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Molecular Characterization and Toxinotyping of a *Clostridium perfringens* Isolate from a Case of Necrotic Enteritis in Indian Kadaknath Fowl

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ABSTRACT

Necrotic enteritis, caused by *Clostridium perfringens*, is an important bacterial disease of poultry. A suspected case of necrotic enteritis was presented for necropsy from an Indian Kadaknath Fowl flock showing diarrhea and progressive debility. Gross examination revealed necrotic to ulcerative lesions in intestine. The organism was isolated from the intestinal contents, tissue and liver under anerobic conditions. The cultural characteristics and Gram staining were suggestive of *C. perfringens*. The sequencing of 16s rRNA gene confirmed the isolate as *C. perfringens* and which was well differentiated from other clostridia associated with avian intestinal tract. This study demonstrates that 16s rRNA gene sequencing can provide rapid and confirmatory identification of *C. perfringens*. Further, Multiplex Polymerase Chain Reaction (mPCR) was performed for toxinotyping and isolate was found to be positive for α toxin (*cpa*) and β 2 toxin (*cpb2*), a feature of *C. perfringens* type A isolates. As some recent studies have highlighted the involvement of NetB toxin in pathogenesis, therefore, PCR was carried out to find the presence of this toxin, the isolate was found to be negative for *netB* gene. This study emphasizes the molecular characterization and toxinotyping as a rapid tool for detection of *C. perfringens* from suspected necrotic enteritis cases. Very few reports regarding molecular characterization are available from India, hence it adds to the available data on this important poultry pathogen. Further investigations are required to understand the exact role of NetB toxin in pathogenesis as various studies including the current one reports NetB negative strains involved in necrotic enteritis.

Key words: Necrotic enteritis, *Clostridium perfringens*, isolation, pathology, PCR, multiplex PCR, molecular characterization, 16s rRNA gene sequencing, phylogenetic analysis, toxinotyping, poultry, Kadaknath fowl

INTRODUCTION

India ranks first in livestock population which contributes 4% to total Gross Domestic Product (GDP) and 27% to agriculture (DAHDF, 2012). Reproductive traits, short productive lifespan, production of good quantity of eggs and worldwide distribution, favour the use of poultry as a major

source of animal protein which maximized the growth of poultry industry in the last two decades. For the growing human population there is much demand for good quantity and quality protein (Godfray *et al.*, 2010). To meet this demand, rearing of poultry by intensive system is practised in most part of the world but which leads to major disease outbreaks, even in indigenous breeds of chickens like Kadaknath, well known for its disease resistance ability. Most alarming among all the diseases is the one which affects the gastrointestinal tract because of production losses, high mortality and risk of contamination of poultry products for human consumption (Dahiya *et al.*, 2006). Necrotic enteritis is a sporadic disease of avian species which has been reported from most areas of world in which poultry are reared (Dahiya *et al.*, 2006). Implementation of legislative restriction on usage of growth promoting antibiotics in animal feed and reduction of using antimicrobial growth promoters due to concern of spreading antimicrobial resistance imparted the increased prevalence of diseases such as necrotic enteritis (Van Immerseel *et al.*, 2009).

Etiological agent of Necrotic enteritis is *Clostridium perfringens* type A and to a lesser extent the type C (Cooper and Songer, 2009), an anaerobic gram positive, spore forming, ubiquitous organism found in the gastrointestinal tract of healthy birds also. Predisposing factors for this condition includes changes in diet, poor hygienic conditions, stress, mucosal damage causing parasites such as Coccidia and the presence of other pathogenic *C. perfringens* strains. Outbreaks occur most frequently in chickens around 2-6 weeks of age when the maternal antibodies ebbed around 2 weeks before attaining maturity of the chicken immune system. Occurrence in broilers up to 11 weeks of age, 3-6 month old commercial layers, 12-16 week replacement pullets have also been reported (Malmarugan *et al.*, 2012). There is also a report in older birds (9 month) from India in 1974. Alpha toxin is considered as the major virulence factor responsible for producing lesions in necrotic enteritis. But alpha toxin null mutant was also equally able to produce disease as compared to wild strains (Keyburn *et al.*, 2008). Recently, a novel pore forming toxin has been described, *Perfringens* necrotic enteritis B-like toxin (NetB). The *netB* gene is located on an approximately 85 kb plasmid (Lepp *et al.*, 2010), associated with pathogenesis causing by damage to host cells. Clinical necrotic enteritis is manifested in two forms, classical acute form characterised by sudden increase in flock mortality without any predictive signs, albeit wet litter is an early indicator of disease. The course of disease is often peracute indicated by death within 1-2 h, sometimes mortality may raise up to 50% (Helmboldt and Bryant, 1971; Riddell and Kong, 1992). The subclinical form is more significant than clinical form due to persistence of disease in flocks without any noticeable clinical signs and mortality. Chronic intestinal damage produced by this form of disease results in poor digestion and absorption, reduced weight gain and increased feed conversion ratio which ultimately leads to poor production performance (Elwinger *et al.*, 1992; Kaldhusdal *et al.*, 2001).

Present study reports disease investigation of an outbreak of deadly necrotic enteritis in a poultry farm of Kadaknath fowl, the isolation and identification of the causative agent (*Clostridium perfringens*), pathology, molecular detection by Polymerase Chain Reaction (PCR) and multiplex PCR, molecular characterization, 16s rRNA gene sequencing, phylogenetic analysis and toxinotyping of the *Clostridium perfringens* isolate.

MATERIALS AND METHODS

Clinical history and necropsy: An adult (8-10 weeks of age) female Kadaknath chicken carcass, representing the desi breed flock, was brought for necropsy examination and diagnosis to Division of Pathology, Indian Veterinary Research Institute, Izatnagar. History revealed that the affected

chicken flock from an organized farm in Bareilly, Uttar Pradesh suffered from coccidiosis a month ago and were treated accordingly. A few birds in the same flock showed signs consisting of inappetence, diarrhoea and progressing debility. Necropsy was carried out for the fowl presented. Samples were collected aseptically which included intestinal contents, pieces of liver and intestinal scrapings for bacteriological examination. These samples were also processed for histopathological studies.

Isolation and identification: Isolation and identification of the causative agent of Necrotic Enteritis (NE) was carried out in the laboratory. Sterile saline was added to the clinical materials such as intestinal contents, liver pieces and scrapings collected from the bird with NE suspected lesions. These were heated at 80°C for 20 min in a water bath. Then the processed samples were initially inoculated in Robertson cooked meat medium and were incubated anaerobically at 37°C for 24 h. The positive cultures were streaked on to blood agar with neomycin antibiotic and incubated in the anaerobic jar at 37°C for 48 h. Gram's staining was carried out for the recovered bacterial isolates. Again, the positive colonies were streaked in egg yolk agar and were kept in the anaerobic jar at 37°C for 48 h.

DNA isolation from the culture: DNA was extracted from the bacterial culture obtained as per the protocol of Wilson (1987). Briefly, bacterial culture of 48 h was transferred to 2 mL collection tube and centrifuge at 12,000 rpm for 5 min for pelleting of bacteria. The pellet was suspended in lysis buffer i.e., 200 µL of 1X TE (pH 8.0), 50 µL 10% SDS and 2 µL proteinase K, vortexed and incubated overnight at 37°C. Next day, 200 µL of 5 M NaCl, 100 µL of 10% CTAB (Cetyl Trimethyl Ammonium Bromide) was added and incubated at 68°C for 10 min in water bath. Then equal amount of chloroform, isoamyl alcohol (24:1) was added, vortexed and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a new microcentrifuge tube and an equal volume of phenol:chloroform, isoamyl alcohol (25:24:1) was added, vortexed and centrifuged at 10,000 rpm for 10 min. Again supernatant was transferred to a new microcentrifuge tube, then 0.6 volumes of 99% isopropanol was added and incubated at room temperature for 1 h, followed by centrifugation at 10,000 rpm for 20 min. The pellet was washed with 70% ethanol, centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and pellet air dried at 37°C in an incubator for 15-20 min. The pellet was resuspended in 30 µL of elution buffer and stored at -20°C.

Amplification of 16s rRNA gene using universal primers: The *C. perfringens* suspected DNA was amplified with 16s rRNA universal primer. Briefly, PCR was carried out in 25 µL reaction mixture containing 10X Dream Taq PCR buffer (MBI Fermentas), 0.2 mM dNTP mixture, 1.25 units Dream Taq DNA Polymerase (MBI Fermentas), 10 pmol of each universal primers SR-FWD 50-AGAGTTTGATYMTGGC-30 (positions 4-19) and SR-REV 50-GYTACCTTGTTAC GACTT-30 (positions 1505-1488) as reported by Davies *et al.* (1996). Agarose gel electrophoresis was carried out to see the results and gel extraction using QIA quick Gel extraction kit was carried out using manufacturer's instruction. The eluted PCR product was submitted for sequencing to Eurofins, Bangalore, India for gene sequencing.

Phylogenetic analysis of 16s rRNA sequence: The 16s rRNA sequence was compared with sequences available in the GenBank (NCBI) database using the Basic Local Alignment Search Tool

(BLAST). The sequence was compared with the 16s rRNA gene sequences of pathogenic avian Clostridia viz., *C. colinum*, *C. fallax*, *C. baratti* producing enteritis in chicken (Cooper *et al.*, 2013) and also with commensal Clostridia associated with avian gastrointestinal tract viz., *C. polysaccharolyticum*, *C. xylanolyticum*, *C. aurantibutyricum*, *C. glycolicum*, *C. leptum*, *C. lituseburense*, *C. saccharolyticum*, *C. thermocellum* and *C. propionicum* (Zhu *et al.*, 2002) using MEGA6 software (Tamura *et al.*, 2013). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

Toxinotyping of the isolate: Multiplex Polymerase Chain Reaction (mPCR) targeting the four major toxin genes namely *cpa*, *cpb*, *etx* and *iap*, the enterotoxin gene *cpe* and all variants of *cpb2* was carried out for toxinotyping the *Clostridium perfringens* isolate obtained using the primers described by Van Asten *et al.* (2009). Agarose gel (1.5%) electrophoresis was carried out to visualize the results after amplification by PCR. Similarly, to know the presence of *netB* gene in the isolate, PCR was carried out targeting the *netB* gene primers as reported by of Toloee *et al.* (2011).

RESULTS

On gross examination, the affected birds of the poultry farm under investigation were found weak and emaciated. Systematic necropsy examination revealed gross pathological changes predominantly in the intestinal segments. The intestinal wall appeared thickened, dark and discoloured to bluish tint. Upon opening the lumen, the mucosa showed nearly circumscribed to oval or elongated shaped necrotic to ulcerative lesions with raised and hyperemic borders (Fig. 1a). The lesions were more severe in duodenum, jejunum followed by ileum, cecum and colo-rectum. No pathologically significant gross lesions in other organs were observed. Histopathology revealed thickened serosal and muscular layers. The submucosa showed varying degrees of degenerative to necrotic changes amidst infiltration by mononuclear cells. The mucosa was characterized by superficial epithelial denudation to complete loss of villi in the necrotic areas (Fig. 1b). The duodenum in presented case consistently presented hyperplasia of submucosal glands, mononuclear cell infiltration and mild fibroplasia (Fig. 1c). Although, no significant pathological changes were observed in the liver on gross examination, histopathology revealed foci of hepatocyte necrosis and severe perivascular infiltration predominated by mononuclear lymphocytic cells (Fig. 1d) followed by a few heterophils.

Bacteriological and biochemical examination were matching characteristics of *C. perfringens*. On blood agar, the bacterial colonies were small, dew drop like and had double zone of haemolysis (Fig. 2). In egg yolk agar, opalescence was observed confirming the presence of alpha toxin of *C. perfringens*. Gram's staining revealed gram positive bacterial rods.

Amplification of 16s rRNA gene yielded expected product size of 994 bp (Fig. 3) and its sequencing and phylogenetic analysis revealed that the isolate belonged to *Clostridium perfringens* type A and evolutionarily related to *C. baratti* and *C. fallax* among *Clostridium* species associated with avian gastrointestinal tract (Fig. 4). Multiplex PCR for toxin typing of *Clostridium perfringens* was positive for α toxin (*cpa*) and β 2 toxin (*cpb2*), a feature of *C. perfringens* type A isolates (Fig. 5). The *C. perfringens* isolate was found to lack *netB* toxin gene as revealed by *netB* gene specific PCR testing.

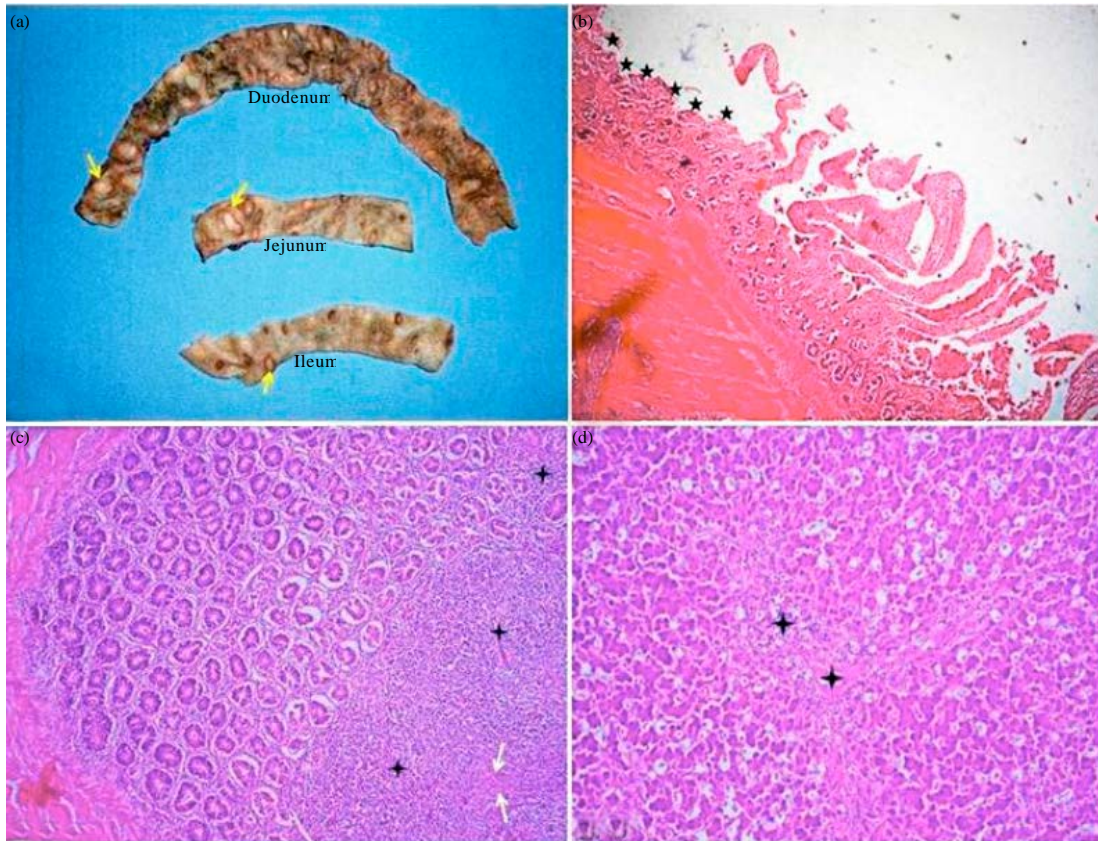


Fig. 1(a-d): (a) Necrotic to ulcerative lesions in the mucosa intestine showing raised and hyperemic borders (arrows), (b) Mucosal ulcer showing epithelial denudation to complete loss of villi (stars), (c) Hyperplastic submucosal glands amidst mononuclear lymphoid infiltration as aggregates (4-point star) in the duodenum and (d) Section of liver showing infiltration of lymphoid cells in the perivascular space (4-point star)



Fig. 2: Blood agar plate showing colonies with double zone of haemolysis

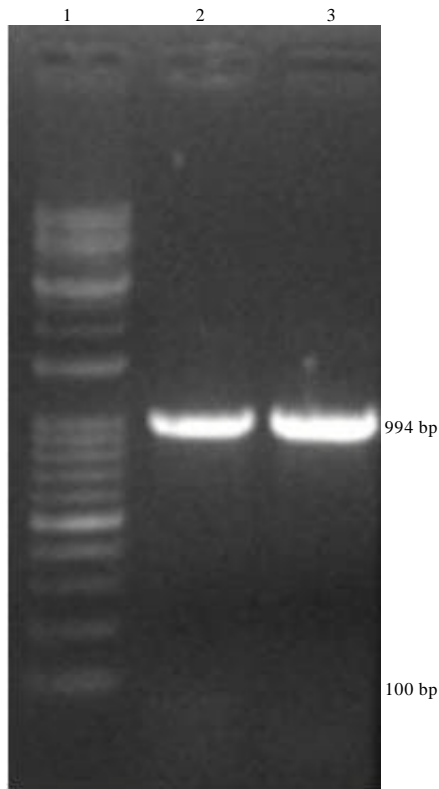


Fig. 3: 16s rRNA PCR amplification: Lane 1: 100 bp ladder (BR biochem life sciences), 2 and 3: Positive sample (replicate)

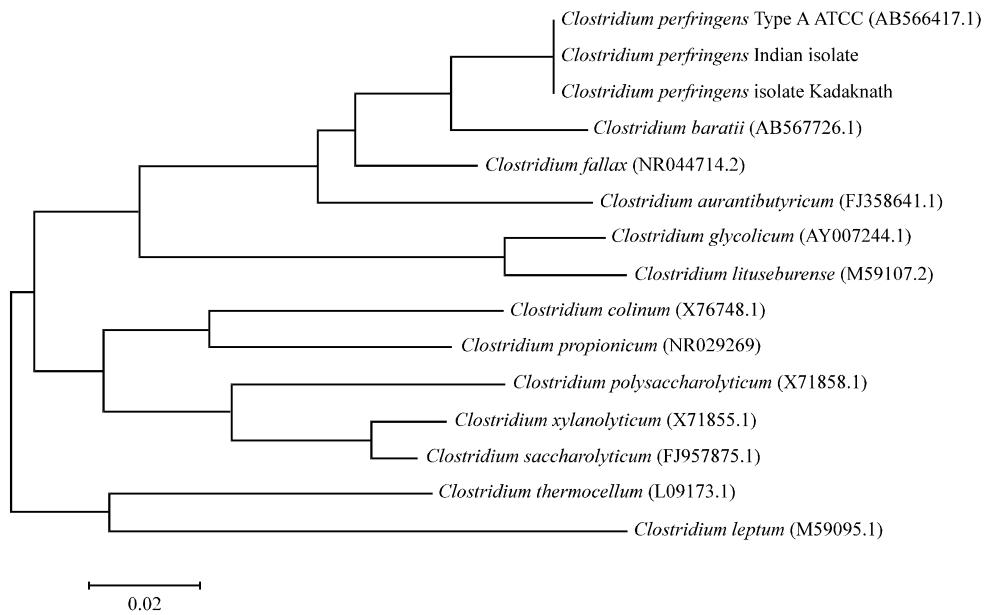


Fig. 4: Phylogenetic analysis of *Clostridium perfringens* isolate

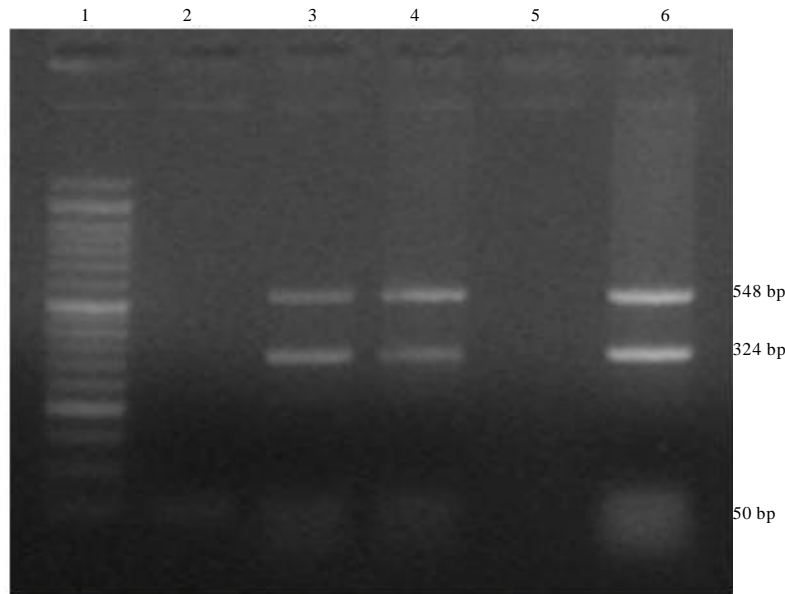


Fig. 5: Gel electrophoresis results of toxin typing of *Clostridium perfringens* by Multiplex PCR: Lane 1: 50 bp ladder (BR biochem life sciences), 2 and 5: Negative control (replicate), 3 and 4: Positive sample (replicate), 6: Positive control

DISCUSSION

Clostridium perfringens, the causative agent of necrotic enteritis in poultry, was isolated and identified from a clinical case of 8-10 weeks old Kadaknath fowl from an organized farm in Bareilly, Uttar Pradesh. Similar findings that necrotic enteritis can occur between 7-16 weeks of birds were reported by earlier workers (Frame and Bickford, 1986; Malmarugan *et al.*, 2012). Gram's staining of intestinal scrapping revealed Gram positive bacterial rods along with oocyst of *Eimeria* spp., which was correlating with the previous report (Malmarugan *et al.*, 2013). The causative agent of necrotic enteritis, *C. perfringens*, though is a commensal organism in healthy poultry but can take upper hand when there is breach in the gut wall and immunity which may be due to infections like coccidiosis (Williams, 2005). In the present study, the poultry flock investigated was recently affected with coccidiosis and hence due to the damage caused by *Eimeria*, *C. perfringens* might have flared up.

Gross pathology revealed gas filled, dilated, thin walled, friable intestine with yellowish brown diphtheritic membrane which was in accordance with earlier reports by Malmarugan *et al.* (2012). Histopathological changes revealed partial to complete loss of villi in the necrotic areas, there was hyperplasia of submucosal glands, mononuclear cell infiltration and mild fibroplasias. Similar histopathological changes were reported by Van Immerseel *et al.* (2009). Bacterial collagenases were thought to be the reason for the abundant damage caused in the intestine (Olkowski *et al.*, 2008).

Bacterial colonies on the blood agar showed double zone of haemolysis and these were small, dew drop like which were similar to the findings reported by Craven *et al.* (2001). The 16s rRNA sequence analysis showed that the sequence was similar to *Clostridium perfringens* type A and the phylogenetic tree based on the 16s rRNA sequence revealed that the isolate was evolutionarily

related to *C. baratti* and *C. fallax* but was well differentiated from the sequences studied, indicating that 16s rRNA and gene sequencing can be employed as an alternate tool for confirmative diagnosis of *C. perfringens* type A. Multiplex PCR for toxin typing of *C. perfringens* was positive for α toxin (*cpa*) and β 2 toxin (*cpb2*), a feature of *C. perfringens* type A isolates.

The isolate obtained was tested for the presence of *netB* gene by PCR amplification but the result revealed that the isolate was negative for *netB*. Recent studies have indicated that *netB* gene is a virulence factor for the causative of necrotic enteritis in chicken (Keyburn *et al.*, 2008, 2010). NetB toxin producing gene was found on a large plasmid (Lepp *et al.*, 2010). Different studies have suggested various opinions regarding the role played by NetB toxin in necrotic enteritis (Chalmers *et al.*, 2008; Martin and Smyth, 2009). NetB toxin positive isolates have been recovered from healthy birds also and similarly NetB negative isolates also got recovered from diseased birds, indicating that necrotic enteritis may be a multifactorial condition (Timbermont *et al.*, 2011). Further studies are required to know the exact role played by NetB toxin and also to find other toxin(s) that are actually the cause of necrotic enteritis as in the case of NetB negative isolates.

CONCLUSION

The present study reports the isolation and identification of *Clostridium perfringens*, causing necrotic enteritis which is an important bacterial disease of poultry, from a farm of Kadaknath fowl in India. It is a thorough disease investigation report based on clinical pathology, molecular detection of *C. perfringens* by PCR and multiplex PCR and its molecular characterization by 16s rRNA gene sequencing, phylogenetic analysis and toxinotyping. The utility of molecular detection, characterization and toxinotyping needs to be explored for rapid detection and confirmation of *C. perfringens* from suspected necrotic enteritis cases. Further studies are also suggested to find out the role of toxins in the development of necrotic enteritis in poultry.

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