SHORT REPORT

The first case of mixed infection of *Clostridium perfringens* and *Escherichia coli* in a calf in Ningxia Hui Autonomous Region of China: A case report

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Received: December 31, 2024; **accepted:** April 8, 2025.

Bovine diarrhea is a prevalent yet serious disease in the livestock industry, significantly impacting cattle growth, development, productivity, and overall health. Among these, bacterial infections, particularly those caused by *Clostridium perfringens* and *Escherichia coli*, play a central role in the development of bovine diarrhea. In October 2023, a cluster of diarrheal outbreaks was identified among 20-day-old neonatal calves at a large-scale beef cattle operation in Ningxia, China. Affected calves (n = 30/30, 100% morbidity) presented with fetid diarrhea, pyrexia, and lethargy, culminating in 20% case fatality (n = 6/30). Tissue samples were collected from two dead calves. Bacterial morphology was first identified using screening media. *Clostridium perfringens* was then typed and identified by TaqMan fluorescence quantitative polymerase chain reaction (PCR), and *E. coli* 16S rRNA was identified by conventional PCR and analyzed for amplified sequences. Pathological sections were made after fixing the diseased tissues and organs to observe the pathological changes in the target organs. Specific colonies were formed in both *Clostridium perfringens* chromogenic medium and erythromycin blue medium. Through molecular biological validation, nine distinct isolates of both *Clostridium perfringens* type A and *Escherichia coli* were successfully identified and characterized. The constructed phylogenetic tree showed the highest support with *E. coli* strains logged in NCBI. Pathological and histological observations showed epicardial inflammatory cell infiltration in cardiac tissue, punctate necrosis of hepatocytes in liver tissue, decreased white medullary lymphocytes and red medullary sludge in splenic tissue, thickening of renal microsomal basement membranes, tubular necrosis, fibrous tissue hyperplasia and inflammatory cell infiltration in renal tissue, spermatogenic tubules atrophy, interstitial vasodilatation congestion, hemorrhage, and inflammatory cell infiltration in testicular tissue. The combination of bacterial morphology and molecular biology results indicated that the diarrhea in this calf herd was caused by a mixed infection of *Clostridium perfringens* type A and *Escherichia coli*, which was the first time that such an infection was reported in Ningxia, China.

Keywords: calves; *Clostridium perfringens*; *Escherichia coli*; isolation; identification; pathological histology.

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Introduction

Bovine diarrhea is a prevalent yet serious disease in the livestock industry, significantly impacting cattle growth, development, productivity, and overall health [1]. This condition not only leads to substantial economic losses but also presents considerable challenges to the sustainable and healthy development of cattle farming [2]. Symptoms typically include loss of appetite, watery or bloody diarrhea, weight loss, and a decline in various production parameters with

severe cases potentially resulting in death [3]. Research indicates that neonatal calves, particularly those between 1 and 4 weeks of age, are at the highest risk. 39% of calf deaths were caused by diarrhea in calves up to 3 weeks of age [4]. The causes of calf diarrhea are multifactorial, involving both infectious and environmental factors [5]. The primary infectious agents include bovine rotavirus, bovine viral diarrhea-mucosal disease virus, bovine Norwalk viruses, bovine coronavirus, *Escherichia coli*, *Clostridium perfringens*, *Salmonella*, *Cryptosporidium parvum*, and *coccidia* [3]. Non-infectious factors primarily include feeding conditions, management practices, water quality, feed, and stress [6]. Among these, bacterial infections, particularly those caused by *Clostridium perfringens* and *Escherichia coli*, play a central role in the development of bovine diarrhea [7].

Clostridium perfringens, an anaerobic, Gram-positive bacterium and opportunistic pathogen, is commonly found in soil, water, and the intestinal tracts of animals. When the intestinal environment in calves is disrupted or subjected to abrupt changes, *Clostridium perfringens* can proliferate rapidly in the gut, producing various toxins that cause gastrointestinal lesions, leading to diarrhea and other clinical symptoms [8]. Typical symptoms include watery stools, sometimes with blood, anorexia, dehydration, fever, and, in severe cases, death [9]. The pathogenicity and epidemiology of *Clostridium perfringens* vary according to the type of toxin it produces. Type A *Clostridium perfringens* is the most associated with calf infections [10]. The bacterium typically enters the animal through contaminated food, water, soil, or feces. Once in the gut, the produced toxins directly damage the gastrointestinal tract, disrupting normal digestion and absorption, which leads to diarrhea. Additionally, these toxins can enter the bloodstream, spreading throughout the body and severely impairing the calf's growth and health [11]. Pathogenic *Escherichia coli* strains primarily produce enterotoxins including enterotoxins, verotoxins, and cytotoxins [12]. This disease significantly affects young animals. Upon invading the intestinal tract, the pathogens adhere to the gut using fimbriae and then secrete toxins that disrupt electrolyte balance in intestinal epithelial cells and damage the intestinal mucosa. These disruptions lead to clinical symptoms such as diarrhea and toxin-induced enteric infections. The disease is characterized by high outbreak potential and mortality rates, posing a serious threat to the healthy development of the livestock industry [13].

As one of the six key industries in the Ningxia Hui Autonomous Region, China, the beef cattle industry plays a crucial role in food safety, livestock development, and increasing the incomes of farmers and herders. However, calf diarrhea remains a significant obstacle to the growth of the cattle farming industry with *Clostridium perfringens* and *Escherichia coli* being common pathogens responsible for diarrhea in calves on farms. No documented cases of *Clostridium perfringens* - *Escherichia coli* coinfection in neonatal calves have been reported in Ningxia Hui Autonomous Region, China. This study combined bacteriological isolation, molecular characterization using polymerase chain reaction (PCR) - based toxin gene detection, and histopathological analysis to investigate the epidemiological patterns and pathogenic mechanisms underlying this dual infection in diarrheic calves. The findings would establish the first regional baseline data and inform evidence-based control strategies for bovine neonatal diarrhea.

Materials and methods

Sample collection

In October 2023, a cluster of diarrheal outbreaks was identified among 20-day-old neonatal calves at Wuzhong Agro-Pastoral Development Co., Ltd., a large-scale beef cattle operation in Ningxia, China. Affected calves (n = 30/30, 100% morbidity) presented with fetid diarrhea, pyrexia, and lethargy, culminating in 20% case fatality (n = 6/30). To identify the causative

pathogens, the farm's veterinarian conducted post-mortem examinations on the deceased calves. The findings included pericardial effusion with fibrinous material, prominent necrotic lesions in the spleen, congestion and enlargement of the liver, kidneys, and mesenteric lymph nodes, as well as intestinal hemorrhaging. Sterile tissue samples were collected from the heart, liver, spleen, kidneys, mesenteric lymph nodes, large intestine, and small intestine of two deceased calves. The samples were collected in sealed sterile containers, and the age, site and other information of the samples were recorded and transported in sampling boxes to the Clinical Veterinary Laboratory at Ningxia Academy of Agriculture and Forestry Sciences (Yinchuan, Ningxia, China) for processing, histopathologic diagnosis, and pathological histological observation.

Bacterial isolation and culture

The surfaces of the organs, lymph nodes, and intestinal tissues from the two deceased calves were sterilized using surface flame. The deep tissues of approximately 5 mm × 5 mm in size were cut and inoculated on *Clostridium perfringens* identification medium (CHROMagar, Paris, France) in an anaerobic bag (Haibo, Qingdao, Shandong, China) and Eosin Methylene Blue (EMB) agar (Haibo, Qingdao, Shandong, China) under aseptic conditions with 2 pieces of each sample in each medium, respectively. The samples were cultured in BSD-YX3200 constant temperature incubator (BoXun, Shanghai, China) at 37°C for 18 - 24 hours before observation of colony growth. 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 isolates were then transferred to 5 mL of Brain-Heart Infusion (BHI) liquid medium with 17.0 g/L tryptone, 3.0 g/L soy peptone, 5 g/L sodium chloride, 2.5 g/L K_2HPO_4, and 2.5 g/L glucose for further cultivation. For *Clostridium perfringens*, the isolates were transferred to BHI medium covered with liquid paraffin.

DNA extraction and PCR

2 mL of strain culture (> 1×10^8 Colony Forming Unit (CFU)/mL) were centrifuged by using Eppendorf 5418R centrifuge (Eppendorf, Hamburg, German) at 12,000 rpm for 2 mins to obtain the precipitation of the strain. Isolates genomic DNA were extracted using TIANGEN TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China) following manufacturer's instructions. The 16S rRNA universal primers and *Clostridium perfringens* toxin typing primers and probes for *cpa*, *cpb*, *etx*, *itx*, and *cpe* toxin genes of *Clostridium perfringens* were synthesized based on our previous research [14, 15] (Table 1). The genes were amplificated by using BIO-RAD S1000 thermal cycler (Bio-Rad, Hercules, California, USA) and Sangon PCR kit (Sangon, Shanghai, China). The PCR reaction mixture was 50 µL with 2 µL of template DNA, 1 µL each of forward and reverse primers, 25 µL of 2×Taq PCR Mix, 21 µL of ddH_2O. The reaction was performed at 95℃ for 5 mins followed by 30 cycles of 94℃ for 50 s, 57℃ for 1 min, and 72℃ for 1.5 mins, then a final extension at 72℃ for 10 mins. The PCR products were electrophoresis analyzed on 1.2% agarose gel and then were purified by using OMEGA.D2500-02 Gel DNA Extraction Kit (Omega, Feiyang, Guangzhou, Guangdong, China) following manufacturer's instructions. The purified PCR products were sent to Shenggong Bioengineering Co., Ltd (Shanghai, China) for sequencing.

Phylogenetic analysis

The sequencing results of the isolates were compared with the standard strains of *E. coli* using BLAST against GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The homologous species with high similarity were searched and compared with the sequences of *E. coli* and some strains with similar sequences using the neighbor-joining (NJ) method in the MEGA 11.0 software (DNAStar, Madison, Wisconsin, USA). Bootstrap confidence values (1,000 replications) were given at the respective nodes.

Table 1. Primer sequence information.

Primer name	Primer sequence	Product length (bp)
16S rRNA	27F: 5′ - AGAGTTTGATCCTGGCTCAG- 3′ 1492R: 5′ - GGTTACCTTGTTACGACTT - 3′	1,465
cpa	F: 5′-ACTCCATATCATCCTGCTAATG-3′ R: 5′- GCAACCTGCTGTGTTTATTT-3′ P: FAM-5′- TTACTGCCGTTGATAGCGCAGGAC -3′-BHQ1	111
cpb	F: 5′- ACAACCCTGTATATGGAAATGA -3′ R: 5′- GGAGCAGTTAGAACTACAGAC -3′ P: HEX-5′- ACGGAAGATATACTAATGTTCCTGCAACTG -3′-BHQ1	145
etx	F: 5′- GCTCTCTTAACTAATGATACTCAAC -3′ R: 5′- GTAAACTTAGCAGTTGCTTGTA-3′ P: Cy5.5-5′- ACTGCAACTACTACTCATACTGTGGGAACT -3′-BHQ3	134
itx	F: 5′- TCCTAATGAACTTGCTGATGTA-3′ R: 5′- GAGTCTAGTTCTGGATTAGGATTAT -3′ P: Texas Red-5′- TGACTATATGCGTGGAGGATATACCGCA-3′-BHQ2	111
cpe	F: 5′- AGCTGCTGCTACAGAAAGATTA-3′ R: 5′- GAGTCCAAGGGTATGAGTTAGAAG-3′ P: Cy5-5′- CTGATGCATTAAACTCAAATCCAGCTGGT -3′-BHQ3	101

Histological observation

The collected tissue samples were immersed in 10% neutral formaldehyde solution fixed for 7 days before dehydration by 75% ethanol for 4 h, 85% ethanol for 2 h, 95% ethanol for 1 h, 4 times of 100% ethanol for 0.5 h, twice xylene for 10 mins, paraffin wax for 1 h, paraffin wax for 2 h, paraffin wax for 3 h in JT-12S automatic dehydrator (Junjie, Wuhan, Hubei, China). The BMJ-A embedding machine (Zhongwei, Changzhou, Jiangsu, China) was used to embed tissues. Leica-2016 rotary microtome (Leica Microsystems, Wetzlar, Germany) was employed for sectioning, and RS36 automatic staining machine (Paisijie, Changzhou, Jiangsu, China) was used for staining of tissues section following the procedures of dewaxing, hematoxylin staining for 10 - 20 mins, rinsing with water for 1 - 3 mins, hydrochloric acid alcohol differentiation for 5 - 10 s, rinsing with water for 1 - 3min, putting into 50℃ warm water or weakly alkaline aqueous solution until blue appears, rinsing with water for 1 - 3 mins, adding 85% ethanol for 3 - 5 mins, eosin staining for 3 - 5 mins, washing with water for 3 - 5 s, gradient ethanol dehydration, xylene transparent, and neutral gum sealing. A digital slice scanner (3DHISTECH, Budapest, Hungary) was used to collect images of the slices.

Results

Colony morphology

After incubation at 37°C in an inverted position, purple-black colonies with a metallic sheen were observed on EMB agar (Figure 1A). On *Clostridium perfringens* chromogenic agar, orange-red single colonies were formed (Figure 1B).

PCR and phylogenetic analysis

A total of 9 bacterial isolates that were obtained on EMB agar and named as NXBZ01 - NXBZ09 in sequence were analyzed for their 16S rRNA amplification products. The results found that the sequence size of the 16S rRNA amplified fragments was about 1,500 bp, which was in line with the expected size. Homology comparison of the sequencing results showed that strains NXBZ01 to NXBZ09 exhibited the highest similarity to the standard *Escherichia coli* strains G (GenBank ID: MK765005.1), BF21-13R (GenBank ID: MZ962379.1), JCM24009 (GenBank

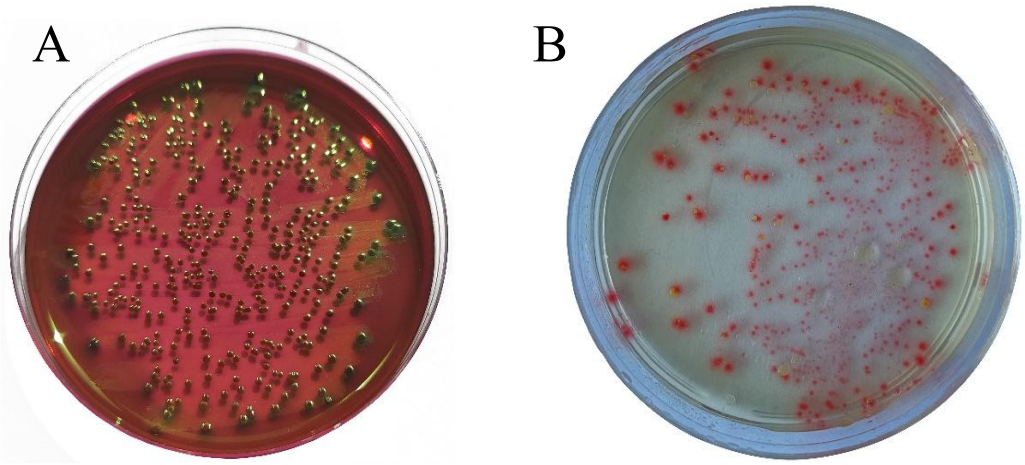

Figure 1. The Morphology of pathogenic isolates on identification media. **A.** Erythromycin blue medium. **B.** *Clostridium perfringens* chromogenic medium.

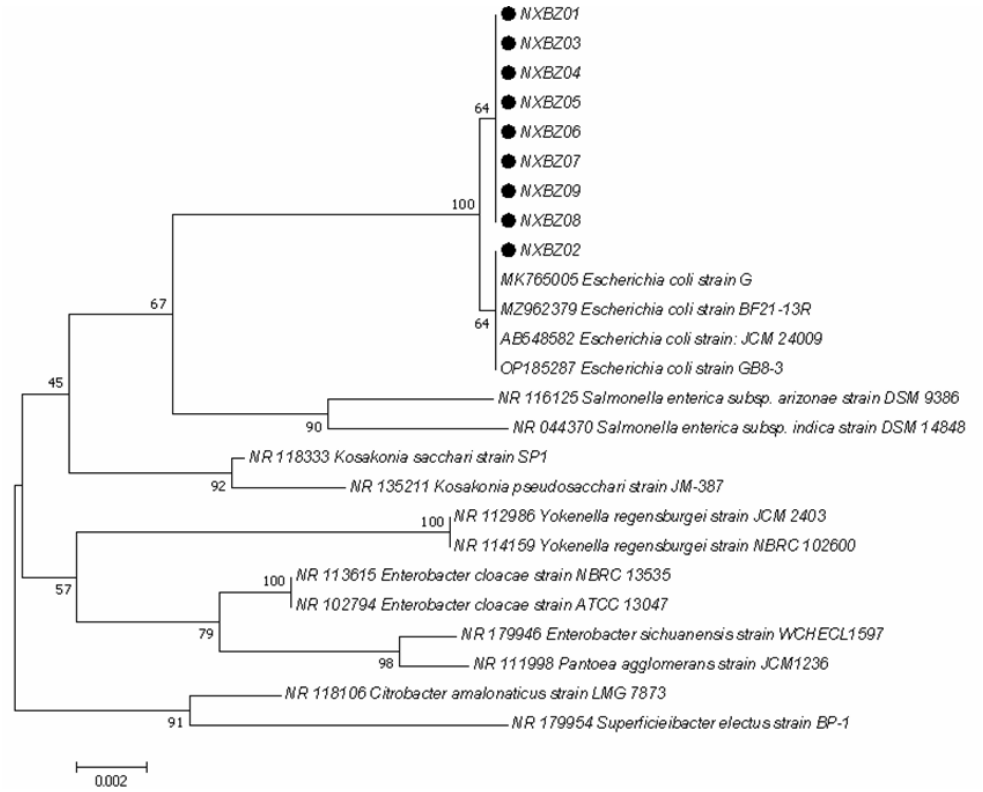

Figure 2. Phylogenetic tree of NXBZ01~NXBZ09 strain based on the gene sequence of 16S rRNA.

ID: AB548582.1), and GB8-3 (GenBank ID: OP185287.1) in the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) database. The phylogenetic tree constructed from these sequences demonstrated that the genetic distance between the NXBZ strains and the reference strains (G, BF21-13R, JCM24009, and GB8-3) were minimal (Figure 2).

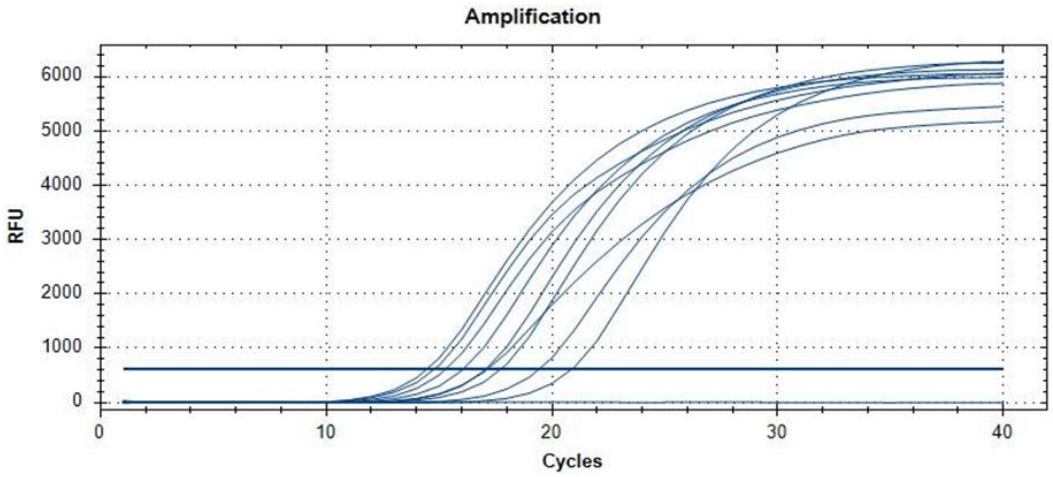

Figure 3. *Clostridium perfringens* toxin gene amplification curve.

Toxin gene detection and typing of *Clostridium perfringens*

A total of 9 strains of *Clostridium perfringens* isolates were obtained from the identification medium and were tested for toxin by fluorescence quantitative PCR with the assigned names of NXBC01 - NXBC09. The results revealed that only the *cpa* toxin gene produced a specific amplification curve, while the other four toxin genes showed no amplification (Figure 3). Based on the *Clostridium perfringens* toxin gene typing method, all nine isolates were identified as Type A.

Histological observation

Examination of the affected tissues revealed that the inflammatory cell infiltration was observed in the epicardium, predominantly composed of round, deeply stained lymphocytes and rod-shaped neutrophils in heart tissue (Figure 4A). Focal hepatocyte necrosis was noted and characterized by condensed and dissolved nuclei in liver tissue. A small number of inflammatory cells, mainly lymphocytes, were present along with extensive hepatocyte dissolution, while liver sinusoids showed congestion (Figure 4B). Cell dissolution was evident in the spleen with a decrease in lymphocytes in the white pulp and congestion in the red pulp (Figure 4C). In lung tissue, alveolar epithelial cells in the respiratory region showed dissolution with congestion and hemorrhaging in the interstitial blood vessels (Figure 4D). The kidney tissues demonstrated extensive cell dissolution in both the glomeruli and renal tubules, while the glomerular basement membrane was slightly thickened. Renal tubular necrosis, structural loss, and partial regeneration of renal tubular epithelial cells were present. Necrotic areas exhibited a large number of inflammatory cells including increased fibroblasts with elongated, oval-shaped nuclei and fibrous cells with spindle-shaped nuclei. Lymphocytes were the predominant inflammatory cells (Figure 4E). Significant atrophy of the seminiferous tubules was observed in testes with a narrowed lumen, widened interstitial space, thinning of the spermatogenic epithelium, and a reduction in spermatogenic cells. Vascular dilation and congestion in the interstitial along with hemorrhaging in the tunica albuginea and local interstitial areas were noted. Red blood cell extravasation and infiltration of inflammatory cells, mainly lymphocytes, were observed (Figure 4F). Mesenteric lymph nodes showed lymphocyte dissolution with a loose arrangement of lymphocytes (Figure 4G). The structural layers of the colon were unclear, and cell nuclei showed signs of dissolution (Figure 4H). Small intestine showed that the villous epithelial cells exhibited dissolution with loss of epithelial cells (Figure 4I). These histopathological changes highlighted the

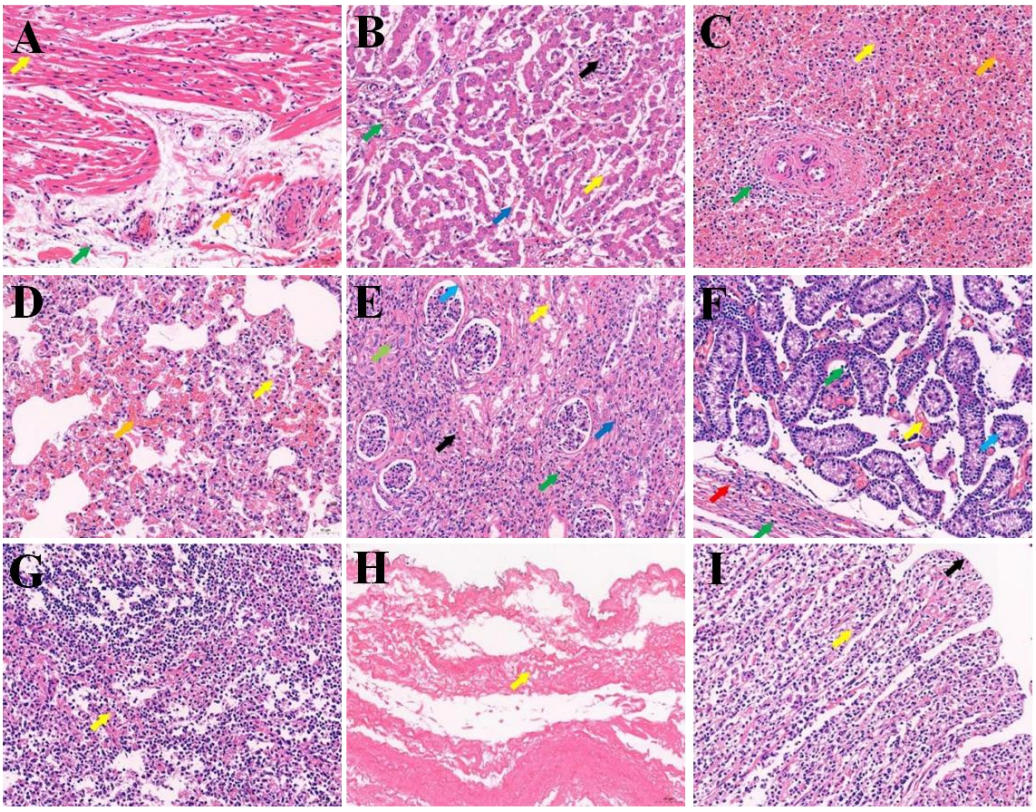

Figure 4. Pathological histological sections of diseased tissues (H.E. staining. 200×). **A.** Heart. **B.** Liver. **C.** Spleen. **D.** Lungs. **E.** Kidneys. **F.** Testes. **G.** Mesenteric lymph nodes. **H.** Large intestine. **I.** Small intestine.

extensive tissue damage associated with the infection, affecting multiple organs and systems.

Discussion

The cattle farming industry is highly vulnerable to various factors including human activity, environmental conditions, and geographical location due to its expansive nature. This complexity increases the challenges associated with pathogenic factors. Both *Clostridium perfringens* and *Escherichia coli* are common opportunistic pathogens, typically found in the intestines of healthy cattle as well as in environments such as livestock housing and the surrounding air. Under normal conditions, they do not cause disease [16]. However, sudden changes in the cattle's growing environment such as fluctuations in temperature, air quality, feed, or water can induce stress in calves, leading to a decline in immune function, which can upset the balance of the normal gut microbiota and break the ecological equilibrium [17]. Under these conditions, *Clostridium perfringens* and *Escherichia coli*, which are already present in the calves' intestines, can proliferate rapidly and produce toxins [18]. These toxins damage the capillary endothelial cells, increasing capillary permeability in intestine and widening the intercellular spaces. As a result, toxins can breach the intestinal barrier and enter the bloodstream, leading to septicemia. This systemic infection can affect vital organs such as the heart, liver, and kidneys, potentially resulting in organ failure and even death [19]. Compared to infections caused by a single pathogen, this mixed infection tends to progress more rapidly, spread more quickly, and affect a wider area. Moreover, the disease can be transmitted through contaminated food, water, feces, and soil, further complicating clinical prevention and treatment efforts [20].

During the pathogen identification process of organ and intestinal tissue samples from diarrheal calves in this study, purple-black round colonies with a metallic sheen were observed on eosin-methylene blue agar, consistent with the colony morphology described by previous study [21]. Following single-colony isolation and PCR amplification, the resulting product was approximately 1,500 bp, matching the expected size. Homology analysis of the sequencing data revealed that the 9 isolates obtained from the eosin-methylene blue agar were most closely related to *Escherichia coli*, confirming the identity of the bacteria through both morphological and molecular biological analysis. On *Clostridium perfringens* chromogenic agar, orange-red colonies were observed. Fluorescence quantitative PCR-based genotyping indicated that all isolates were of *Clostridium perfringens* Type A, a strain commonly associated with pathogenicity. These results were consistent with findings reported by previous research [22]. Pathological observations revealed that mixed infections of *Clostridium perfringens* and *Escherichia coli* caused varying degrees of damage to the organs and intestinal tissues of the calves. Notably, inflammatory cell infiltration was observed in the epicardium of the heart, while focal necrosis of hepatocytes was seen in the liver. Lymphocytic infiltration indicated that a significant number of inflammatory cells had accumulated at the site of pathogen invasion, where they could effectively phagocytize the pathogens. However, the inflammatory mediators and lytic enzymes released by these cells might also damage normal cells and tissues, leading to organ enlargement and partial cell degeneration and necrosis [23]. Notably, the intestinal tissue exhibited both damage and inflammation, consistent with previous studies on the impact of toxins on intestinal morphology [24]. In the spleen, a reduction in lymphocytes in the white pulp and congestion in the red pulp were observed. In the kidneys, the glomerular basement membrane was thickened, renal tubular necrosis occurred, and there was an increase in fibrous tissue proliferation and inflammatory cell infiltration. In the testes, significant atrophy of the seminiferous tubules was evident, accompanied by vascular dilation, congestion in the interstitial tissue, hemorrhage, and inflammatory cell infiltration. These pathological changes aligned with the previous findings [20].

Ningxia's geographical location makes it particularly prone to cold and dry conditions during the winter and spring, which can easily trigger calf diarrhea and predispose calves to secondary infections. Additionally, in October, as the region transitions from late autumn to early winter, the sudden drop in temperature combined with the open layout of farms and high wind speeds significantly impacts water sources, feed, and bedding. Therefore, special attention must be given to drinking water and housing conditions for cattle. Based on the findings of this study, it is recommended that the farm implement comprehensive disinfection procedures. Probiotics should be used to regulate the gastrointestinal health of uninfected calves, improving their digestion and disease resistance. Furthermore, in daily management, environmental sanitation and disinfection should be strengthened, using the rotation of different disinfectants to prevent the development of pathogen resistance and halt the spread of disease [25]. Regular monitoring and management practices should also be enhanced, ensuring that sick animals are promptly treated and isolated, and potential transmission routes are thoroughly cleaned and disinfected.

Acknowledgements

This work was funded by National Key R&D Program (Grant No. 2022YFD1602501), National Beef Cattle and Yak Industry Technology System Funding Program (Grant No. CARS-37), Key Research and Development of Ningxia Hui Autonomous Region (Grant No. 2022BBF03024), and Tongxin County Science and Technology Program Project (Grant No. 2023KJJH002).

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