatA*, and *aatB* (*EAEC*); *ipaH* (*EIEC*); *elt* and *estA* (*ETEC*). Primers were designed and synthesized by Sangon Biotech (Shanghai, China), and their sequences are listed in Table 3. The PCR reaction mixture (25 μ L) consisted of 12.5 μ L 2 \times Taq Master Mix, 1 μ L each of forward and reverse primers, 1 μ L DNA template, and 9.5 μ L ddH₂O. PCR products were analyzed on a 1% agarose gel, and band positions were visualized and recorded using a gel documentation system.

Statistical analysis: The prevalence was calculated by the formula given below. The frequency of resistance to different antimicrobial classes and the distribution of virulence genes were expressed as percentages. Phylogenetic analysis was done by the ClustalW algorithm with the help of MEGA version 11.0 (Molecular Evolutionary Genetics Analysis).

Prevalence (%) = No. of sample positive for bacteria/
Total No. of samples tested \times 100

RESULTS

Isolation and identification of *E. coli*: Nineteen *E. coli* isolates were recovered from 32 fecal samples of diarrheic sheep, with a prevalence of 59.37%. On MacConkey agar, the isolates formed characteristic pink colonies, followed by EMB agar for further confirmation, where they exhibited black colonies with a distinctive metallic green sheen. PCR amplification of the 16S rRNA gene yielded approximately a 1,500bp product (Fig. 1). Sequencing and BLAST analysis confirmed all isolates as *Escherichia coli*. Sequences were submitted to NCBI GenBank under accession numbers PV848920–PV848938.

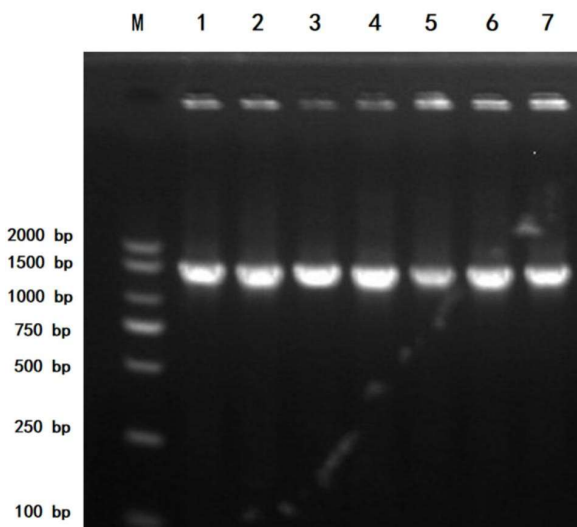


Fig. 1: Agarose gel electrophoresis of PCR amplification products from 19 isolated strains. M represent DL2000 DNA Marker; lanes 1–7 indicate the amplified 16S rRNA gene from isolated strains.

16S rRNA genes phylogenetic analysis: To further elucidate phylogenetic relationships among the *E. coli*, Neighbor-Joining phylogenetic tree was constructed based on the 16S rRNA gene sequences (Fig. 2), with *Staphylococcus aureus* (NR_118997.2) used as the outgroup.

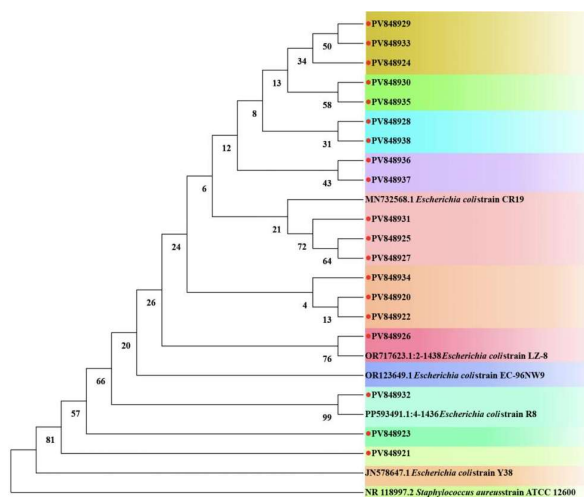


Fig. 2: Phylogenetic tree based on 16S rRNA gene sequences of *Escherichia coli* strains constructed using the Neighbor-Joining method. The number of nodes indicates the bootstrap values.

All 19 isolates (PV848920–PV848938) were clustered within the *Escherichia coli* clade. Among them, PV848929 and PV848933 formed a distinct cluster, as did PV848930 and PV848935. Another well-supported cluster included PV848931, PV848925, and PV848927, suggesting a close genetic relationship among these strains. Notably, PV848925, PV848927, and PV848931 clustered closely with *E. coli* CR19 (MN732568.1), a strain isolated from sheep feces in the Chinese plateau region. PV848926 grouped with *E. coli* LZ-8 (OR717623.1), while PV848932 was highly similar to *E. coli* R8 (PP593491.1), with a bootstrap value of 99, indicating a close phylogenetic relationship.

Additionally, PV848921 and PV848923 formed a separate branch with *E. coli* Y38 (JN578647.1), which originated from sheep in Henan Province. This suggests possible geographic or host-specific adaptations contributing to their divergence from other isolates.

Antimicrobial susceptibility and resistance gene detection: To assess the antimicrobial resistance profile of ovine diarrheic *E. coli* in Ningxia, both disk diffusion testing and PCR-based detection of 20 common resistance genes were performed on 19 isolates. The results revealed widespread resistance and a high prevalence of resistance gene carriage among the isolates.

As shown in Fig. 3, all 19 *E. coli* strains (100%) were resistant to ampicillin and trimethoprim-sulfamethoxazole. High resistance was observed to ciprofloxacin (94.74%, 18/19), streptomycin (89.47%, 17/19), gentamicin (89.47%, 17/19), and tetracycline (89.47%, 17/19). Resistance to amikacin was moderate (63.16%, 12/19), while resistance to ceftazidime and cefotaxime was relatively low, 5.3% (1/19) and 31.6% (6/19), respectively. All isolates were highly sensitive to polymyxin B (100%, 19/19). These findings indicate a serious multidrug resistance (MDR) burden, particularly against commonly used broad-spectrum antibiotics.

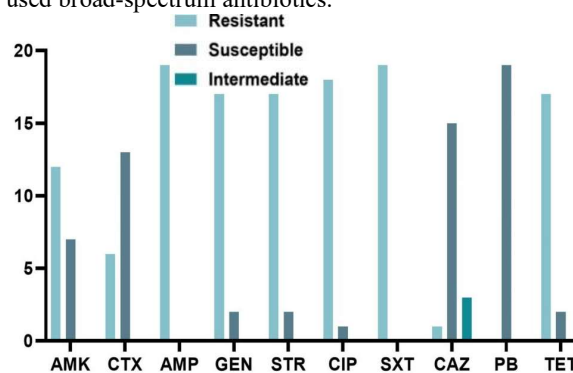
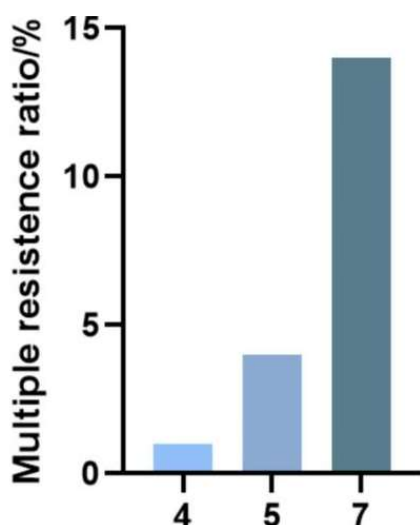


Fig. 3: Statistical analysis of the antimicrobial susceptibility of *Escherichia coli* to commonly used antibiotics (β -lactams, quinolones, aminoglycosides, tetracyclines, polymyxins, and sulfonamides).

Multidrug resistance was observed in all isolates, with resistance patterns ranging from 4 to 7 antibiotics (Fig. 4). Among these, 7-drug resistance was the most prevalent (73.68%, 14/19), followed by 6-drug resistance (21.05%, 4/19), and 4-drug resistance was observed in only one isolate (5.26%). The most frequently observed core MDR pattern was CIP+AM+SXT+CN, which was shared across different MDR profiles.



Number of multiple drug resistant species

Fig. 4: Percentage of *Escherichia coli* strains exhibiting resistance to 10 different antimicrobial agents.

PCR analysis revealed that most isolates carried multiple antimicrobial resistance genes (Fig. 5). Among tetracycline resistance genes, *tetA* had the highest detection rate at 89.47% (17/19), followed by *tetD* (42.11%, 8/19) and *tetB* (15.79%, 3/19). For sulfonamide resistance, *sul2* and *sul1* were detected in 94.74% (18/19) and 68.42% (13/19) of isolates, respectively, while *sul3* was only found in 10.53% (2/19) of isolates. Among aminoglycoside resistance genes, *strA* and *strB* showed high prevalence at 84.21% (16/19) and 78.95% (15/19), respectively; *aph(3')-VI* was detected in 47.37% (9/19), and *aadA* in only 10.53% (2/19). For β -lactam resistance, *blaTEM* was highly prevalent (89.47%, 17/19), followed by *blaOXA* (42.11%, 8/19), while *blaCTX-M-1* was found in only one isolate (5.26%). *blaSHV* was not detected. Regarding quinolone resistance genes, *aac(6)-Ib* was detected in 63.16% (12/19), while *qnrS* and *aac(3)-II* were each present in 21.05% (4/19). *qnrA* and *qnrB* were not detected. Overall, the results demonstrated a high level of resistance gene carriage, with significant potential for multidrug resistance in the *E. coli* isolates.

Detection of virulence genes in diarrheagenic *E. coli* isolates: PCR-based detection of 11 virulence genes was conducted on 19 *E. coli* isolates. The *eaeH* gene showed the highest detection rate at 100% (19/19), indicating the widespread presence of enteropathogenic (*EPEC*) or enterohemorrhagic (*EHEC*) *E. coli*-related virulence genes. Additionally, *aata* and *aggR*, which are associated with enteroaggregative *E. coli* (*EAEC*), were respectively detected in three isolates (15.79%). Other virulence genes such as *elt*, *stx1*, *ipaH*, and *bfpA* were not detected. Notably, one isolate (PV848932) harbored both *eaeH* and *aata* virulence genes (Fig. 6).

Overall, results suggest that *E. coli* strains isolated from diarrheic sheep in this farm primarily carried virulence genes related to *EAEC*, *EHEC*, and *EPEC*, implying that these pathotypes may be the major contributors to diarrhea in the local sheep population.

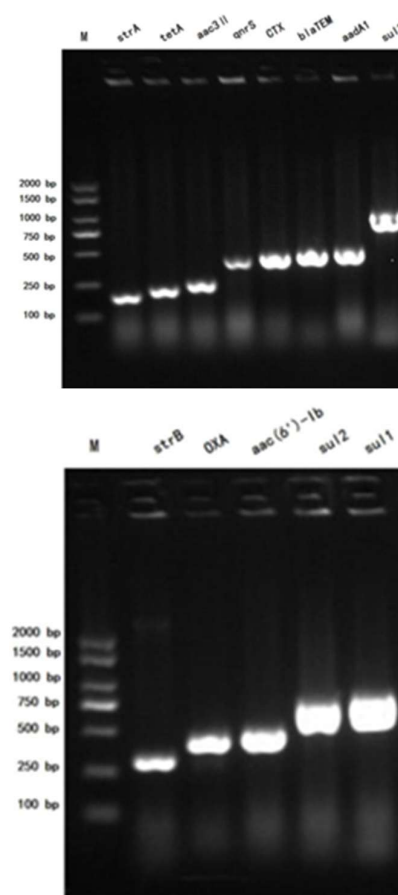


Fig. 5: Agarose gel electrophoresis of PCR amplification products of partial resistance genes in *Escherichia coli*. These genes are amplified from 19 isolated strains of *Escherichia coli*. The names of the target genes are indicated above each corresponding lane. M represents the DL2000 DNA marker.

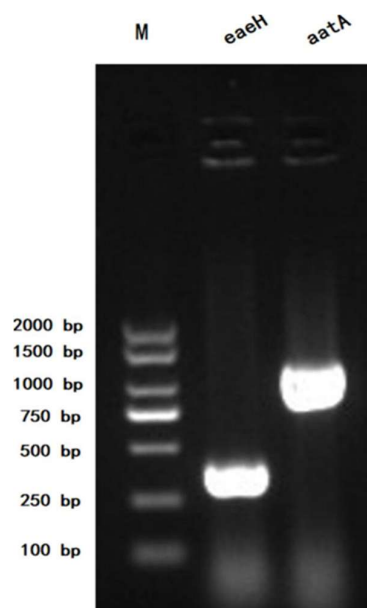


Fig. 6: Agarose gel electrophoresis of PCR amplification products of partial virulence genes in *Escherichia coli* strain PV848932. The names of the target genes are indicated above each corresponding lane. M: DL2000 DNA marker.

DISCUSSION

The results revealed widespread multidrug resistance (MDR) and the presence of diarrhea-associated virulence genes, indicating that these isolates may be significant etiological agents of sheep diarrhea, posing potential risks to both animal and public health. The prevalence of *E. coli* isolates is consistent with other studies conducted by Kumar *et al.* (2015). The most common bacterial organism in GIT problems of sheep carcasses, according to Shabana *et al.* (2017), is *E. coli*, which is followed by *Proteus species*, *Klebsiella species*, *Salmonella* Typhimurium, *Staphylococcus species*, and *Pseudomonas aeruginosa*. Research by Ding *et al.* has demonstrated that environmental, dietary, and age-related variables continuously alter microbial composition. The findings of this study indicated that the two prevalent phyla were Proteobacteria and Firmicutes (Ding *et al.*, 2023).

Phylogenetic trees based on 16S rRNA gene sequences provide a reliable approach for inferring evolutionary relationships and genetic relatedness among *E. coli* strains from different hosts and geographical regions (Touchon *et al.*, 2020). In this study, the 16S sequences clustered all the isolates within the *E. coli* lineage. Notably, PV848925, PV848927, and PV848931 formed a distinct clade with *E. coli* CR19, a strain isolated from sheep in the Chinese plateau region, suggesting potential genetic connections between *E. coli* strains from Ningxia and other high-altitude ecological zones. Such clustering may reflect the influence of regional factors such as climate, farming practices, and sheep breeds on *E. coli* population structure (Escobar-Paramo *et al.*, 2004). PV848921 and PV848923 formed a separate branch with *E. coli* Y38 from Henan Province, suggesting possible host adaptation or geographic isolation. These geographic evolutionary patterns are commonly observed in animal-derived pathogens and are often influenced by host specificity, environmental pressures, and antibiotic selection (Lagerstrom and Hadly, 2021). Further whole-genome-based analyses are warranted to validate these evolutionary associations.

The increase of antibiotic resistance is a global health risk affecting human and veterinary medicine and highlighted within the One Health framework (Zychska *et al.*, 2025). In livestock production, antibiotics are randomly used for prophylactic, therapeutic, and growth promotion purposes. The overuse of antibiotics in livestock production has led to the development of AMR in bacterial strains in animals, which spread to humans and cause diseases and threaten the health of humans and animals (Enshaie *et al.*, 2025). In terms of antimicrobial resistance, this study revealed severe resistance among the isolates. All strains are resistant to ampicillin and trimethoprim-sulfamethoxazole, with high resistance rates also observed for ciprofloxacin, streptomycin, and gentamicin (~90%), reflecting a classical MDR phenotype. Similar resistance trends have been reported in sheep and cattle from the UK, where β -lactam and tetracycline resistance exceed 70%, often driven by *tet* and *bla* gene carriage (Martínez-Vázquez *et al.*, 2021). These antibiotics are among the most widely used in Chinese livestock production (Yang *et al.*, 2019), and their long-term or improper use may exert persistent selective pressure favoring resistance

development and dissemination (Pang *et al.*, 2019). In contrast, all isolates were sensitive to polymyxin B, which may be attributed to its known toxicity and stringent usage restrictions. Moreover, its cost is higher than that of alternative agents such as colistin sulfate, yet its therapeutic efficacy is slightly lower (Wang *et al.*, 2024), which explains its limited use in farming contexts. Selective bacteria, particularly *E. coli* and other bacteria, including *Staphylococcus aureus*, *Klebsiella spp.*, and *Salmonella spp.*, have been the main focus of AMR investigations in dairy farm environments. The investigations encompassing 78 antimicrobials revealed patterns of antimicrobial resistance, with gentamicin accounting for 70.8%, ampicillin for 58.3%, and tetracycline for 58.3% of the total (Velloo *et al.*, 2025).

The resistance genotype–phenotype correlation was generally consistent. For instance, the high resistance to aminoglycosides corresponded with high detection rates of *strA* (84.21%) and *strB* (78.95%); tetracycline resistance matched the widespread presence of *tetA* (89.47%). For β -lactams, the high ampicillin resistance was aligned with the 89.47% detection rate of *bla*TEM. Sulfonamide resistance was likely driven by *sul2* (94.74%) and *sul1* (68.42%). Such genotype–phenotype concordance has been reported previously; Hwang *et al.* (2023) found consistency between *E. coli* resistance genotypes and phenotypes to range between 89–100%. The results suggest that acquired resistance genes have a significant role in resistance dissemination and are likely to be transmitted via mobile genetic elements such as plasmids (Xu *et al.*, 2022; Youngblom *et al.*, 2023), enhancing adaptability and transmission potential. *E. coli* isolated from goats raised under intense and semi-intensive farming practices showed clustering of resistance features and an antimicrobial resistance mechanism. The study found that compared to their healthy counterparts, isolates from diarrheal animals are probably more ACBL (AmpC β -lactamase)-positive ($P < 0.001$), and are resistant to tetracycline (Banerjee *et al.*, 2022).

Regarding virulence, the *eaeH* gene was detected, indicating the widespread presence of EPEC or EHEC-associated attaching and effacing (A/E) factors. *eaeH* encodes intimin, a key adherence factor that mediates tight attachment to intestinal epithelial cells and disrupts mucosal integrity, forming the molecular basis for pathogenesis in EPEC and EHEC (Blanco *et al.*, 2005). Although *stx1/stx2* genes were not detected, similar findings were reported in Spain, where AECC strains from diarrheic lambs were *Stx*-negative but still induced A/E lesions in rabbit intestinal models via intimin-mediated mechanisms (Cid *et al.*, 2001). High prevalence of *eaeH* suggests that these isolates may retain strong pathogenic potential despite lacking Shiga toxins. Moreover, several strains carried EAEC-related genes such as *aggR* and *aata*, reflecting the virulence diversity among ovine *E. coli* isolates. Notably, one isolate (PV848932) carried both *eaeH* and *aata*, suggesting possible horizontal gene transfer or plasmid recombination (Garcia *et al.*, 2016). This virulence gene diversity could suggest evolutionary adaptation in the complex intestinal ecosystem of livestock and highlights the need for more stringent disease control measures in farms. Furthermore, 66% of *E. coli* isolates had the *eaeA* gene; all of them had the St1 and St2 virulence

genes (Wani *et al.*, 2009). These "high-virulence, high-resistance" strains may possess enhanced pathogenicity and environmental persistence (Feng *et al.*, 2025; Lazar *et al.*, 2023). Some studies suggest that plasmids co-carrying resistance and virulence genes can be stably maintained under antibiotic pressure, which results in the appearance of very strong and resistant strains (Beceiro *et al.*, 2013). Once introduced into the human population through foodborne transmission, such strains may pose significant public health threats (Elbehiry *et al.*, 2025). Given that Ningxia is a key livestock production region in northwest China, with widespread distribution of sheep products, strengthening surveillance of resistance and virulence in animal-derived *E. coli* is urgently needed.

Conclusions: This study elucidated the phylogenetic relationships, antimicrobial resistance patterns, and virulence gene profiles of *E. coli* from diarrheic sheep in Ningxia, China. The isolates exhibited high rates of multidrug resistance and carried key virulence genes such as *eaeH* and *aggR*, indicating their potential role in both diarrheal disease and resistance dissemination. The occurrence of strains harboring high-level virulence and antimicrobial resistance represents a potential public health threat through the food chains. The outcomes point out the significance of prudent antibiotic use and continuous microbial surveillance and provide valuable data for regional disease control and antimicrobial resistance risk assessment. The study has limitations, as the sample size was small and confined to diarrheic sheep in Ningxia, China, which may not indicate the complete epidemiological situation. The drivers of antimicrobial resistance in these strains were not clarified fully because the detailed usage of farm-level antibiotics and comprehensive genetic analysis of virulence and resistance genes were not available. Future studies require targeting novel genes in *E. coli*, and further focus on similar grounds for bacterial and viral pathogens responsible for diarrhea.

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