SHORT REPORT

Isolation, identification, and antibiotic resistance analysis of diarrheal pathogens in calves from a cattle farm in Ningxia Hui Autonomous Region

Shenghu Li[1, †], Yanan Guo[2, †, *], Beibei Yan[3], Xiaohao Niu[4], Xicheng Shao[5], Xueyi Wang[5], Shilin Bai[5]

[1]Ecological Farm Training Center, Ningxia Vocational and Technical College, Yinchuan, Ningxia Hui Autonomous Region, China. [2]Institute of Animal Science, Ningxia Academy of Agriculture and Forestry Sciences, Yinchuan, Ningxia Hui Autonomous Region, China. [3]Ningxia Guyuan Agricultural Product Quality and Safety Supervision Center, Guyuan, Ningxia Hui Autonomous Region, China. [4]Pingluo County Agriculture and Rural Bureau, Pingluo, Ningxia Hui Autonomous Region, China. [5]Xiji County Animal Disease Prevention and Control Center, Xiji, Ningxia Hui Autonomous Region, China.

Received: November 6, 2025; **accepted:** February 6, 2026.

Neonatal calf diarrhea is a major disease that leads to morbidity, mortality, and growth retardation in newborn calves. This condition not only increases the culling rate of calves but also significantly raises rearing costs, thereby directly impacting the economic performance of dairy farms. In Yinchuan, Ningxia Hui Autonomous Region, China, the incidence of calf diarrhea remains high, influenced by factors such as climate and husbandry management practices. Among these factors, bacterial infections are a primary cause. These infections often result in acute diarrhea, posing a serious threat to the health of calves. To identify the primary pathogens and their antimicrobial resistance profiles in diarrheic calves from a large-scale farm in Ningxia, this study initially screened 40 fecal samples using the BoviD-5 Ag Test Kit. Positive samples were subsequently cultured on Eosin Methylene Blue Agar and MacConkey Agar for bacterial morphological identification followed by antimicrobial susceptibility testing of the isolated bacteria. Conventional polymerase chain reaction (PCR) was then performed to detect *E. coli* 16S rRNA and coronavirus from the isolates. The results showed that the isolates exhibited typical phenotypic characteristics of *E. coli*. Molecular verification successfully identified both *E. coli* and coronavirus isolates. Phylogenetic analysis revealed the highest homology with *E. coli* CP119739.1 and coronavirus BCoV isolate BCo/YNLP/2023 (Yunnan). Antimicrobial susceptibility test demonstrated that all 25 *E. coli* strains exhibited multidrug resistance, showing the highest resistance rate to streptomycin (52.00%, 13/25) followed by ampicillin (40.00%, 10/25) with most susceptible strains displaying intermediate sensitivity or lower. The isolates showed susceptibility rates of 80.00% (20/25) to florfenicol, gentamycin, and norfloxacin; 72.00% (18/25) to sulfaguanidine; 68.00% (17/25) to cefotaxime; and 64.00% (16/25) to both tetracycline and vancomycin. The combination of bacteriological morphology and molecular biology demonstrated that the diarrhea in this calf herd was caused by a co-infection of *E. coli* and coronavirus with all 25 isolated *E. coli* strains exhibiting multidrug resistance. The identification and analysis results of this case report revealed the cause of calf diarrhea for the farm and provided a basis for selecting appropriate and effective drugs to treat diarrhea diseases. Meanwhile, it also provided a basis for the protection of calf diarrhea in the cattle breeding industry in the region.

Keywords: calf diarrhea; pathogen detection; *Escherichia coli*; drug resistance; coronavirus.

[*]**Corresponding author:** Yanan Guo, Institute of Animal Science, Ningxia Academy of Agriculture and Forestry Sciences, Yinchuan, Ningxia Hui Autonomous Region 750002, China. Email: gyn330@126.com.

[†]These authors contributed equally to this work.

Introduction

Calf diarrhea is one of the important diseases leading to morbidity, mortality, and growth retardation of newborn calves. Due to its high morbidity and mortality, it has become a major disease factor restricting the economic benefits of the cattle industry and poses a major threat to the sustainable development of animal husbandry [1]. The disease is most common in calves around 2 weeks of age and can occur throughout the year. In clinical practice, sick calves often have increased mortality due to untimely diagnosis and treatment. Even if the calf survives, improper treatment will seriously affect its later growth and development. The clinical manifestations of calf diarrhea are mainly loose or paste stool, watery diarrhea, and dehydration. Studies have shown that infection factors are the key causes of calf diarrhea. The main pathogens include *Escherichia coli*, *Salmonella*, coronavirus, and rotavirus [2]. In addition, *E. coli* K99 is one of the most important pathogens that cause diarrhea in calves. It is a serotype of *E. coli*. The mortality of calf diarrhea caused by *E. coli* can sometimes reach 40 - 50% [3]. Currently, antibiotic treatment is commonly used in clinical practice for calf diarrhea caused by *Escherichia coli*. However, for viral diarrhea, antibacterial therapy is ineffective, and viral diarrhea often leads to acute death of calves. On the other hand, the current abuse of antibiotics is serious. In the treatment of diarrhea, the extensive use of antibiotics can easily lead to pathogen resistance, thereby reducing or making the treatment ineffective. In addition, there is currently no effective treatment for viral diarrhea. The purpose of this study was to investigate the causes of diarrhea in a large number of calves in the cattle farm to provide antibiotic resistance data and a basis for the selection of appropriate therapeutic drugs for the cattle farm. It also provided a reference for the prevention and control of calf diarrhea in animal husbandry in the region.

Materials and methods

Sample collection

A total of 40 anal swab samples from diarrhea milk and meat calves were collected from large-scale breeding farms around Yinchuan, Ningxia Hui Autonomous Region, China and transported to the Clinical Veterinary Laboratory of the Academy of Sciences (Yinchuan, Ningxia, China) for processing by using BoviD-5 Ag Test Kit (Haibo Biotechnology Co., Ltd., Qingdao, Shandong, China) following manufacturer's instructions to obtain positive samples.

Bacterial isolation

The initial screened positive samples were cultured simultaneously with Eosin methylene blue agar and McKangkai agar (Haibo Biotechnology Co., Ltd., Qingdao, Shandong, China) in a BSD-YX3200 constant temperature incubator (Shanghai Boxun Company, Shanghai, China) at 37°C for 18 to 24 hours. Typical single colonies were picked for further purification on fresh selective media using the streak plate method. The plates were incubated at 37°C until single colonies were isolated. The purified isolate was then transferred to 5 mL of LB broth and incubated at 37℃, 180 rpm, for 18 - 24 h.

Polymerase chain reaction (PCR) amplification

2 mL of strain culture with the concentration greater than 1×10^8 CFU/mL was centrifuged at 12,000 rpm for 2 minutes to obtain a strain precipitate. The genomic DNA of the isolate was extracted using TIANGENTIANamp Bacterial DNA Extraction Kit (Beijing Tiangen Company, Beijing, China) following the manufacturer's instructions. The PCR primers for *E. coli* were designed according to the report of Ok *et al*. with the sequences of K99-F as 5'-TAT TCT TAG GTA TGG-3' and K99-R as 5'-GGT ATC CTT TAG CAG CAG TAT TTC-3') and were synthesized by Shanghai Shenggong Bioengineering Co., Ltd. (Shanghai, China). The PCR reaction included 2 µL of template DNA, 1 µL of each primer, 25 µL of 2X Taq PCR mixture (Beijing Jumei Biotechnology Co., Ltd., Beijing, China), and 21 µL of ddH_2O. PCR amplification was performed at 95℃ for 5 min followed by 25 cycles of 95℃ for 30 s, 50℃ for 45 s, 70℃ for 90 s before 72℃ for 10 mins using BIO-

RAD S1000 thermal cycler (Bio-Rad, Hercules, CA, USA). The PCR products were analyzed on 1.2% agarose gel and then purified by using OMEGA D2500-02 Gel DNA Extraction Kit (Omega, Feiyang, Guangzhou, Guangdong, China). The purified PCR products were sent to Shenggong Bioengineering Co., Ltd (Shanghai, China) for sequencing. Viral RNA was extracted from fecal samples using Total RNA extraction kit (Tiangen Biochemical Co., Ltd., Beijing, China). The RNA was then reverse transcribed into cDNA using HiScript II Reverse Transcriptase kit (Nanjing Novozymes Bioscience Co., Ltd., Nanjing, Jiangsu, China). PCR primers for bovine coronavirus detection were designed according to the report of He *et al.* with the sequences of BCoV-F as 5'-CGA GTT GAA CAC CCA GAT-3' and BCoV-R as 5'-GAG ACG GGC ATC CAC T-3') [5]. The PCR reaction mixture contained 1.5 µL of template cDNA, 1 µL of each primer, 12.5 µL of 2X Taq PCR mixture, and 9 µL of ddH_2O. PCR amplification was performed at 94℃ for 5 min followed by 35 cycles of 94℃ for 30 s, 49℃ for 30 s, 72℃ for 30 s before 72℃ for 10 mins. The PCR products were analyzed on 1.2% agarose gel and then purified using OMEGA D2500-02 Gel DNA Extraction Kit before sequencing by Shenggong Bioengineering Co., Ltd (Shanghai, China).

Phylogenetic analysis

The sequencing results were analyzed by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against GenBank database. The homologous species with high similarity were searched by using the neighbor-joining (NJ) method in the MEGA 11.0 software (DNAStar, Madison, Wisconsin, USA). The data were then uploaded through the Interactive Tree of Life (ITOL) (https://itol.embl.de/) to make a gene tree.

Preparation of drug-sensitive tablets

The circular filter paper with a diameter of 6.0 mm was sterilized in a high-pressure sterilizer for 15 min. The corresponding concentrations of testing drugs were prepared according to the criteria of the "2010 Antimicrobial Susceptibility Test Implementation Standard". 1 mL of each testing drug was added to each piece of sterilized circular filter paper with a total of 100 filter papers being prepared. The filter paper was placed at 4℃ for 1 h to ensure fully absorption of the drug liquid before transferred to the sterile culture dish for drying at 37°C for 12 - 24 h. The dried self-made drug sensitive tablets were refrigerated and stored in sterilized penicillin bottles for short-term use.

Drug resistance detection

100 µL of *E. coli* K99 positive bacteria isolated from 40 fecal samples of this study and enriched in nutrient broth were evenly coated on McConkey agar medium [4]. The in-house laboratory-made drug susceptibility tablets including streptomycin, ampicillin, tetracycline, entamycin, sulfamonomethoxine, florfenicol, norfloxacin, cefotaxime, and vancomycin were pasted on the bacterial coated agar medium and inverted cultured at 37°C for 24 - 48 h following the instructions of American Committee for Clinical Laboratory Standards (NCCLS) Drug Susceptibility Disk Diffusion Method.

Results

Sample testing results

A total of 25 *E. coli* positive and 5 coronavirus positive samples were identified through all 40 samples, while rotavirus, *Cryptosporidium*, and *Giardia* were not detected. The calves infected with *E. coli* exhibited diarrhea symptoms for about a week with feces that were yellowish-gray, yellow, or off-white, thin and viscous. They also had rough and disheveled hair, poor appetite, abdominal contractions, and lethargy. Calves infected with coronavirus developed watery diarrhea at approximately 4 days of age with yellow-white paste watery stool and gastrointestinal mucosa shedding. Cattle with severe cases of the disease might experience urinary and fecal incontinence and loss of control over their bodily functions. The sick calves were lethargic, drowsy, and fatigued with a poor appetite. The gastric mucosa of the deceased calves showed atrophy and shedding with large areas of patchy bleeding.

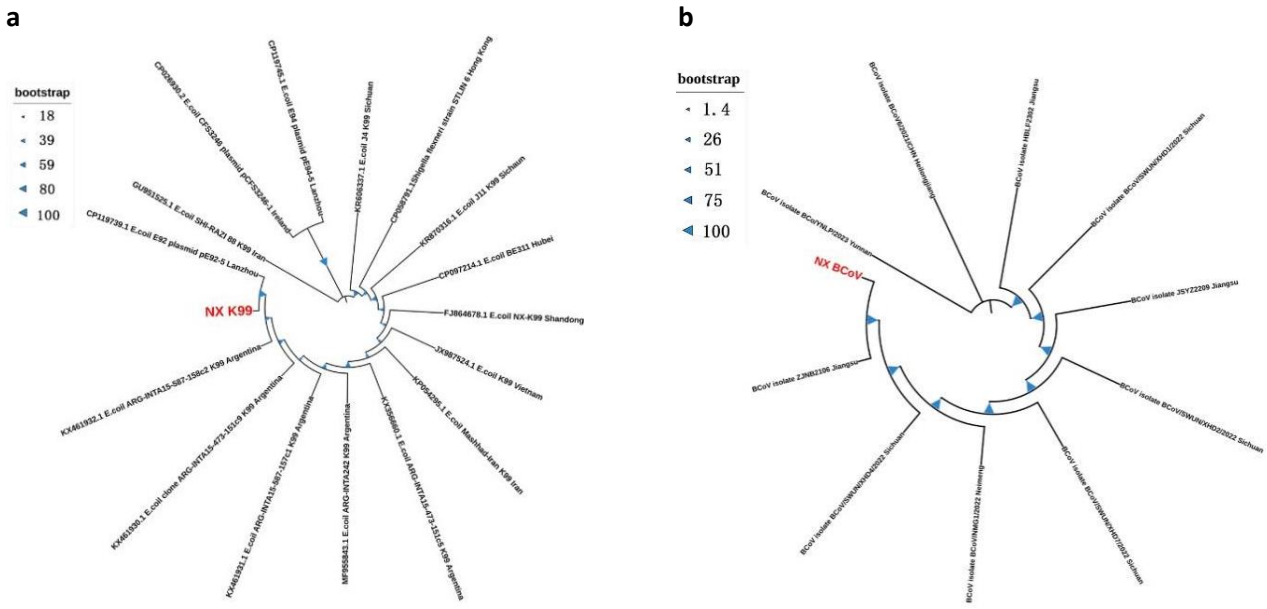

Figure 1. Phylogenetic trees of *E. coli* K99 (a) and bovine coronavirus (b).

Observation of colony morphology

The purple-black metallic luster colonies and red circular colonies were observed on Eosin methylene blue agar and MacConkey agar medium, respectively. The microscopic examination showed Gram-negative short bacilli. Combined with morphological, culture, staining characteristics, the isolated bacterial colony was identified as *E. coli*.

Phylogenetic analysis

The result showed that the isolated *E. coli* K99 demonstrated high homology to CP119739.1 *E. coli* E92 plasmid pE92-5 Lanzhou strain with the bootstrap value of 94, while the bovine coronavirus gene detected in this study had high homology to BCoV isolate BCo/YNLP/2023 Yunnan, which belonged to the same branch as the gene carried by BCoV isolate ZJNB2106 Jiangsu strain with a self-expansion value of 100 (Figure 1).

Drug sensitivity test

The results of drug sensitivity test showed that the highest resistance rates of 25 strains of *E. coli* to *streptomycin* and *ampicillin* were 52.00% (13/25) and 40.00% (10/25), respectively. The sensitivity rates of 25 strains to *florfenicol, gentamicin, norfloxacin, sulfaguanidine, cefotaxime, tetracycline*, and *vancomycin* were 80.00% (20/25), 80.00% (20/25), 80.00% (20/25), 72.00% (18/25), 68.00% (17/25), 64.00% (16/25), and 64.00% (16/25), respectively (Table 1). The results showed that the isolated strains had multiple drug resistance.

Discussion

The immune function of newborn calves is not perfect and is susceptible to factors such as environment, feeding management, and stress [7]. The causes of calf diarrhea are complex and diverse. The disease is widespread in the world, causing huge economic losses to the cattle industry. Currently, the clinical treatment of calf *E. coli* diarrhea mainly depends on antibiotics, but its long-term broad use has exacerbated the problem of bacterial resistance [8]. Drug resistance not only seriously affects efficacy but also threatens the safety of animal food [9]. However, the occurrence of viral diarrhea in calves is often due to the lack of reliable antibiotics and other drugs. In clinical practice,

Table 1. Drug sensitivity test of isolated strains.

Medicines vocabularies	Drug sensitivity results statistics			
	Sensitive	Moderately sensitive	less sensitive	Resistance
Streptomycin	0	2	10	13
Ampicillin	2	2	11	10
Tetracycline	16	4	3	2
Gentamycin	20	3	1	1
Sulfaguanidine	18	3	2	2
Florfenicol	20	3	1	1
Norfloxacin	20	4	1	0
Cefotaxime	17	4	2	2
Vancomycin	16	6	2	1

symptomatic treatment is mainly used for calf diarrhea, but the efficacy is poor, resulting in a high mortality rate among calves due to diarrhea. This poses a continuous and serious threat to the livestock industry and hinders its high-quality development.

This study identified 5 coronavirus positive and 25 *E. coli* positive samples from 40 calf diarrhea samples, indicating that *E. coli* was the main pathogen causing diarrhea in calves in the area. The results of drug sensitivity test showed that most strains had multiple drug resistance. The isolated strains were sensitive to *gentamycin*, *florfenicol*, *norfloxacin*, tetracycline, *cefotaxime*, and *vancomycin*, which suggested that sensitive drugs could be selected for prevention and treatment. The results also demonstrated that the closest genetic relationship in the phylogenetic tree of isolated *E. coli* K99 was the bacteria of Lanzhou, which indicated that the bacteria with similar geographical location might be closer in evolution. The main branches of the evolutionary tree of bovine coronavirus (BCoV) composed of native virus genes in China, suggesting that the virus had not yet formed the molecular epidemiological characteristics of cross-border transmission. This phenomenon might be related to the biosafety quarantine measures implemented in international trade that effectively blocked the spread of pathogens and also was limited by the inherent geographical distribution limitations of BCoV natural hosts (mainly cloven-hoofed animals). Studies have shown that the spread of BCoV has significant regional aggregation and seasonal fluctuations. The scientific prevention and control of BCoV should be based on regional and seasonal characteristics [10].

Due to the limitation of production conditions, farms generally do not apply vaccinations to against *E. coli*, diarrhea mucosal disease virus, coronavirus, and other diseases. Meanwhile, within the enclosure, problems also existed such as inadequate cleaning of feces and timely replacement of bedding, insufficient early detection of sick calves, and inappropriate use of medication. It is suggested that the farms should strengthen the management of breeding, clean the feces, replace the litter in time, and disinfect the enclosure regularly. Newborn calves should be vaccinated promptly, and a strict immunization schedule should be established. The calves' condition should be monitored regularly, and if any abnormalities are observed, testing and diagnosis should be carried out as soon as possible. If a calf is experiencing diarrhea, the pathogen causing the diarrhea should be identified promptly, and appropriate treatment measures should be taken. Depending on the severity of the calf's diarrhea, lactase tablets can be used to regulate the intestinal flora and eliminate harmful pathogens in the gastrointestinal tract. For calves suffering from dehydration due to diarrhea, gentamicin and norfloxacin should be used in the later stages, and fluids should be replenished promptly.

Acknowledgements

This work was supported by Ningxia Hui Autonomous Region Agricultural Science and Technology Independent Innovation Fund Project (Grant No. NKYG-25-03), Xiji County Science and Technology R&D Plan Project (Grant No. XKYF2025-003), Ningxia Hui Autonomous Region Innovation Team Project (Grant No. 2024CXTD009), Ningxia Hui Autonomous Region Science and Technology Infrastructure Construction Project (Grant No. 2025DPC05017).

References

1. Xu GD, Cao R, Luo W, Cao JP. 2024. Analysis and summary on three kinds pathogens investigation of calf diarrheic in China. Sci Technol Tianjin Agric For. 2024(1):41-46.
2. Liu L, Yang D. 2014. Classification, clinical features and control strategies of calf infectious diarrhea. China Dairy Cattle. 2014(14):53-56.
3. Sun Y, Wang Q, Mao W, Wang B, Dong HY, Fan HL, *et al*. 2023. Drug resistance analysis and virulence gene detection of *Escherichia coli* causing calf diarrhea in Inner Mongolia. China Anim Husb Vet Med. 50(9):3811-3822.
4. Ok M, Güler L, Turgut K, Ok U, Sen I, Gündüz IK, *et al*. 2009. The studies on the etiology of diarrhea in neonatal calves and determination of virulence gene markers of *Escherichia coli* strains by multiplex PCR. Zoonoses Public Health. 56(2):94-101.
5. He QF, Guo ZJ, Li R, Zhou J, Yue H, Zhang B, *et al*. 2018. Establishment and application of a RT-PCR assay for detecting bovine coronavirus. Acta Vet Zootech Sin. 49(10):2292-2298.
6. Cao F, Wei Y, Wu Y, Zhao PF, Chen ZM, Ren JJ, *et al*. 2024. Isolation, identification and drug resistance detection of *Escherichia coli* from calves diarrhea in a large-scale dairy farm. China Dairy. 2024(2):71-75.
7. Wu J, Liu HQ, Bai T, Wang F, Yang CH, Feng MD, *et al*. 2022. Investigation on main pathogens of calf diarrhea in some areas of Xinjiang Uygur Autonomous Region and analysis of drug resistance of the isolates. Heilongjiang Anim Sci Vet Med. 2022(20):86-92, 143.
8. Wu RB, Gao YM, Wang PX, Yang Z, Zhang JY, Wang HS. 2015. Detection of antibiotic resistance and distribution of resistance determinants in *Escherichia coli* isolated from beef. China Anim Husb Vet Med. 42(2):452-458.
9. Liang YY, Li K, Jia L, Hou MY, Tian MY, Zhang T, *et al*. 2022. Pathogenic analysis and treatment of diarrhea of calves caused by pathogenic *Escherichia coli*. J Vet Med China. 42(3):477-482, 495.
10. Wang MJ, Jiang Q, Ma XJ, Xia RY, Guo XP, Sun L, *et al*. 2023. Molecular epidemiological investigation of bovine coronavirus in calf diarrhea in main cattle producing areas of Xinjiang. J Anim Husb Vet Med. 54(12):5125-5133.