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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 include 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). The intestinal bacteria produce a variety of metabolites that affect animal health, especially 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 entero-pathogenic or extra-intestinal pathogenic. Extra-intestinal pathogenic *E. coli* (ExPEC) has evolved the capacity to colonize extra-intestinal areas through adaptive modifications. It result in infections in humans as well as pets and 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; Xu *et al*., 2025; Zafar *et al*., 2024). 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 therapeutic 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 development and spread of AMR (de Man *et al*., 2000). It exacerbate the potential for zoonotic transmission of resistant strains to humans and posing 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 retain 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. While, 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 is 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, 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 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 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 5 mL LB broth and incubated at 36 ± 1 °C with shaking at 200 rpm for 24 hours. A 1 mL aliquot was centrifuged at 8,000 rpm 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. 25 µL: 12 µL of 2× Taq MasterMix (Dye plus), 1 µL each of forward and reverse primers (10 µmol/L), 2 µL of DNA template, and 9 µL of ddH_2O. 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.

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 using ClustalW algorithm with the help of MEGA version 11.0 (Molecular Evolutionary Genetics Analysis). The neighbor-joining (NJ) method was applied on a phylogeny tree and genetic distances were estimated along Kimura 2-parameter model. Bootstrap analysis was done with a number of 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 μL PCR reaction mixture consisted of 12.5 μL of 2× Taq Master Mix, 1 μL each of forward and reverse primers, 1 μL of DNA template, and 9.5 μL of ddH$_2$O. 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 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

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 μL) consisted of 12.5 μL 2× Taq Master Mix, 1 μL each of forward and reverse primers, 1 μL DNA template, and 9.5 μL ddH$_2$O. 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 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 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 × 100

Table 2: Primer information of resistance genes

Antibiotics	Genes	Sequences (5′→3′)		Size/ bp	Annealing temperature/°C
β-lactams	*blaTEM*	F:TCGCCGCATACACTATTCTCAGAATGA	R:ACGCTCACCGGCTCCAGATTTAT	445	61
	blaSHV	F:ATGCGTATATTCGCCTGTG	R:CCTCATTCAGTTCCGTTTCC	502	54
	blaCTX-M	F:CGCGCTACAGTACAGCGATA	R:CCTTAGGTTGAGGCTGGGTG	434	57
Aminoglycosides	*aac(3)-II*	F:ACTCTGATCGGATACCCGTC	R:CTCCGTCAGCGTTTCAGCTA	237	57
	aac(6')-Ib	F:CATGACCTTGCGATGCTCTA	R:GCTCGAATGCCTGGCGTGTT	470	57
	aadA	F:TTTGCTGGTTACGGTGAC	R:GCTCCATTGCCCAGTCG	499	54
	strA	F:TGCTCCTCTTCTCCATCC	R:CGCCGCCAATATGTTCTA	197	54
	strB	F:ATGATGCAGATCGCCATGTA	R:CGGTCGTAGAGGCAATCTG	326	54
	aph(3')-VI	F:ATACAGAGACCACCATACAGT	R:GCACAATCAATAATAGCAAT	234	48
	blaOXA	F:TTTTCTGTTGTTTGGGTTTC	R:TTTCTTGGCTTTTATGCTTG	447	48
Quinolones	*qnrA*	F:AGAGGATTTCTCACGCCAGG	R:TGCCAGGCACAGATCTTGAC	580	57
	qnrB	F:GGCATCGAAATTCGCCACTG	R:TTTGCCGCCCGCCAGTCGAA	264	61
	qnrS	F:GCAAGTTCATTGAACAGGGT	R:TCTAAACCGTCGAGTTCGGCCG	428	54
Sulfonamide	*sul1*	F:TGGTGACGGTGTTCGGCATTC	R:GCGAGGGTTTCCGAGAAGGTG	789	61
	sul2	F:CGGCATCGTCAACATAACC	R:GTGTGCGGATGAAGTCAG	722	54
	sul3	F:CATTCTAGAAAACAGTCGTAGTTCG	R:CATCTGCAGCTAACCTAGGGCTTTGGA	990	57
Tetracyclines	*tetA*	F:GCTACATCCTGCTTGCCTTC	R:CATAGATCGCCGTGAAGAGG	210	57
	tetB	F:TTGGTTAGGGGCAAGTTTTG	R:GTAATGGGCCAATAACACCG	659	54
	tetC	F:CTTGAGAGCCTTCAACCCAG	R:ATGGTCGTCATCTACCTGCC	418	57
	tetD	F:AAACCATTACGGCATTCTGC	R:GACCGGATACACCATCCATC	787	54

Table 3: Primer information of virulence genes

Genes	Sequences (5′→3′)		Size/bp	Annealing temperature/°C
aatA	F:CATAGGCGTTTCTCTTTCCGAT	R:CCTGTCGTTCATACAGATTCGTT	1226	54
Let	F:GGCGTTACTATCCTCTCTAT	R:TGGTCTCGGTCAGATATGT	272	54
stx1	F:ACACTGGATGATCTCAGTGG	R:CTGAATCCCCCTCCATTATG	614	54
aggR	F:ACGCAGAGTTGCCTGATAAAG	R:AATACAGAATCGTCAGCATCAGC	400	54
aatB	F:GAATGCAGAGCAAGCGTTCAAT	R:GCCGACTTTAGCCCTCCCATA	40	57
stx2	F:CAGTCGTCACTCACTGGTTTCATCA	R:GGATATTCTCCCCACTCTGACACC	283	57
ipaH	F:TGAGAGCTGTGAGGACCGT	R:CATGCCATGGTCCCCAGAGG	383	61
eaeH	F:TGTTCCGCAAGACACTAATGGT	R:CGCCGTAGAGCCGAGAGTAA	400	57
bfpA	F:GATAAAACTGATACTGGGCAGC	R:AGTGACTGTTCGGGAAGCAC	826	54
eaeA	F:GGAACGGCAGAGGTTAATCTGCAG	R:GGCGCTCATCATAGTCTTTC	346	57
estA	F:CAACTGAATCACTTGACTCTT	R:TTAATAACATCCAGCACAGG	158	49

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 1,500 bp product (Fig. 1). Sequencing and BLAST analysis confirmed all isolates as *Escherichia coli*. Sequences were submitted to NCBI GenBank under accession numbers PV848920–PV848938.

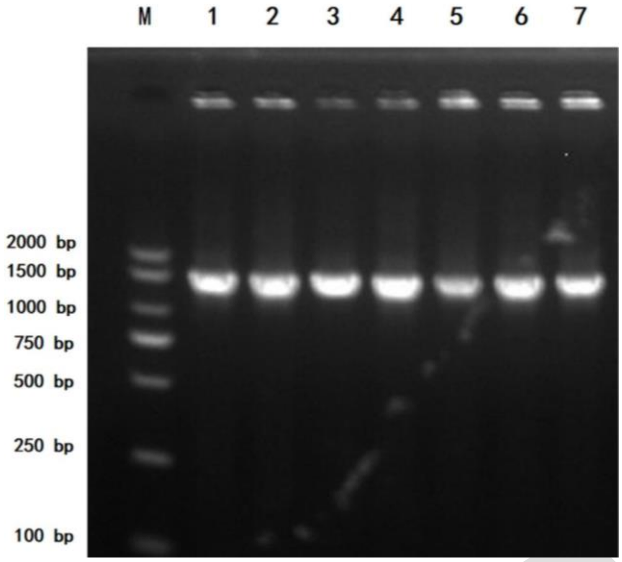

Fig. 1: Agarose gel electrophoresis of PCR amplification products from 19 isolated strains. M represent DL2000 DNA Marker; lanes 1–7 indicating the amplified 16S rRNA gene from isolated strains.

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.

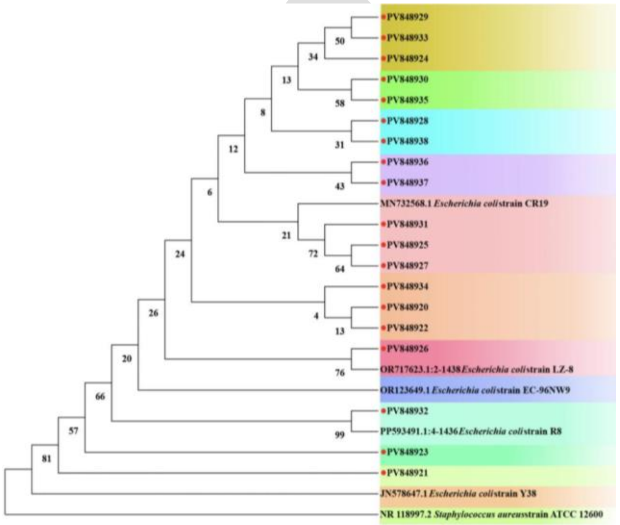

Fig. 2: Phylogenetic tree based on 16S rRNA gene sequences of *Escherichia coli* strains constructed using the Neighbor-Joining method. The number of nodes indicates the bootstrap values.

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 were 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.

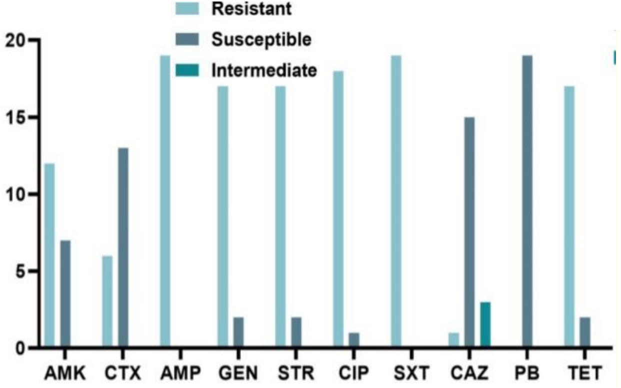

Fig. 3: Statistical analysis of the antimicrobial susceptibility of *Escherichia coli* to commonly used antibiotics (β-lactams, quinolones, aminoglycosides, tetracyclines, polymyxins, and sulfonamides).

Multidrug resistance was observed in all isolates, with resistance patterns ranging in 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.

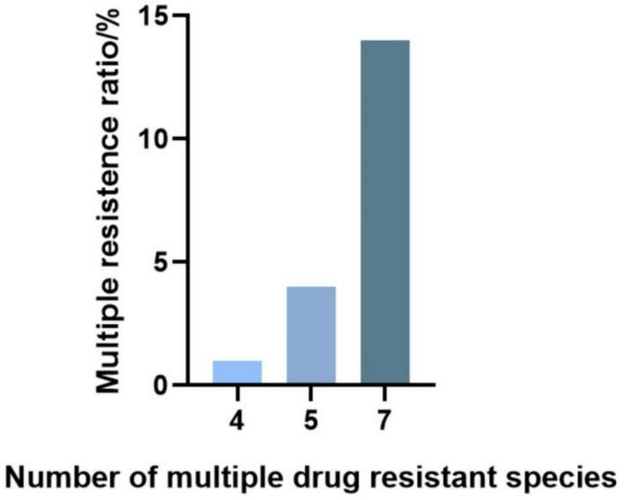

Fig. 4: Percentage of *Escherichia coli* strains exhibiting resistance to 10 different antimicrobial agents.

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.

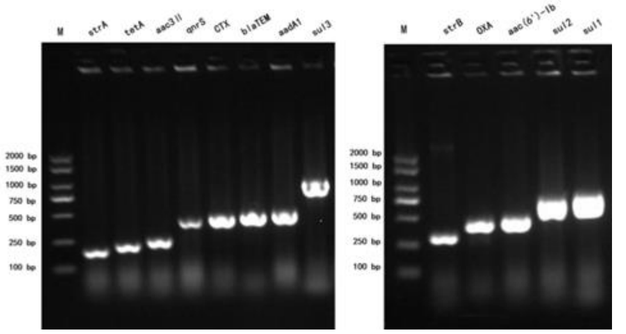

Fig. 5: Agarose gel electrophoresis of PCR amplification products of partial resistance genes in *Escherichia coli*. These genes are amplified from 19 isolated strains of *Escherichia coli*. The names of the target genes are indicated above each corresponding lane. M represent the DL2000 DNA marker.

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).

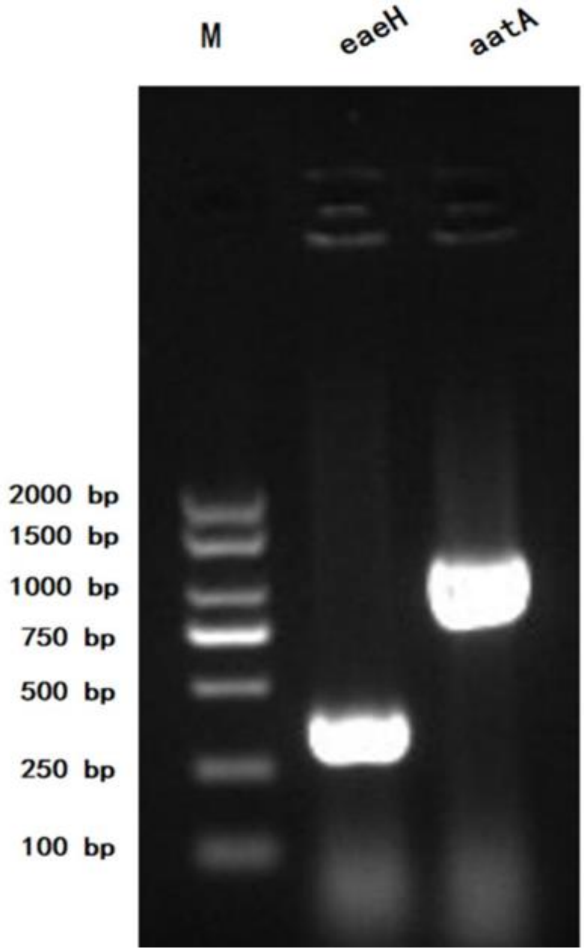

Fig. 6: Agarose gel electrophoresis of PCR amplification products of partial virulence genes in *Escherichia coli* strain PV848932. The names of the target genes are indicated above each corresponding lane. M: DL2000 DNA marker.

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.

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 *E. coli* isolates prevalence 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, that 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 (Veloo *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 *blaTEM*. Sulfonamide resistance was likely driven by *sul2* (94.74%) and *sul1* (68.42%). Such genotype–phenotype concordance has been reported previously; Hwang *et al*. found consistency between *E. coli* resistance genotypes and phenotypes to range between 89–100% (Hwang *et al.*, 2023). The results suggest that acquired resistance genes have significant role in resistance dissemination and 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 AEEC 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 Slt1 and Slt2 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 result 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 represent 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 samples size was less 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.

Ethics approval and consent to participate: All the experiment procedures in this study were under the ethics committee of Nanjing Agricultural University (NJAU.No20240910161).

Data availability: NCBI GenBank under accession numbers PV848920–PV848938.

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REFERENCES

Ali S, and Alsayeqh AF, 2022. Review of major meat-borne zoonotic bacterial pathogens. Frontiers in public health, 10: 1045599.

Banerjee J, Bhattacharyya D, Habib M, *et al.*, 2022. Antimicrobial Resistance Pattern, Clustering Mechanisms and Correlation Matrix of Drug-Resistant *Escherichia coli* in Black Bengal Goats in West Bengal, India. Antibiotics (Basel) 11(10): 1344.

Beceiro A, Tomas M and Bou G, 2013. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clinical Microbiology Reviews 26(2): 185-230.

Blanco M, Schumacher S, Tasara T, *et al.*, 2005. Serotypes, intimin variants and other virulence factors of eae positive *Escherichia coli* strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (eae-eta2). BMC Microbiology 5: 23.

Cao H, Pradhan AK, Karns JS, *et al.*, 2019. Age-Associated Distribution of Antimicrobial-Resistant *Salmonella enterica* and *Escherichia coli* Isolated from Dairy Herds in Pennsylvania, 2013-2015. Foodborne Pathogens and Disease 16(1): 60-67.

Cid D, Ruiz-Santa-Quiteria JA, Marín I, *et al.*, 2001. Association between intimin (eae) and EspB gene subtypes in attaching and effacing strains isolated from diarrhoeic lambs and goat kids. Microbiology 147: 2341-53.

de Man P, Verhoeven BA, Verbrugh HA, *et al.*, 2000. An antibiotic policy to prevent emergence of resistant bacilli. Lancet 355(9208): 973-8.

Ding X, Jiang HY, Zhang RR, *et al.*, 2023. Comparative Analysis of Nasal Microbial Community between Tibetan Sheep with different Ages. Pakistan Veterinary Journal 43(4): 723-31.

Elbehiry A, Marzouk E, Abalkhail A, *et al.*, 2025. Microbial Food Safety and Antimicrobial Resistance in Foods: A Dual Threat to Public Health. Microorganisms 13(7): 1592.

Ema FA, Shanta RN, Rahman MZ, *et al.*, 2022. Isolation, identification, and antibiogram studies of *Escherichia coli* from ready-to-eat foods in Mymensingh, Bangladesh. Veterinary World 15(6): 1497-505.

Enne VI, Cassar C, Sprigings K, *et al.*, 2008. A high prevalence of antimicrobial resistant Escherichia coli isolated from pigs and a low prevalence of antimicrobial resistant *E. coli* from cattle and sheep in Great Britain at slaughter. FEMS Microbiology Letters 278(2): 193-9.

Enshaie E, Nigam S, Patel S, and Rai V, 2025. Livestock antibiotics use and antimicrobial resistance. *Antibiotics*, *14*(6), 621.

Escobar-Paramo P, Grenet K, Le Menac'h A, *et al.*, 2004. Large-scale population structure of human commensal *Escherichia coli* isolates. Applied and Environmental Microbiology 70(9): 5698-700.

Feng C, Jia H, Yang Q, *et al.*, 2025. Genetic evolution of antibiotic resistance and virulence genes in *Escherichia coli* isolates from a Chinese hospital over a 12-year period. Microorganisms 13(4): 954.

Fidan I, Kalkanci A, Yesilyurt E, *et al.*, 2009. Effects of Saccharomyces boulardii on cytokine secretion from intraepithelial lymphocytes infected by *Escherichia coli* and *Candida albicans*. Mycoses 52(1):29-34.

Garcia BG, Ooka T, Gotoh Y, *et al.*, 2016. Genetic relatedness and virulence properties of enteropathogenic *Escherichia coli* strains of serotype O119:H6 expressing localized adherence or localized and aggregative adherence-like patterns on HeLa cells. International Journal of Medical Microbiology 306(3): 152-64.

Hwang M, Choi H, Navarathna T, *et al.*, 2023. Genotype-to-Phenotype Agreement of Antimicrobial Resistance in *Escherichia coli*. Open Forum Infectious Diseases 10 (Supplement2).

Kaur P and Dudeja PK, 2023. Pathophysiology of Enteropathogenic *Escherichia coli*-induced Diarrhea. Newborn (Clarksville, Md.) 2(1): 102-13.

Kumar S, Jakhar KK, Nehra V, *et al.*, 2015. Pathomorphological and microbiological studies in sheep with special emphasis on gastrointestinal tract disorders. Veterinary World 8(8): 1015-20.

Lagerstrom KM and Hadly EA, 2021. The under-investigated wild side of *Escherichia coli*: genetic diversity, pathogenicity and antimicrobial resistance in wild animals. Proceedings of the Royal Society B: Biological Sciences 288(1948): 20210399.

Lazar V, Oprea E and Ditu LM, 2023. Resistance, Tolerance, Virulence and Bacterial Pathogen Fitness-Current State and Envisioned Solutions for the Near Future. Pathogens 12(5): 746.

Li J, Liu P, Menguy N, *et al.*, 2022. Identification of sulfate-reducing magnetotactic bacteria via a group-specific 16S rDNA primer and correlative fluorescence and electron microscopy: Strategy for culture-independent study. Environ Microbiol 24(11):5019-5038.

Martínez-Vázquez AV, Vázquez-Villanueva J, Leyva-Zapata LM, *et al.*, 2021. Multidrug Resistance of Strains Isolated From Bovine Feces and Carcasses in Northeast Mexico. Frontiers in Veterinary Science 8: 643802.

Nawaz Z, Aslam B, Zahoor MA, *et al.*, 2021. Frequency of Extended Spectrum Beta Lactamase Producing *Escherichia coli* in Fresh and Frozen Meat. Pakistan Veterinary Journal, 41(1): 102-106.

O'Brien TF, 2002. Emergence, spread, and environmental effect of antimicrobial resistance: How use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. Clinical Infectious Diseases 34: S78-S84.

Osterberg J, Wingstrand A, Nygaard Jensen A, *et al*., 2016. Antibiotic Resistance in *Escherichia coli* from Pigs in Organic and Conventional Farming in Four European Countries. PLoS One 11(6): e0157049.

Pang Z, Raudonis R, Glick BR, *et al*., 2019. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. Biotechnology Advances 37(1): 177-192.

Pinheiro REE, Chaves TP, Melo ES, *et al*., 2020. Modulatory-antibiotic activity of the essential oil from *Eucalyptus citriodora* against MDR bacterial strains. Cellular and Molecular Biology 66(4): 60-64.

Ramatla T, Tutubala M, Motlhaping T, *et al*., 2024. Molecular detection of Shiga toxin and extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolates from sheep and goats. Molecular Biology Reports 51(1): 57.

Rychlik A, 2025. Fecal microbiome transplantation in the treatment of chronic enteropathies. Polish Journal of Veterinary Sciences, 28(4), 691-700.

Shabana, II, Bouqellah NA and Zaraket H, 2017. Investigation of viral and bacterial enteropathogens of diarrheic sheep and goats in Medina, Saudi Arabia. Tropical Biomedicine 34(4): 944-55.

Smith JL, Fratamico PM, Gunther NW, 2007. Extraintestinal pathogenic *Escherichia coli*. Foodborne Pathogens and Disease 4(2):134-63.

Touchon M, Perrin A, de Sousa JAM, *et al*., 2020. Phylogenetic background and habitat drive the genetic diversification of *Escherichia coli*. PLoS Genetics 16(6): e1008866.

Veloo Y, Thahir SSA, Zakaria Z, *et al*., 2025. A scoping review unveiling antimicrobial resistance patterns in the environment of dairy farms across Asia. Antibiotics (Basel) 14(5): 436.

Wang Y, Yu L, Zhu J, *et al*., 2024. Cost-effectiveness analysis of polymyxin B versus colistin for treating patients with carbapenem-resistant gram-negative bacterial infections. Scientific Reports 14(1): 23635.

Wani SA, Hussain I, Fayaz I, *et al*., 2009. Subtype analysis of stx1, stx2 and eae genes in Shiga toxin-producing *Escherichia coli* (STEC) and typical and atypical enteropathogenic *E. coli* (EPEC) from lambs in India. The Veterinary Journal 182(3): 489-90.

Xu C, He Q, Zhu Z, *et al*., 2025. Propolis improves intestinal barrier function against *Cryptosporidium parvum* via NLRP6 inflammasome. mBio 16(11):e0231725.

Xu C, Kong L, Liao Y, *et al*., 2022. Mini-Review: Antibiotic-Resistant *Escherichia coli* from Farm Animal-Associated Sources. Antibiotics (Basel) 11(11): 1535.

Yang H, Paruch L, Chen X, *et al*., 2019. Antibiotic Application and Resistance in Swine Production in China: Current Situation and Future Perspectives. Frontiers in Veterinary Science 6: 136.

Yaseen K, Ali S, Rahman SU and Sajid MS, 2025. Comparative molecular virulence typing and antibiotic resistance of *Campylobacter* species at the human–animal–environment interface. Foodborne pathogens and disease 22(2): 109-117.

Youngblom MA, Shockey AC, Callaghan MM, *et al*., 2023. The Gonococcal Genetic Island defines distinct sub- populations of Neisseria gonorrhoeae. Microbial Genomics 9(5): mgen000985.

Zafar S, Sarfraz MS, Ali S, *et al*., 2024. Recapitulation of peste des petits ruminants (PPR) prevalence in small ruminant populations of Pakistan from 2004 to 2023: A systematic review and meta-analysis. Veterinary Sciences 11(6): 280.

Zaib H, Kanwar R, Zafar N, and Ali S, 2019. Prevalence and multidrug resistance profiles of several bacterial pathogens isolated from hospital inanimate surfaces in Faisalabad, Pakistan. Novel Research in Microbiology Journal 3(6): 526-534.

Zhang S, Gao H, Zhang G, *et al*., 2024. Metavirome analysis of domestic sheep in Shaanxi, Gansu, and Ningxia, China. Frontiers in Veterinary Science 11: 1508617.

Żmudzki J, Piątkowska M, and Jedziniak P, 2012. Residues of veterinary medicinal products and coccidiostats in eggs–causes, control and results of surveillance program in Poland. Polish Journal of Veterinary Sciences, 15(4): 803-812.

Zychska M, Rzewuska M, Kizerwetter-Swida M, *et al*., 2025. Antimicrobial resistance in bacteria isolated from diseased horses in Poland, 2010-2022. Polish Journal of Veterinary Sciences, 28(2): 291-302.