RESEARCH ARTICLE

Type identification and histopathological analysis of *Clostridium perfringens* type D infection in Suffolk rams

Jingsong Wang[1, †], Yanan Guo[1, †, *], Xin Li[2, †], Wei Wang[3], Jidong Li[3], Jiandong Wang[1, †, *]

[1]Institute of Animal Science, Ningxia Academy of Agricultural and Forestry Sciences, Yinchuan, Ningxia, China. [2]Ningxia Veterinary Drug and Feed Supervision Institute, Yinchuan, Ningxia, China. [3]School of Animal Science and Technology, Ningxia University, Yinchuan, Ningxia, China

Received: July 18, 2023; **accepted:** August 25, 2023.

Clostridium perfringens **(*C. perfringens*) is widely distributed in soil and in the human and animal intestines and is the causative agent of gas gangrene and enterotoxaemia in humans and animals. When *C. perfringens* infect domestic animals, it causes diarrhea, multiple-organ bleeding, and nervous system disorders by releasing toxins. In this study, to identify the etiology and pathology of the acute cause of death in a Suffolk ram, 12 different tissues including heart, liver, spleen, lung, kidney, testis, mesenteric lymph nodes, cecum, colon, middle colon, ileum, and rectum from the dead sheep were collected and pathogen isolation was performed by using identification medium of *C. perfringens* and 5% defibrated sheep blood agar medium. 16S rRNA gene of the isolated strain was amplified and sequenced. Phylogenetic trees of isolates were constructed based on 16S rRNA sequences and genetic evolution was analyzed. The specific primers for toxin typing of cpa, cpb, etx, itx, and cpe were used to identify the isolated strains. The pathological changes of tissues and organs were observed by histopathology. The results showed that 12 *C. perfringens* type D strains were isolated from different tissues. Histopathological observation showed endocardial hemorrhage and muscle fiber degeneration and necrosis, liver tissue capsule hemorrhage and liver sinusoids dilatation and congestion, white pulp atrophy and lymphocytopenia in spleen, renal glomerular necrosis with interstitial inflammatory cell infiltration and fibrous tissue hyperplasia in kidney, exfoliation of intestinal villous epithelial cells from the ileum tissue with inflammatory cell infiltration and vasodilation and congestion, mucosal layer extensive necrosis and hemorrhage of the colon tissue with necrosis and exfoliation of epithelial cell and inflammatory cell infiltration and vascular dilation and congestion, inflammatory cell infiltration and vasodilatation congestion in cecal tissue, spermatogenic tubule atrophy in testis tissue and necrosis and reduction of spermatogenic cells serous with exudation of serous substances and other pathological changes. The results showed that the pathogenic bacteria that caused the death of the flock was *C. perfringens* type D, which was found in all visceral tissues. This study also proved that *C. perfringens* could invade the reproductive organs of rams and cause pathological damage.**

Keywords: Suffolk ram; infection; *C. perfringens*; toxin typing; histopathology.

[*]**Corresponding authors:** Jiandong Wang and Yanan Guo, Animal Science Institute, Ningxia Academy of Agriculture and Forestry Sciences, Yinchuan, Ningxia 750021, China. Emails: jiandongwang668@126.com (Wang J). gyn330@126.com (Guo Y).

[†]These authors contributed equally.

Introduction

Clostridium perfringens (*C. perfringens*) also called *Clostridium welchii* is a kind of gram-positive spore-forming anaerobic bacteria that is widely distributed in soil and human and animal intestines. *C. perfringens* is the pathogen of human and animal gas gangrene and

enterotoxaemia [1]. At present, it is believed that *C. perfringens* can produce more than 20 kinds of toxins, mainly including α toxin (*C. perfringens* alpha toxin, cpa), ε toxin (*C. perfringens* epsilon toxin, etx), β toxin (*C. perfringens* beta toxin, cpb), ι toxin (*C. perfringens* iota toxin, itx), enterotoxin (CPE), and NetB toxin (necrotic enteritis B-like toxin, NetB). *C. perfringens* include seven types depending on the toxin types including A (α toxin), B (α toxin, ε toxin, β toxin), C (α toxin, β toxin, partly CPE), D (α toxin, ε toxin, partly CPE), E (mainly α toxin, ι toxin, some CPE), and F (mainly α toxin and CPE) [2]. *C. perfringens* A to E type causes disease in cattle and sheep, and *C. perfringens* G type is mainly associated with necrotizing enteritis in chickens [3].

C. perfringens disease is characterized by short incubation period, rapid onset, and rapid death, which seriously endangers human health and the development of animal husbandry. Food poisoning caused by *C. perfringens* is one of the most major foodborne diseases in the world. In the United States, there were more than one million cases of foodborne diseases caused by *C. perfringens* poisoning every year. According to statistics, there were about 5 million outbreaks of *C. perfringens* related food poisoning in the member states of the European Union every year [4]. C. perfringens has been listed as the second factor causing foodborne diseases in South Korea, and many foodborne diseases caused by *C. perfringens* have also been reported in Beijing, Zhejiang, and other places in China [5]. *C. perfringens* can cause severe enterotoxemia, enteritis, and other diseases in cattle, sheep, pigs, and poultry [6]. Although the incidence is low (2-8%), the fatality is very high (100%). Gurjar *et al*. collected a total of 307 fecal samples from 7 dairy herds in central Pennsylvania in USA and found that the positive rate of *C. perfringens* was as high as 78.50% (241/307), of which type A accounted for 93.36% (225/241), type B, C, and E all accounted for 2.49% (6/241), and type D accounted for 1.66% (4/241) [6]. Nazki *et al*. checked *C. perfringens* in sheep and goats in Kashmir Himalayan, India with 177 samples (152 sheep, 25 goats) collected from healthy, diarrheal animals, and animals suspected of dying of enterotoxemia [1]. The results showed that the positive rate of *C. perfringens* was 70.62% (125/177), of which type A accounted for 60.00% (75/125), type D accounted for 40.00% (50/125), and no B, C, and E strains were detected. Ashley A *et al*. collected 513 diarrhea samples (333 from large pig farms and 180 from regional farms) from 11 large pig farms and 16 regional farms in the Midwest of the United States to detect *C. perfringens* with the positive rate as high as 91.81% [7]. It has been estimated that necrotizing enteritis caused by *C. perfringens* cost the global poultry industry up to 6 billion dollar per year [8]. ε toxin (etx) produced by type D *C. perfringens* in the intestine is the main toxin inducing enterotoxemia in sheep and goats [3]. Mass propagation of *C. perfringens* in the intestines can cause intestinal flora disorders and release α toxins and ε toxins that change mucosal permeability and promote the absorption of the toxins by intestinal mucosa, and with the circulation of blood to various organs. Toxins in the blood vessels can also lead to vascular damage and increased vascular permeability, and can cause edema of lungs, kidneys, and other organs [9]. When a certain amount of ε toxins accumulates in the brain, it damages cerebral vascular endothelial cells, leading to nervous system disorders, inducing nervous system syndromes, such as antinotaxis, dyskinesia, near-death struggle, rapid death, *etc*. [10].

With rapid development, sheep farming has become an important industrial pillar of the livestock industry. Therefore, the healthy development of sheep farming is very important. However, the morbidity and mortality of *C. perfringens* infected disease in sheep have gradually increased, which seriously affects the breeding efficiency and the sustainable development of sheep farming. *C. perfringens* with different toxin types can cause different diseases, which consist of four common types including sudden death of sheep, sheep melioidosis, sheep enterotoxemia, and lamb dysentery. The clinical symptoms of these diseases are very similar. Therefore, it is

necessary to determine the toxin genotype of this bacterium quickly and accurately to effectively treat, prevent, and control *C. perfringens*. In the clinical setting, a preliminary diagnosis of *C. perfringens* is usually made after taking a history, clinical symptoms, and a pathologic profile. However, a laboratory diagnosis is needed to confirm the causative agent. This study was conducted for pathogen isolation and pathohistological changes of *C. perfringens* type D infection in Suffolk ram, aiming to provide a scientific basis for the accurate clinical diagnosis and timely prevention and control of the disease.

Materials and Methods

Sample Collection

In March 2022, a large number of sheep died acutely in a Suffolk ram farm in Wuzhong, Ningxia, China. The records showed that the farm owner purchased 100 Suffolk rams in the age of 6- to 8-month-old from Gansu HeLiShan GuangXin Animal Husbandry Co., Ltd. in Zhangye City, Gansu, China about 10 days ago, and brought them back to the sheep farm. The new arrivals were replaced with the new feed formula, and drug dewormed with albendazole on the second day of arriving at the dose of 30 mg/kg of body weight once a day for 3 days. After stopping the drug, most of the sheep appeared to have swollen abdomen, depressed spirit, lying on the ground. With the development of the disease, the animals demonstrated the symptoms of head and neck tilted backward, constantly circling, respiratory difficulties, collapse, and other symptoms, and finally died of exhaustion in about 2 to 3 days. A small portion of the sheep immediately appeared to be collapsed, the whole-body spasms, and lasted for about one hour and then died. A total of 67 deaths were reported. During this period, the owner applied a one-time intramuscular injection of 0.025 mL of 10% enrofloxacin per kg of body weight, twice daily and continuously, but no efficacy appearing. Upon arrival at the site, 14 sheep were still showing symptoms such as abdominal swelling and lying down, and one of them showed neurological symptoms of head and neck tilting back and died after about 1 hour. The autopsy of dead sheep was performed immediately after death. The dissection revealed severe hemorrhage of the intestinal mucosa. The samples from 12 tissues including heart, liver, spleen, lung, kidney, testis, mesenteric lymph nodes, cecum, colon, middle colon, ileum, and rectum were collected in sealed sterile containers with two samples from each tissue. The samples were then placed in 4°C sampling boxes and transported to the laboratory.

Isolation of *C. perfringens*

Two (2) copies of each sample from 12 different tissues were inoculated into the plates with eosin methylene blue agar (Hopebiol, Qingdao, Shandong, China), defibrated sheep blood (G-CLONE, Beijing, China), and chromogenic medium of *C. perfringens* (CHROMagar, Paris, France) aseptically. The chromogenic medium of *C. perfringens* plate was put into an anaerobic package and placed in an anaerobic bag. After being incubated at 37°C, upside down, for 24 h, the growth characteristics and shape of the bacterial colonies were observed. A single colony was picked for Gram staining and microscopic examination. Another single bacterial colony was picked and smeared on a new plate by using the three-character streak method, and then was incubated at 37°C, upside down, for 24 h. A newly growing single colony from the new plate was picked again and amplified in the brain heart immersion broth liquid medium (Hopebiol, Qingdao, Shandong, China).

16S rRNA gene amplification and sequencing

Two (2) mL of the above bacterial culture was centrifuged at 13,000 rpm for 2 mins to obtain the bacterial precipitate. Bacterial genomic DNA was then extracted by using SteadyPure Bacterial Genomic DNA extraction kit (Accurate Biology, Changsha, Hunan, China). The 16S rRNA universal primers (Table 1) were applied for 16S rRNA gene polymerase chain reaction (PCR) amplification in a reaction system of 50 µL with 25 µL of 2× Taq PCR Mix (Accurate Biology, Changsha, Hunan,

Table 1. Primer sequence information of *C. perfringens* gene fragment.

Name	Primer sequences (5′ - 3′)	Clip size
16S rRNA	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	1,465 bp [11]
cpa	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	325 bp [12]
cpb	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAG	236 bp [12]
etx	AGTATCTAATGAAATGTCCA TTCCACTTACTTGTCCTAC	585 bp [12]
itx	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	445 bp [12]
cpe	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233 bp [13]

China), 2 µL of each 10 µmol /L 16S rRNA forward and reverse primers, 2 µL of DNA template, and 19 µL of ddH_2O. The PCR reaction was performed by using BIO-RAD T100 Thermal Cycler (Bio-Rad, Hercules, California, USA) with the program of 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 90 s. The PCR products were analyzed by 1.2% agarose gel electrophoresis and sequenced by Sangon Bioengineering Technology Service Co., Ltd., Shanghai, China).

Toxin genotyping of *C. perfringens* isolates

C. perfringens toxin genes of cpa, cpb, etx, and itx were detected by using multiplex PCR detection method and toxin typing specific primers (Sangon Bioengineering Co., Ltd., Shanghai, China) (Table 1). The PCR reaction system was 25 µL with 12.5 µL of 2× Taq PCR Mix, 0.2, 0.2, 0.8, and 0.8 µL of each 10 µmol/L forward and reverse primers of cpa, cpb, etx, and itx, respectively, 2 µL of template DNA, 6.5 µL of ddH_2O. The cpe toxin gene was detected by using single PCR detection method with the reaction system of 25 µL including 12.5 µL of 2× Taq PCR Mix, 1 µL of each 10 µmol/L forward and reverse primers, 2 µL of template DNA, 8.5 µL of ddH_2O and the PCR program as 35 cycles of 95°C for 30 s, 52.6°C for 30 s, and 72°C for 50 s. The PCR products were analyzed by 1.2% agarose gel electrophoresis.

Genetic evolution analysis of pathogenic bacteria

The sequencing results of bacterial isolates were compared with the standard strain sequence in GenBank (https://www.ncbi.nlm.nih.gov/) by using DNA Star software (DNASTAR, Inc., Madison, Wisconsin, USA). The phylogenetic tree was constructed based on 16S rRNA sequences by using the Neighbor-joining (NJ) adjacency method in MEGA 11.0 software (Mega Limited, Auckland, New Zealand), and genetic evolution analysis was performed.

Observation of pathologic histology

Twelve (12) different tissue samples were collected and immersed in 100 mL/L formalin solution. After fixation for 7 days, the fixed tissues were dehydrated by JT-12S Automatic Dehydrator (Wuhan Junjie Electronics Co., Wuhan, Hubei, China), paraffin-embedded by using BMJ-A Embedding Machine (Changzhou Suburbs Zhongwei Electronic Instrument Factory, Changzhou, Jiangsu, China), sectioned by using Leica-2016 Rotary Microtome (Leica Microsystems, Wetzlar, Germany), and then H.E. stained by using RS36 Automated Stainer (Paisijie Medical Equipment Co., Changzhou, Jiangsu, China). After staining, images for specific lesions were acquired by using Pannoramic 250 Digital Slice Scanner (3DHISTECH, Budapest, Hungary).

Results

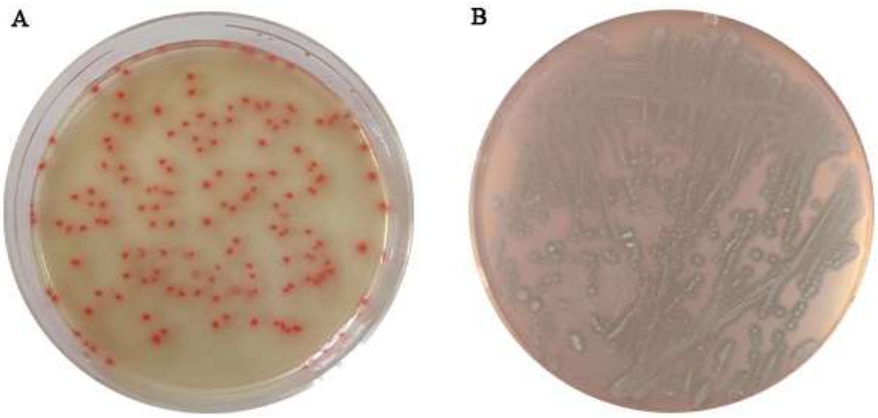

Figure 1. Morphology of *C. perfringens* isolates on *C. perfringens* chromogenic medium (**A**) and 10% sheep blood agar plates (**B**).

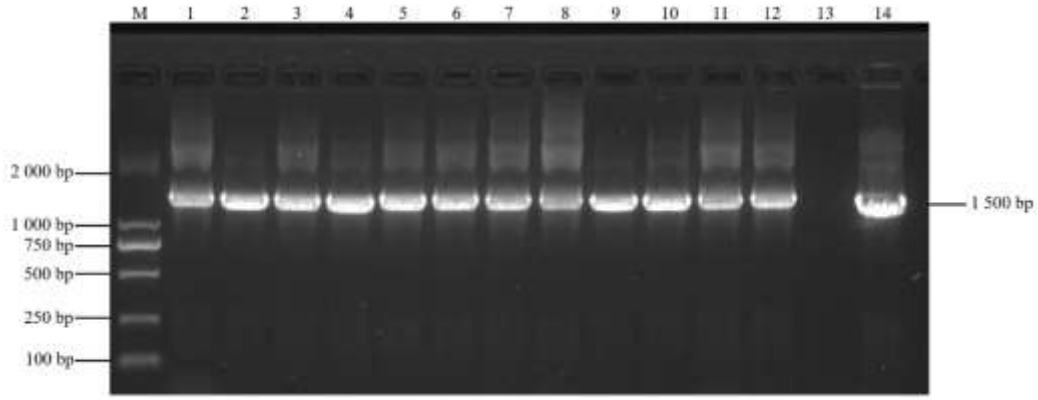

Figure 2. 16S rRNA PCR amplification results. **Lane M:** DNA Marker DL 2000. **Lanes 1-12:** isolated strains. **Lane 13:** negative control. **Lane 14:** positive control (standard strains *C. perfringens* CVCC81).

Morphology of bacterial colony

A large number of red single colonies were observed on the chromogenic medium of *C. perfringens* after incubating at 37°C for 24 h (Figure 1A). There was smooth, gray, round, double hemolytic ring colonies on the blood plate (Figure 1B). No colony was found in levine eosin-methylene blue agar medium.

Genetic evolution analysis of *C. perfringens* isolates

Analysis of 16S rRNA amplification products isolated from 12 strains showed that the sizes of 16S rRNA amplification fragments were all 1,465 bp (Figure 2). The 16S rRNA sequence homology comparison results showed that the 12 samples had the highest homology with standard strains of *C. perfringens* ATCC 13124, 9784, and 8205. The results of the constructed phylogenetic tree demonstrated that the 12 samples were the closest to the strains of *C. perfringens* ATCC 13124, 9784, and 8205, and the support rate was up to 100% (Figure 3).

Toxin genotyping of isolates

The results of toxin gene PCR were shown in Figure 4. The genes of α and ε toxins were amplified in the heart, liver, spleen, lung, kidney, testis, mesenteric lymph nodes, cecum, colon, middle colon, ileum, and rectum. The PCR results of the other toxin genes were negative, indicating that the Suffolk ram was infected with *C. perfringens* type D.

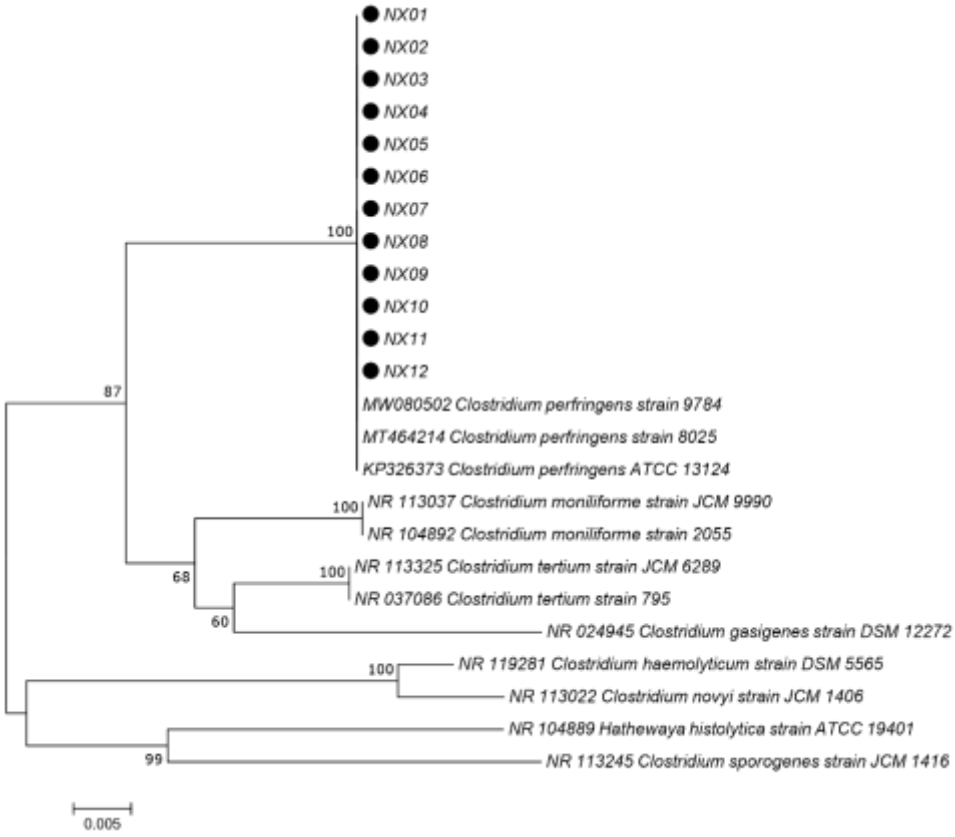

Figure 3. Phylogenetic tree of 12 isolated strains (NX01 to NX12) based on the gene sequence of 16S rRNA.

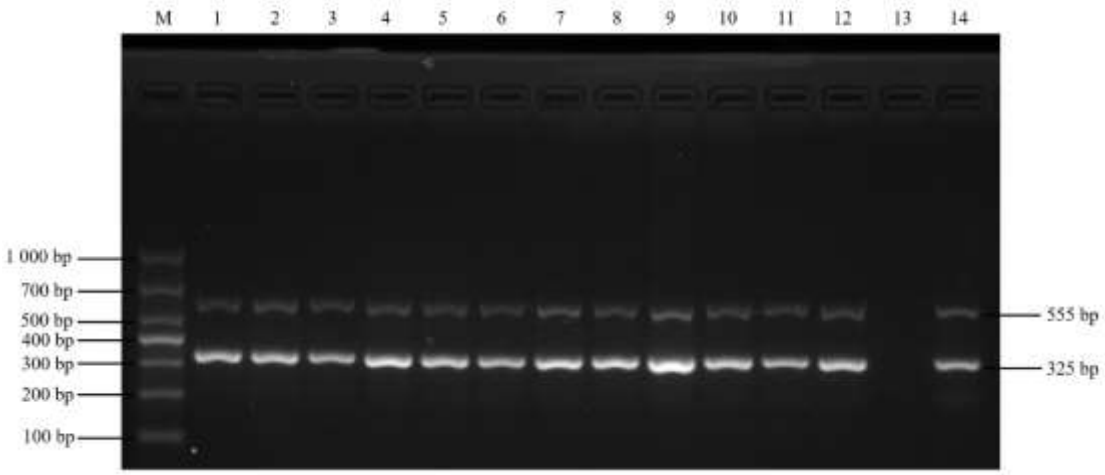

Figure 4. *C. perfringens* toxin genotyping PCR amplifications. **Lane M:** DNA Marker DL 2000. **Lanes 1-12:** isolated strains. **Lane 13:** negative control. **Lane 14:** positive control (standard strains *C. perfringens* CVCC81).

Histopathological findings

Histopathological observation of the viscera revealed local hemorrhage of the endocardium with the degeneration, necrosis, and dissolution of the surrounding myocardial fibers (Figure 5A and 5B). The liver tissue capsule bled slightly and

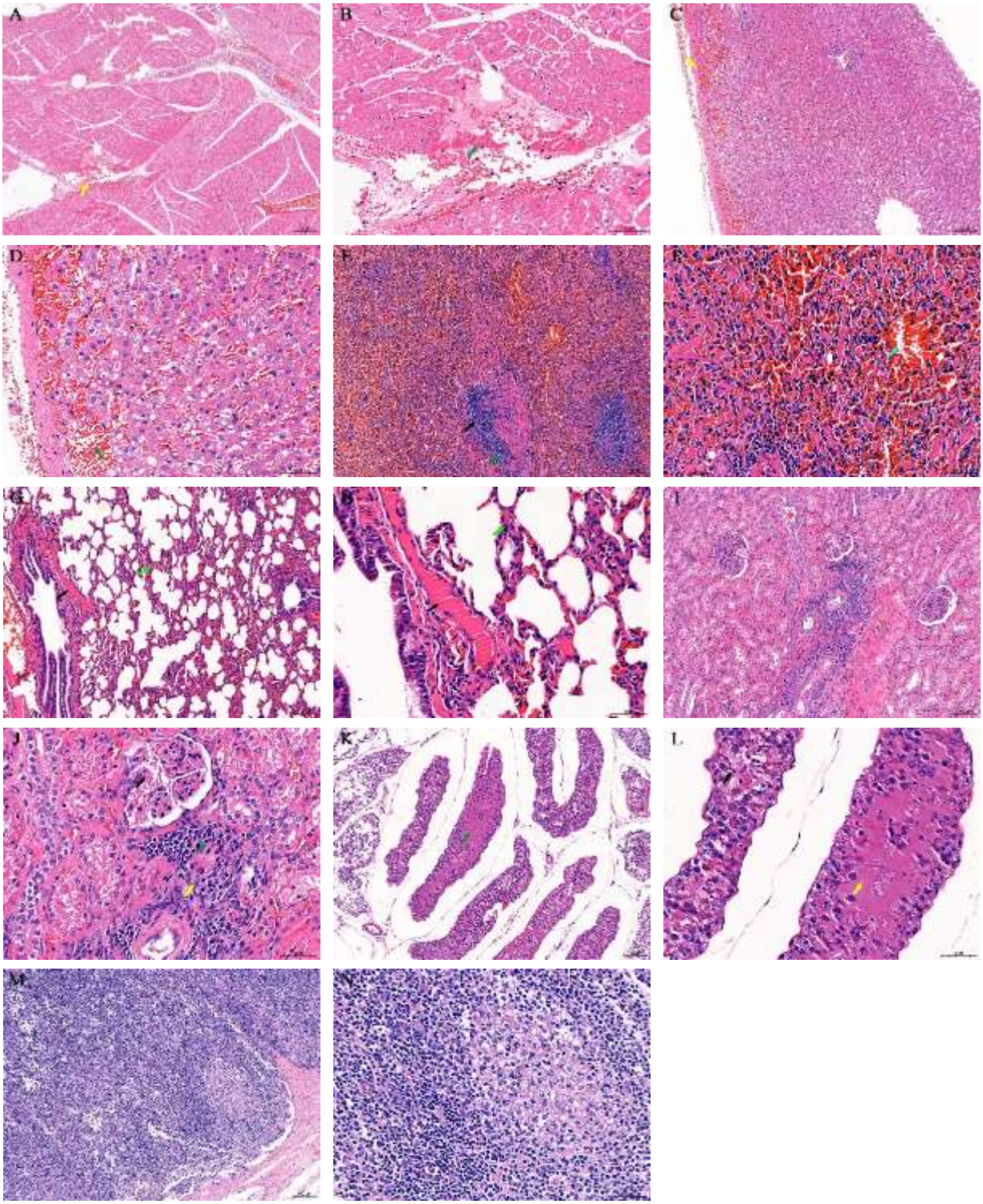

Figure 5. Histopathological sections of organ tissues (H.E. staining). **A (100×):** heart endocardial hemorrhage (yellow arrow). **B (400×):** heart myofibrosis necrosis (green arrow). **C (100×):** liver capsular hemorrhage (yellow arrow). **D (400×):** liver dilated sinusoid congestion (green arrow). **E (100×):** spleen white pulp atrophy (black arrow) and lymphocytes decreased (green arrow). **F (400×):** spleen splenic sinusoidal congestion (cyan arrow). **G (100×) and H (400×):** thickened alveolar septa in the lung (green arrow) and hemorrhage (black arrow). **I (100×) and J (400×):** kidney glomerular necrosis (black arrow), lymphocytic infiltration (green arrow), and fibroblastic proliferation (yellow arrow). **K (100×):** testis seminiferous tubule atrophy (green arrow). **L (400×):** testis spermatogenic cell necrosis (black arrow) and exudation of serous material (yellow arrow). **M (100×) and N (400×):** increased lymphocytes, plasma cells, and macrophages in mesenteric lymph nodes.

the liver lobulation was not obvious. The hepatic sinusoids were dilated and congested to varying degrees, and the subcapsular hepatic sinusoids were significantly dilated (Figure 5C and 5D). The spleen tissue capsule was intact, the boundary between white and red pulps was clear. However, the white pulp was atrophic, and the number of lymphocytes was significantly reduced. There was no obvious proliferation or reduction of various cellular components in the red pulp. Congestion of the splenic blood sinus was observed (Figure 5E and 5F). The bronchial structure at all levels of lung tissue was relatively normal with no obvious inflammatory cell infiltration and fibrosis in the interstitial. However, a severe hemorrhage was observed in the alveolar septum (Figure 5G and 5H). The renal tissue capsule was relatively intact with some glomeruli that were locally necrosis. The glomus structure was fuzzy, and some cells were cytolysis. Local interstitial fibrous tissue proliferation was observed with an increase in fibroblasts containing long oval or oval nuclei accompanied by a small amount of inflammatory cell infiltration that was mainly lymphocytes with round and dark-stained nuclei (Figure 5I and 5J). The lumen sizes of testicular seminal tubules were different with more local tubules atrophy, reduced lumen diameter, more spermatogenic cells in the lumen, nucleus sequestration and disintegration, less spermatogenic cells, and some exudation of strong eosinophilic serous substances (Figure 5K and 5L). The connective tissue envelope of mesenteric lymph node tissue was intact. There were many cellular components in the medulla including lymphocytes, plasma cells, and macrophages (Figure 5M and 5N).

The histopathological observation of intestinal tissue showed that the ileal tissue was locally decomposed by necrosis of intestinal villous epithelial cells. The nuclei of necrotic cells were seen to consolidate and disintegrate with many inflammatory cells, mainly lymphocytes, infiltrating the interstitial layer of the mucosa. The vascular dilatation and bruising were also shown (Figure 6A and 6B). The mucosal surface of the cecum tissue was covered by a single layer of columnar epithelium and smooth without villi. The intestinal glands in the lamina propria were densely arranged. The interstitial blood vessels were significantly dilated and congested, and inflammatory cells were infiltrated, mainly lymphocytes (Figure 6C and 6D). The mucosal layer of colon tissue was necrotic in a large area with many necrotic cell debris, many inflammatory cells, mainly neutrophils, infiltration, and a lot of bleeding. There were different degrees of inflammatory cell infiltration and vasodilation and congestion in the lamina propria of the middle colon, and the inflammatory cells were mainly lymphocytes (Figure 6E - 6H). Necrosis and exfoliation of epithelial cells were observed on the mucosal surface of rectum tissue. The intestinal glands in the lamina propria were densely arranged. Some cells were necrotic with necrotic cell sequestration and disintegration and a large number of cell debris. Interstitial fibrous tissue proliferated, and inflammatory cells infiltrated with increased fibroblasts. The inflammatory cells were mainly lymphocytes and plasma cells (Figure 6I and 6J).

Discussion

C. perfringens is a kind of Gram-positive bacterium presenting straight rod shape, round at both ends, single or double arrangement, no flagella, unable to move. *C. perfringens* type A strain can cause gas gangrene in humans and animals, enterotoxemia in domestic and wild animals [14, 15], while type B strain causes dysentery in lambs, multiple sclerosis in humans, enterotoxemia or necrotic enteritis in domestic animals, ruminants, and pigs [14, 16, 17]. Type C strain can cause enterotoxemia and necrotic enteritis in ruminants, piglets, horses, and necrotic enteritis in humans [3, 18], while type D strain can cause enterotoxemia in ruminants. Type E strain can cause enterotoxemia in calves and lambs. Type F strain can cause food poisoning in humans and is associated with non-foodborne gastroenteritis [2]. Type G strain can

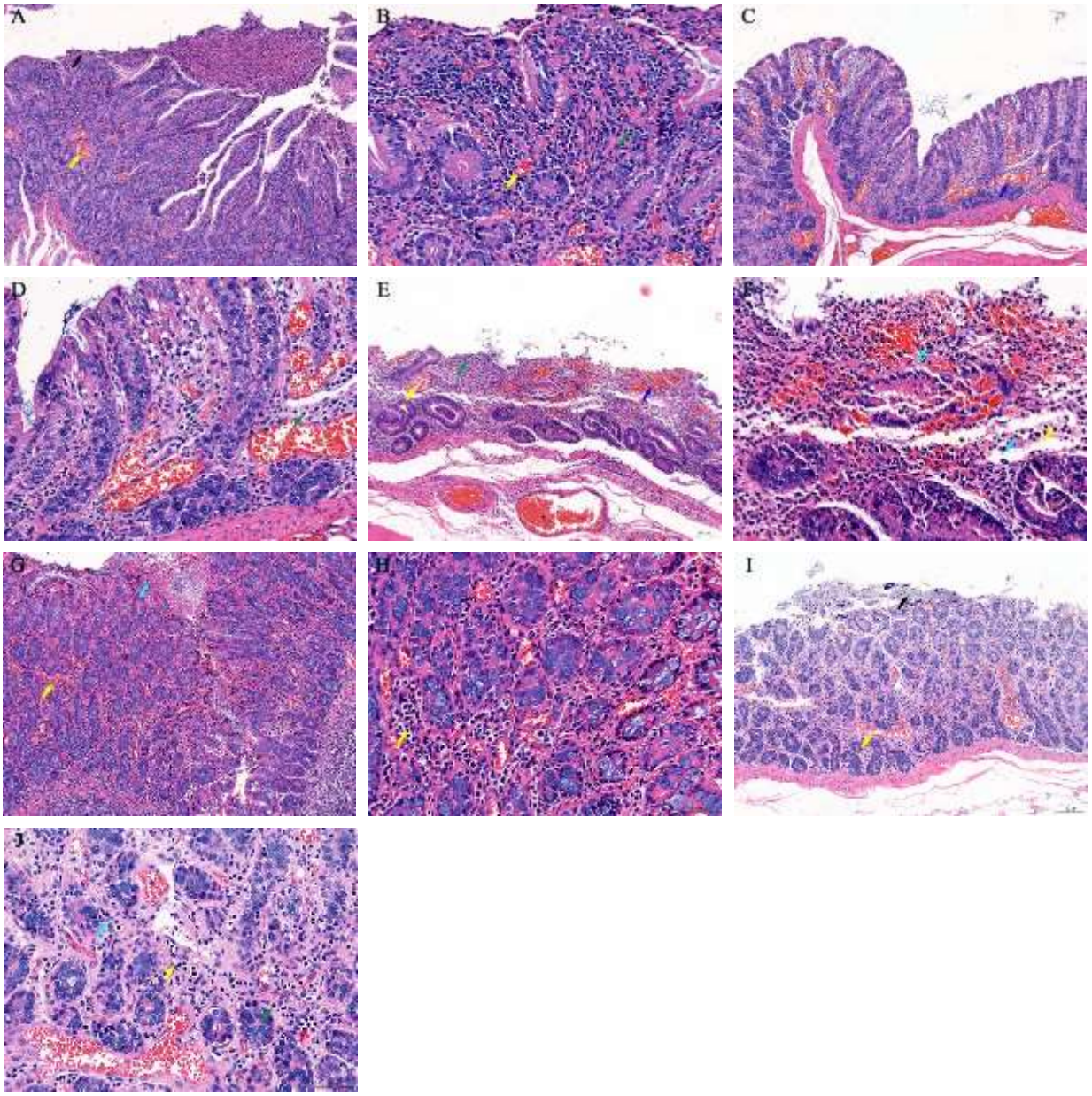

Figure 6. Histopathological sections of intestinal tissues (H.E. staining). **A (100×):** ileum epithelial detachment (black arrow) and vasodilator congestion (yellow arrow). **B (400×):** ileum lymphocytic infiltration (yellow arrow) and vasodilator congestion (green arrow). **C (100×):** cecum vasodilator congestion (blue arrow). **D (400×):** cecum lymphocytic infiltration (green arrow). **E (100×):** colon extensive necrosis of mucosal layer (green arrow), vasodilator congestion (yellow arrow), and bleeding (blue arrow). **F (400×):** colon lymphocytic infiltration (yellow arrow) and neutrophil infiltration (cyan arrow). **G (100×):** middle colon epithelial detachment (cyan arrow) and vasodilator congestion (yellow arrow). **H (400×):** middle colon neutrophil infiltration (yellow arrow). **I (100×):** rectum epithelial detachment (black arrow) and vasodilator congestion (yellow arrow). **J (400×):** rectum necrotic cell (yellow arrow), lymphocytes (red arrow), plasma cell (green arrow), and fibroblast (blue arrow).

cause necrotic enteritis in birds [2]. In 2020, Mohiuddin *et al*. isolated and identified *C. perfringens* from sheep in seven regions of Pakistan [19]. The results showed that type A and type D strains were the main endemic strains. In 2021, Hayati *et al*. isolated 124 *C. perfringens* strains from sheep in Fars province, Iran and confirmed that type A and D strains were the main endemic strains and β2-toxin was the main pathogenic toxin [17]. Cheng *et al*. also isolated 175 *C. perfringens* strains from sheep in Jiangsu province, China and found that type A and type D

strains were the main endemic strains [20]. In addition, Alsaab *et al*. isolated 13 *C. perfringens* strains from sheep in Qasim region of Saudi Arabia and all of which were type D strains, indicating that the endemic strain in this region was type D strain [21]. In this study, only α-toxin and ε-toxin were produced by *C. perfringens* detected in the visceral tissues and intestinal contents of Suffolk rams, which indicated that the Suffolk rams were infected by type D *C. perfringens*. The results of this study and other recent research confirmed that *C. perfringens* type D was the main epidemic strain in sheep. It is necessary to strengthen the research and prevention of *C. perfringens* type D infection.

Sheep are more susceptible to *C. perfringens* infection, especially lambs. *C. perfringens* type D strain multiplies and produces toxins in both the small and large intestines. The epsilon toxin is absorbed in all intestinal segments, but to a higher degree in the colon. Epsilon toxin is the third strongest bacterial toxin known after botulinum toxin and tetanus toxin [22]. In this study, epsilon toxin was detected in the cecum, colon, ileum, and rectum. At the same time, epsilon toxin was detected in the outer and middle part of the colon, indicating that epsilon toxin was absorbed more in the colon. Although epsilon toxin induced intestinal lesions, resulting in changes in organ function and making the toxin absorbed, these lesions are not often observed in cases of enterotoxemia caused by *C. perfringens* type D strain infection [23]. Epsilon toxin enters the blood circulation system through the intestinal tract and reaches tissues or organs such as the lung, kidney, liver, and brain. The toxin has also been found in the spinal cord, eyes, and turbinate [23]. In this study, the organs or tissues of chest and abdomen from dead sheep were collected. Epsilon toxin was detected by PCR in the heart, lung, liver, spleen, kidney, testis, intestinal lymph nodes, large intestine, and small intestine. In the intestine, the effects of the epsilon toxin can cause fluid accumulation and mucosal hemorrhage. Histopathological changes in the small and large intestines included mucosal hyperemia and erosion, hemorrhage affecting the mucosa, lamina propria, and submucosa, and moderate polymorphonuclear leukocyte infiltration [24]. Epithelial cells exfoliation and necrosis, lamina propria hemorrhage, and polymorphonuclear leukocyte infiltration were observed on the ultrastructure [23]. In this study, the lesions included necrosis and exfoliation of small intestine epithelial cells, infiltration of a large number of inflammatory cells, vasodilatation, necrosis in the large intestinal mucosa, necrosis and exfoliation of epithelial cells, infiltration of inflammatory cells were observed, which was similar to the results of previous studies, and provided a certain basis for the study of *C. perfringens* D type induced enterotoxemia in sheep. Epsilon toxin enters the organs through the blood circulation, damaging the tissues and organs by accumulation. In this study, the lesions were found by histological observation of the heart, lung, spleen, kidney, and intestinal lymph nodes, showing local endocardial hemorrhage, degeneration, necrosis and dissolution of cardiac muscle, atrophy and hemorrhage of spleen, and severe hemorrhage in the interalveolar septum of the lung. A previous report confirmed that *C. perfringens* type D could cause pulmonary edema in chronic cases [3]. However, no obvious pulmonary edema was found in this study. The discoveries of heart and spleen in this study could provide some references for the diagnosis of *C. perfringens* type D and the course of enterotoxemia in ruminants.

C. perfringens type D can cause enterotoxemia in sheep, also known as soft kidney disease or "fast disease", which was characterized by kidney swelling, bleeding, and soft texture, presenting typical "soft kidney" symptoms [25]. The results of this study demonstrated the same pathological changes in kidneys. In chronic enterotoxemia, ε-toxin enters the circulatory system through the blood and affects endothelial cells, resulting in perivascular edema, degeneration, and necrosis of brain parenchyma. No parenchymal organ degeneration was found in histopathological observation of this case, which might be due to acute death of the animal.

Acknowledgement

This work was funded by Key R & D project of Ningxia Hui Autonomous Region, China (2021BEF02026).

References

1. Nazki S, Wani SA, Parveen R, Ahangar SA, Kashoo Z A, Hamid S, *et al*. 2017. Isolation, molecular characterization and prevalence of *Clostridium perfringens* in sheep and goats of Kashmir Himalayas, India. Vet World. 10(12):1501-1507.
2. Rood JI, Adams V, Lacey J, Lyras D, Mcclane BA, Melville SB, *et al*. 2018. Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe. 53:5-10.
3. Uzal FA, Songer JG. 2018. Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. J Vet Diagn Invest. 20(3):253-265.
4. Da Silva Felício MT, Hald T, Liebana E, Allende A, Hugas M, Nguyen-The C, *et al*. 2015. Risk ranking of pathogens in ready-to-eat unprocessed foods of non-animal origin (FoNAO) in the EU: initial evaluation using outbreak data (2007-2011). Int J Food Microbiol. 195:9-19.
5. Zhang SH, Wang SL. 2021. Research progress on antimicrobial resistance of *Clostridium perfringens* of animal origins. Xu Mu Shou Yi Xue Bao. 52(10):2762-2771.
6. Gurjar AA, Hegde NV, Love BC, Jayarao BM. 2008. Real-time multiplex PCR assay for rapid detection and toxintyping of *Clostridium perfringens* toxin producing strains in feces of dairy cattle. Mol Cell Probes. 22(2):90-95.
7. Baker AA, Davis E, Rehberger T, Rosener D. 2010. Prevalence and diversity of toxigenic *Clostridium perfringens* and *Clostridium difficile* among swine herds in the midwest. Appl Environ Microbiol. 76(9):2961-2967.
8. Mwangi S, Timmons J, Fitz-Coy S, Parveen S. 2019. Characterization of *Clostridium perfringens* recovered from broiler chicken affected by necrotic enteritis. Poult Sci. 98(1):128-135.
9. Finnie JW, Navarro MA, Uzal FA. 2020. Pathogenesis and diagnostic features of brain and ophthalmic damage produced by *Clostridium perfringens* type D epsilon toxin. J Vet Diagn Invest. 32(2):282-286.
10. Dorca-Arévalo J, Soler-Jover A, Gibert M, Popoff MR, Martín-Satué M, Blasi J. 2008. Binding of epsilon-toxin from *Clostridium perfringens* in the nervous system. Vet Microbiol. 131(1-2):14-25.
11. Petti CA. 2007. Detection and identification of microorganisms by gene amplification and sequencing. Clin Infect Dis. 44(8):1108-1114.
12. Bao CL, Fu MZ, He YP, Bai T, Wei DX, Zhang YM, *et al*. 2017. Establishment and preliminary application of multiplex PCR for detecting toxin typing of goat *Clostridium perfringens* strains. Chin J Vet Sci. 37(08):1523-1527.
13. Yonogi S, Kanki M, Ohnishi T, Shiono M, Iida T, Kumeda Y. 2016. Development and application of a multiplex PCR assay for detection of the *Clostridium perfringens* enterotoxin-encoding genes cpe and becAB. J Microbiol Methods. 127:172-175.
14. Omer SA, Al-Olayan EM, Babiker SEH, Aljulaifi MZ, Alagaili AN, Mohammed OB. 2020. Genotyping of *Clostridium perfringens* Isolates from domestic livestock in Saudi Arabia. Biomed Res Int. 2020:9035341.
15. Milton AAP, Agarwal RK, Priya BG, Saminathan M, Aravind M, Reddy A, *et al*. 2017. Prevalence and molecular typing of *Clostridium perfringens* in captive wildlife in India. Anaerobe. 44:55-57.
16. Munday JS, Bentall H, Aberdein D, Navarro M, Uzal FA, Brown S. 2020. Death of a neonatal lamb due to *Clostridium perfringens* type B in New Zealand. N Z Vet J. 68(4):242-246.
17. Hayati M, Tahamtan Y. 2021. Toxin typing of *Clostridium perfringens* associated with enterotoxaemia in sheep in Fars Province. Arch Razi Inst. 76(3):691-697.
18. Diab SS, Kinde H, Moore J, Shahriar MF, Odani J, Anthenill L, *et al*. 2012. Pathology of *Clostridium perfringens* type C enterotoxemia in horses. Vet Pathol. 49(2):255-263.
19. Mohiuddin M, Iqbal Z, Siddique A, Liao S, Salamat MKF, Qi N, *et al*. 2020. Prevalence, genotypic and phenotypic characterization and antibiotic resistance profile of *Clostridium perfringens* type A and D isolated from feces of sheep (*Ovis aries*) and goats (*Capra hircus*) in Punjab, Pakistan. Toxins (Basel). 12(10):657-670.
20. Cheng C, Zhang TY, Cheng DR, Cao SY, Tao JP, Xing H. 2021. Surveillance of *Clostridium perfringens* infection in clinical healthy meat sheep in Jiangsu province. Yang Zhou Da Xue Xue Bao Nong Ye Yu Sheng Ming Ke Xue Ban. 42(04):34-38.
21. Alsaab F, Wahdan A, Saeed EMA. 2021. Phenotypic detection and genotyping of *Clostridium perfringens* associated with enterotoxemia in sheep in the Qassim Region of Saudi Arabia. Vet World. 14(3):578-584.
22. Harkness JM, Li J, Mcclane BA. 2012. Identification of a lambda toxin-negative *Clostridium perfringens* strain that processes and activates epsilon prototoxin intracellularly. Anaerobe. 18(5):546-552.
23. Alves GG, Machado De Ávila RA, Chávez-Olórtegui CD, Lobato F C. 2014. *Clostridium perfringens* epsilon toxin: the third most potent bacterial toxin known. Anaerobe. 30:102-107.
24. Morris WE, Dunleavy MV, Diodati J, Berra G, Fernandez-Miyakawa ME. 2012. Effects of *Clostridium perfringens* alpha and epsilon toxins in the bovine gut. Anaerobe. 18(1):143-147.
25. Gao J, Zhang CM, Liu W, Zhao YL, Zhao BH. 2013. Progress on ε Toxin of *Clostridium perfringens* Type D. Prog Vet Med. 34(10):101-105.