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RESEARCH ARTICLE

A Comprehensive Diagnosis of Deer Enterotoxaemia Caused By *Clostridium perfringens A* in Shandong Province, China

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ABSTRACT

We report the outbreak of deer enterotoxaemia that was collected since February 2014 from Jining, Shandong province, China. The dead deer were characteristic of enterotoxaemia symptoms and necrotic features. The histopathology of intestinal mucosa and serosa was severe hemorrhagic necrosis and the infiltration of *Clostridium perfringens* with the diameter of 1~3mm and obtuse ends. The intestinal content was inoculated into differential medium. Round, middle-black bacterium on the TSC agar plate and gray to grey-yellow, hemi-translucent colonies on the egg yolk agar plate was also seen. The isolated deer *Clostridium perfringens* type A could lead mice to death and SPF chicken to ill, and there was no distinctive species difference. The CPA gene of *Clostridium perfringens* was amplified by using the multiple PCR. The sequence was compared with the genomic sequences in the GenBank nucleotide database found to share 99.4% sequence similarity with the *Clostridium perfringens*. The final *Clostridium perfringens* type A infected diagnosis was based on comprehensive analysis of epidemiological evidences, clinical signs, necrotic examinations, accompanied by histopathological as well as microbiological findings. A comprehensive analysis of the outbreak of the deer *Clostridium perfringens* was also discussed.

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INTRODUCTION

Clostridium perfringens was described as bacillus aerogenes capsulatus, which was first isolated by Welchii and Nuttad in 1892. The causative agent was the genus clostridium of the family bacillaceae, belonging to mild anaerobic bacteria (G^+) with obtuse ends. This microorganism, which was the conditional pathogen, could be a normal inhabitant of the intestine of the most animals including humans, but while the intestinal microenvironment was disrupted by sudden changes in diet or other adverse factors, it would quickly proliferate and produce toxins acting locally or being absorbed into the general circulation, and then causing necrotic enteritis or enterotoxaemia of swine, chicken, rabbit and lamb *et al*.. *C. perfringens* was divided into 5 serotype (A, B, C, D, and E) based on the major lethal toxins, namely alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) (Niilo, 1980; Rood, 1998). However, *C. perfringens* can produce up to fifteen toxins (α, γ, ε, θ, κ, μ and ν) (Garmory *et al*., 2000; Sakurai, 1995; Songer, 1996).

C. perfringens was prevalent in bovine (Cho *et al*., 1990; Niilo, 1978), swine (Cho *et al*., 1991; Mackinnon, 1989; Johnson *et al*., 1992; Taylor, 1984; Yeh *et al*., 1993), and chicken (Al-Shikhly and Truscott, 1977; Niilo, 1978; Truscott and Al-Sheikhly, 1977) reported it as a cause of necrotic enteritis. However, the outbreak of clostridial enterotoxemia had seldom taken place in some sika deer farms in the past years. The disease of the infected flock resulted in acute and fast death accounting for >60% total mortality, which led to tremendous economic losses (Fengxiang *et al*., 1997; Liming *et al*., 1992; Naisheng *et al*., 1994). The initial case was reported in the deer production facility located in Zhong-wei County, Ningxia, where 156 deer were infected with *C. perfringens* causing 23 deer death (Jiaming *et al*., 2003).

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In 2006-2007, the co-infection of *C. perfringens* and *Pasteurella* resulted in acute death of 200 deer (Yongjiang *et al*., 2008). Some results (Chuanfang *et al*., 2004; Naisheng *et al*., 1994; Jiaming *et al*., 2003) showed that *C. perfringens* (A, C and D) were the major causative agents causing deer enterotoxaemia. However, until now the report of sika deer enterotoxaemia has less and less. In the present study, a diagnosis of *C. perfringens* infection was confirmed in deer enterotoxaemia cases via comprehensive analysis of epidemiological data, clinical signs, necrotic features, histopathological examinations, bacterium isolation and identification together with pathogenicity test.

MATERIALS AND METHODS

Clinical diagnosis

The primary clinical diagnosis of the diseased deer was based on the clinical symptoms, the necrotic findings, and the analysis of the epidemiological data. For the histopathological analysis, the tissue samples (intestinal canal, liver, spleen, kidney, mesenteric lymph nodes) were fixed in 10% formalin. The fixed samples were embedded in paraffin, and 4-μm sections were prepared. The thin sections were stained with hematoxylin and eosin (H&E). The stained sections were observed using light microscopy, and evaluated for the histopathological diagnosis.

Microscopic examination

The intestinal contents taken from the posterior fornix and the organs including liver, kidney and spleen were smeared for Gram's staining and oil microscopy. The visceral organ and mesenteric lymph nodes were collected. The intestinal contents were respectively incubated on TSC agar (casein - ferrous sulfate - cycloserine agar) with anaerobic culture in 37°C for 16-24 h. Susceptible colonies were smeared on egg yolk agar cultured for 24h by anaerobic tank, observing and staining the growth of bacteria.

Pathogenicity test

The single colony from egg yolk agar plate was inoculated into the nutrient broth with anaerobic culture in 37°C, and then the broth culture was transferred into the Gordon broth with anaerobic culture using a shaking table at 37°C constant temperature for 12 h. The broth culture was centrifuged at 10,000 × g at 4°C for 30 min and the supernatant was collected. Ten weanling mice were randomly divided into two groups of five mice each. The mice in each group received an abdominal injection of 1 mL supernatant or phosphate-buffered saline (negative control group). One-day-old specific pathogen-free (SPF) chickens were obtained from the Poultry Institute at the Shandong Academy of Agricultural Sciences in Jinan City. Five chickens were inoculated with 1 mL of the broth culture or phosphate-buffered saline (negative control) per chicken via the oral route and rising for 8 days in isolators maintained under negative pressure. Their clinical symptoms were monitored continuously. Organ lesions were observed in the experimentally inoculated mouse and chicken.

Multiplex PCR identification

Uzal (2008) reported that all toxin genes were simultaneously amplified by a multiplex PCR method with combinations of the primers, which can differentiate the all toxin genes of *C. perfringens* from no-specific amplification of other bacteria. Four pair primers were designed(Table 1)according to the sequence published in Genbank and the primers in public (Aschfalk and Muller, 2002; Xiaorong *et al*., 2002; Yoo *et al*., 1997), which could be helpful to differentiate four toxins (cpa, cpb, etx and iA). The PCR program was performed using an initial denaturation step of 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1min, with a final elongation at 72°C for 10 min.

RESULTS

Epidemiology

The majority of sika deer farms were located in Xinjiang, Nei menggu, Liaoning, Jilin and Heilong province, but there were few raising in Shandong province in the past year. However, there is the rapid expansion of special economic animal including deer in the recent year. In April 2006, Deer production facility was located in Jining County in northwestern Shandong province, where a farmer purchased approximately 60 fawns from Helong province. Until now, 160 healthy livestock is raising in this farm. Since there had been no prevalence of enterotoxaemia, the farmer vaccinated the flock against this bacterium. Nevertheless, due to the sudden alternation of concentrated fodder, the deer flocks were infected with *C. perfringens* in the cold winter in February 2014. The disease of the infected flocks was acute with mortality of >50%. What's more, the majority of the diseased were adult stag and hind. The therapy of tetracycline and streptomycin produces no obvious effect, leading tremendous economic losses.

Clinical symptom

The major clinical signs were depressed physical activity, anorexia, diarrhea and pyrexia (41°C to 42°C). Animal characterized by profuse, watery diarrhea (containing desquamated intestinal mucosa fragments and blood clots) would be in death 4-5h after the occurrence of those typical symptoms. The morbidity rate was > 25% (40/160). The majority of the diseased were adult robust stag and doe, while there was usually abdominal discomfort with distended tympanic abdomen.

Necropsy

The blood coagulation abnormality, systemic sepsis, mucous cyanosis and no rigor mortis was observed after necropsy was completed. Light edema and hyperemia was detected in lung. Enlarged, pale, and friable liver and spleen was also seen. Numerous soybean- to pea-sized hemorrhagic ulcers were observed in the mucosa of the abomasum (Fig. 1A).The disease resulted in similar intestinal serosa lesions consisting of multifocal or diffuse haemorrhagia (Fig. 1B,1C), predominantly in jejunum and ileum, which can be observed with blood- and fibrin-filled loops in its mucosa (Fig. 1D,1E). Anal relaxation was covered with brown blood. Kidney became enlarged, gray, and scattered hemorrhage in the medulla (Fig. 1F).

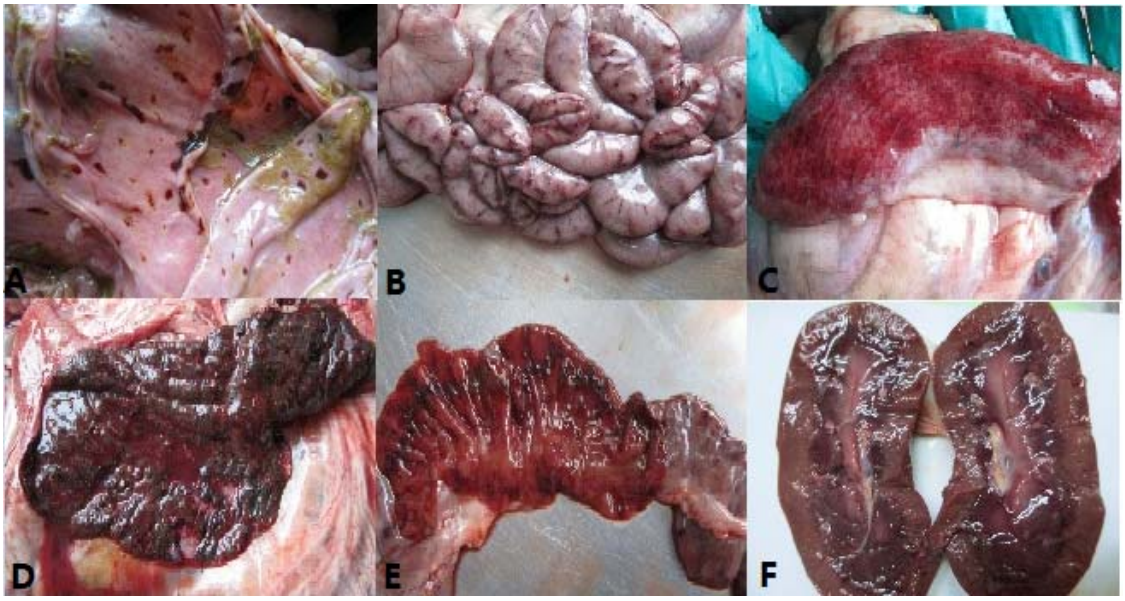

Fig. 1: A. The scattered hemorrhagic ulcer of gastric mucosa. B. The hemorrhagic ulcer of jejunum serosa. C. The diffuse haemorrhage of ileum serosa. D. The diffuse hemorrhagic necrosis of ileum mucosa. E. The hemorrhagic necrosis of jejunum mucosa. F. Swelling of the kidney, cortex soft, gray and black.

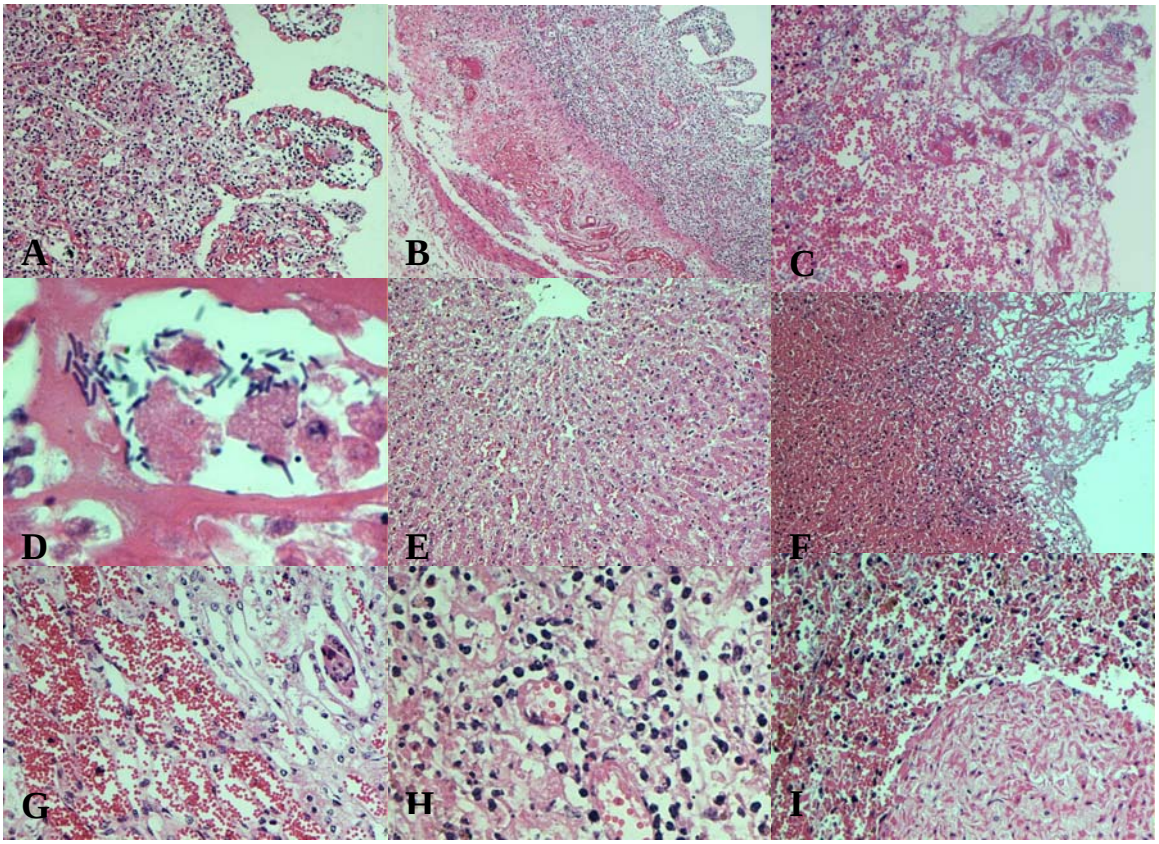

Fig 2: A. The incrassation of the jejunum mucosa lamina propria with the acidophilic leukocytes and lymphocytic infiltration (100×). B. Capillary hyperemia of the jejunum lamina propria and mucosa (200×).C. Severely profuse necrosis was observed in the cecum mucosa with intestinal gland necrosis, mucosal epithelial cells desquamation and inflammatory cell infiltration (200×). D. Bacterium with 3-5 nm in diameter, grayish, circular was also seen in the necrotizing lesions (1000×). E. Granular degeneration and vascular degeneration of hecapocyte (200×). F. The cells of mucosa layer necrosis and shed, lamina propria hemorrhage (100×) G. The degeneration and necrosis of renal tubular epithelial cells, hemorrhage and a little lymphocyte infiltration of renal interstitium(400×). H. Necrotic lymphocytes with homogeneous and red-stained necrotic foci were detected within a mesenteric lymph nodes tissue specimen (400×). I. Lymphocyte necrosis was observed with white pulp and red pulp in spleen (200×).

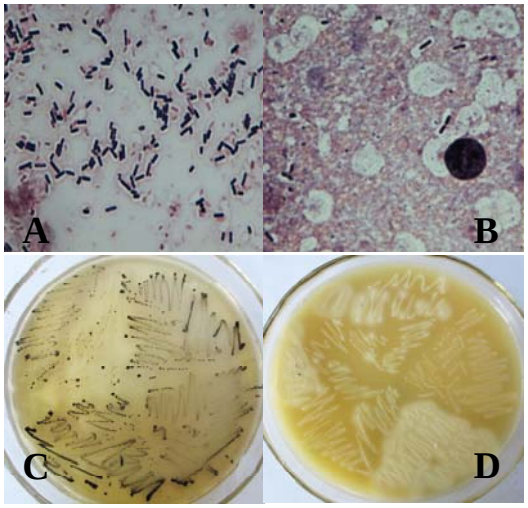

Fig 3: A. Gram-positive bacilli with capsule and rounded ends on the smear of intestinal content (1000×).B. Gram-positive bacterium was present in the liver smears (1000×). C Black bacterium with metallic luster on the TSC agar plate. D. Gray to grey-yellow and smooth hemi-translucent colonies on the egg yolk agar plate.

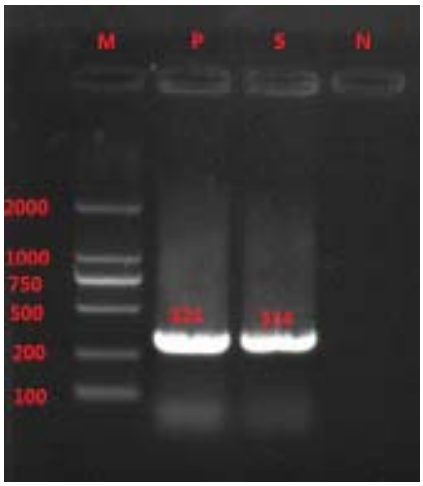

Fig. 4: PCR amplification of isolated bacterium; M: Marker DL2000; P: positive control; S: sample; N: negative control

Histopathological diagnosis

Intestinal villus was necrotized and desquamated with hyperemia, edema, hemorrhage, acidophilic leukocytes and lymphocytic infiltration of lamina and propria muscularis (Fig. 2A, 2B). Severely profuse necrosis was observed in the cecum mucosa with intestinal gland necrosis, mucosal epithelial cells desquamation and inflammatory cell infiltration. Bacterium which 3-5 nm in diameter, grayish, circular was also seen in the necrotizing lesions (Fig. 2C, 2D). Hepatocyte lesions were associated with granular degeneration, vascular degeneration and steatosis (Fig. 2E). Hepatic sinusoid was filled with erythrocytes and a little inflammatory cell, while focal necrosis was detected in hepatic lobule edge. Mucosal layer of abomasums showed a large number of cell necrosis and shedding, lamina propria hemorrhage (Fig. 2F). Expansion and hyperemia of glomerular capillary resulted into swelling of the glomerulus and renal capsule stenosis. Histopathological changes in kidney included degeneration and necrosis of renal tubular epithelial cells, hemorrhage and a little lymphocyte infiltration of renal interstitium (Fig. 2G). More common hemoglobin particles or casts were found in the renal tubular cavity. Necrotic lymphocytes with homogeneous and red-stained necrotic foci were detected within a mesenteric lymph node tissue specimen (Fig. 2H). Lymphocyte necrosis was observed in white pulp and red pulp of spleen (Fig. 2I).

Isolation and identification of bacterium

Gram-stained smears of intestinal content revealed large numbers of Gram-positive bacilli with capsule and rounded ends. Further, another Gram-negative bacillus was seen in the smear (Fig. 3A). Gram-positive bacterium was present in the liver smears (Fig. 3B), but it was absent in the kidney and spleen.

Round, protruding, smooth, middle-black bacterium with neat edge and moist surface were observed in the TSC agar plate (Fig. 3C). Gray to grey-yellow and smooth hemi-translucent colonies with the diameter of 1~3mm and neat edges were also seen on the egg yolk agar plate (Fig. 3D). The opalescent aureole appeared around/at the bottom of the colonies. Gram-positive bacilli with capsule were present in the smears.

Animal pathogenicity test results

The major clinical signs of mouse inoculated with the supernatant were depressed physical activity, anorexia, and death within 24h. No mice died in the negative group. SPF chicken showed the clinical symptom of the transient bloody stool at 5 days post-inoculation (PI), but No chicken died in the whole breeding process. After necropsy, incrassated intestinal wall without bleeding was observed.

PCR identification

The genomic DNA was PCR-amplified and the PCR products were electrophoresed on 1.2% agarose gels. A DNA fragment with an expected size of 324bp was detected (Fig. 4).

Similarity and phylogenetic analyses

The results of sequence similarity analyses using the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/) showed that the DNA sequences from the isolated bacteria were more than 99.4% identical to their *Clostridium perfringens* counterparts (Fig.5,6), suggesting that the isolated strain was indeed *Clostridium perfringens A.*

DISCUSSION

This *Clostridium perfringens A* outbreak causing deer enterotoxaemia was the first reported in Shandong province, although occasional scattered cases have previously occurred in the northeast and northwest regions in China. The scale of special economic animal such as deer developed quickly with the absence of a scientific and standardized management and necessary disease prevention and control measures. This disease was associated with introduction of animal to feedlots without progressive adaptation to grains or fodder and with the

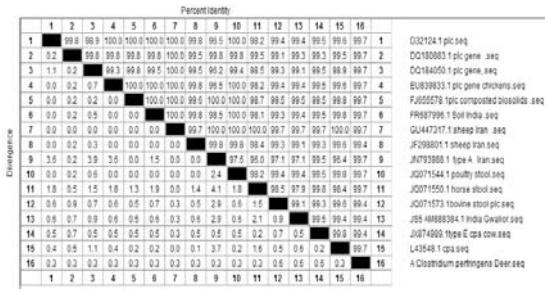

Fig. 5: Similarity analysis of nucleotide sequences between the isolated strain and others

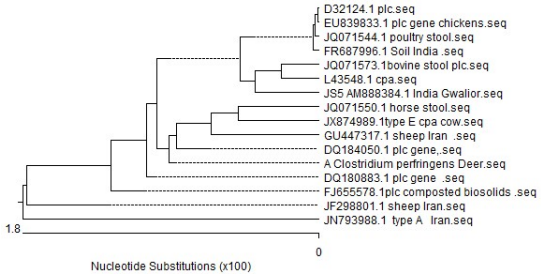

Fig. 6: Analysis of genetic relationship between the isolated strain and others

Table 1: the sequences of PCR primers

Promer	Nucleotide sequence (Forward/Reverse)	Size
cpa	5'-GCTAATGTTACTGCCGTTGA-3'	324bp
	5'-CCTCTGATACATCGTGTAAG-3'	
cpb	5'-GC GAATATGCTGAATC ATCTA-3'	196bp
	5'-GCAGGA ACATTAGTATATCTTC-3'	
etx	5'-GCGGT G ATATCCATCTATTC-3'	665bp
	5'-CCACTTACTTGTCCTACTAAC-3'	
iA	5'-ACTACTCTCAGACAAGAC AG-3'	446bp
	5'-CTTTCCTTCTATTACTATACG-3'	

weather sudden change, which can lead to alteration of intestinal flora and the proliferation of pathogenic *Clostridium perfringens*. All the factors strengthened physical deterioration of deer and the susceptibility to the bacteria, and then brought the deer into morbidity and death.

324bp PCR product (CPA gene) made a further identification for the *Clostridium perfringens A* infected deer. The similarity analysis of nucleotide sequence results show that the deer *Clostridium perfringens* isolate, shared a higher level of sequence homology (>99.4% sequence similarity) with other strains. The results of the present study were consistent with those reported by Xijun Y (2002), detecting seven kinds of economic animal *Clostridium perfringens* by using serum neutralization test and indicated that the serotype of *Clostridium perfringens* causing deer enterotoxemia and sudden death was serotype A, accounting for 85.4% (35 /41). Type A could produce several powerful toxins such as alpha toxin, which was believed to be a major factor responsible for the organism's tissue pathology. Some results (Chunhua *et al*., 1992; Fuwu *et al*., 1997; Wenjing *et al*., 1997; Yuguo, 1998) showed that *Clostridium perfringen*s type A was more common compared with others. Information about pathogenesis of type A enteric infection in ruminants is different and often contradictory, but it is generally assumed that most clinical signs and lesions are due to the effects of CPA (Uzal and Songer, 2008). In 2001, the British public health laboratory reported that 66 percent of 1426 food poisoning events were associated with *Clostridium perfringens* (beef 34% and pork 32%), with type A accounting for 43%. In 2005, Hairong W *et al*. (2005) research indicated that *C. perfringens* type A was detected in every barn air and manure in some cities, in Shandong province.

The deer *Clostridium perfringens* type A could cause mouse death and SPF chicken to be diseased, and there was no distinctive species difference. However, the SPF chicken showed mild clinical symptoms, and without acute enterotoxemias and sudden death. According to McGowan *et al*. (1958), sheep type A enterotoxemia is similar to this case, characterized by liver, spleen and intestine. Histopathological changes include periacinar necrosis of the liver, splenic congestion, hemorrhagic necrotic enteritis due to blood-borne toxin.

Conclusions

The final diagnosis was based on comprehensive analysis of epidemiological data, clinical signs, necrotic tissue features, examinations, accompanied by histopathological as well as microbiological findings. The sick deer was characterized by typical *Clostridium perfringens A* symptoms and necrotic features. The outbreak was acute with high morbidity that was attributed to a serious *Clostridium perfringens A* infection. Taking the emergent *Clostridium perfringens* vaccine for the other deer, combined with adding neomycin sulphate and penicillin in fodder, achieved a favorable effect and brought the disease under control.

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