RESEARCH ARTICLE

Characterization of *net*B positive *Clostridium perfringens* Type A Recovered from Necrotic Enteritis in Commercial Broiler Flocks

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Abstract

Necrotic enteritis (NE) is a significant GI tract disease in chickens, predominantly caused by type A strains of *Clostridium perfringens*. This condition presents a substantial financial burden on the farmers, jeopardizing flock health and productivity. Recent research highlights the crucial role of the netB toxin, as a primary pathogenic factor in the development of NE. Present study details an outbreak of NE recorded in a commercial broiler flock raised in deep litter system. Dead birds were presented for necropsy with a history of sudden heavy mortality. Significant pathological features in the intestines and viscera of the affected birds were suggestive of NE. Microbiological evaluation including cultural examination and molecular characterization targeting 16s rRNA sequences revealed presence of *C. perfringens*. Toxinotyping of the isolate under study revealed that the predominant toxin produced was alpha toxin and that it belongs to *C. perfringens* type A. The isolate was also found to produce *net*B toxin. Heavy mortality recorded in the present investigation may be attributed to *net*B gene.

Keywords: α toxin, *Clostridium perfringens*, Necrotic enteritis, *Net*B toxin, Toxinotyping.

Ind J Vet Sci and Biotech (2025): 10.48165/ijvsbt.21.3.12

Introduction

Clostridium perfringens is the primary instigating factor in the development of Necrotic enteritis (NE), first documented in Australia in 1930 (Parish, 1961). Thereafter, NE was recorded sporadically across the globe (Timbermont *et al.,* 2011). *C. perfringens* is an anaerobic, spore forming Gram positive bacterium and is a constituent of normal flora in GIT of poultry, cattle, sheep and goat (Hatheway, 1990; Ficken, and Wages, 1997). NE in chicken leads to substantial financial setback to the poultry industry by reducing feed efficiency and growth rate. NE affects the broilers in the age group of 2 to 6 weeks and layers of 3-6 months, and is mostly observed either in clinical or subclinical form (Skinner *et al.*, 2010). Clinical form of NE occurs as an outbreak with clinical signs like depression, ruffled feathers, and diarrhea with high mortality. Subclinical NE is usually manifested as impaired growth rate and inefficient feed utilization. Peracute form has also been described where the affected birds succumb in few hours, and mortality rising upto 50%. Sudden feed changes, poor hygiene, stress, mucosal damage caused by parasites like *Coccidia* spp., and other immunosuppressive diseases often predispose chickens to NE (Shojadoost *et al.,* 2012; Moore, 2016). Chronic intestinal damage caused by necrotic enteritis, combined with coccidial infections, can impair gut absorption, reduce body weights and lower feed efficiency, leading to inadequate returns.

C. perfringens produces variety of toxins that contributes to its virulence. Four different toxins produced by *C. perfringens* are alpha, beta, iota and epsilon. Based on the toxins produced, *C. perfringens* is subdivided into five biotypes, type A secretes alpha toxin, type B secretes alpha, beta and epsilon toxins, type C secretes alpha and beta toxins, type D secretes alpha and epsilon toxins, and type E secretes alpha and iota toxins (Schlegel *et al.,* 2012). Of the four toxins, alpha is appraised as the crucial element responsible for heavy mortality that occur during NE. Keyburn *et al.* (2010) found a novel virulence factor, *Net*B (Necrotic enteritis B-like toxin) in a *C. perfringens* mutant unable to produce α-toxin, yet still causing NE. *Net*B gene encodes a pore-forming toxin and is located on a plasmid of size 85 Kbp. Current investigation reports an outbreak of NE in a commercial broiler flock caused by type A strains of *C. perfringens*.

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How to cite this article: Balam, D., Maddireddy, H., Moturi, S., & Kumari P., M. (2025). Characterization of *net*B Positive *Clostridium perfringens* Type A Recovered from Necrotic Enteritis in Commercial Broiler Flocks. Ind J Vet Sci and Biotech, 21(3), 58-62.

Source of support: Nil

Conflict of interest: The authors state that they have no competing interests.

Submitted 24/02/2025 **Accepted** 19/03/2025 **Published** 10/05/2025

Materials and Methods

Clinical History and Necropsy

History revealed that a commercial broiler farm in Andhra Pradesh with a capacity of 40,000 birds experienced heavy mortality during May, 2024 in one of its broiler stocks aged 4-6 weeks. The birds were raised in deep litter system. Over a period of 15 days, the affected stock showed a mortality percentage of 7.08% (620/ 8750). A total of thirty birds were presented for postmortem examination. Before death, the affected birds exhibited clinical signs like ruffled feathers, decreased appetite, severe depression, reluctancy to move, diarrhea, dehydration and progressing debility. Necropsy was performed, and samples, including intestinal scrapings and liver tissue, were aseptically collected for microbiological evaluation. Additionally, the tissues were subjected to histopathological examination.

Bacteriological Examination

Intestinal scrappings suspended in sterile normal saline were heated in water bath at 80 °C for 20 min. The treated samples were inoculated into thioglycolate broth and incubated in an anaerobic jar with gas generating packs at 37° C for 18-24 h. Broth culture was subsequently spread onto Perfringens agar base (Tryptose Sulphite Cycloserine -TSC) supplemented with 5% egg yolk and incubated in an anaerobic jar with gas generating packs at 37° C for 24 h. The identity of *C. perfringens* was confirmed based on lecithinase activity and haemolysis pattern on TSC agar and 5% blood agar, respectively, Gram staining and lactose fermentation.

Isolation of DNA and PCR

Boiling and snap-chilling method was used to isolate DNA. Briefly, 2 mL of overnight *C. perfringens* culture was spinned at 10,400 g for 10 min. PBS was added to the pellet and spinned once again at the same speed for 10 min. The pellet obtained was then solubilized in 200 µL of nuclease free water and boiled at 100 °C for 10 min, immediately followed by snap chilling for 6-7 min. The lysate was spinned for 10 min at 10,400 g and the supernatant was transferred into fresh tubes and preserved at -20 °C.

The extracted DNA was used to identify *C. perfringens* using primers targeting 16s rRNA gene producing a predicted amplicon of 481 bp (Tonooka *et al.,* 2005). PCR was performed in 10 µL reaction mixture , 2X master mix (M/s ProMega) and 10 pmol of each primer. DNA amplicons were electrophoresed on 1% agarose gel with ethidium bromide and visualized using UV light.

Toxinotyping of *C. perfringens*

C. perfringens toxinotyping was accomplished by multiplex PCR to identify the presence of genes encoding alpha, beta, iota and epsilon toxins using previously described primers (Van Asten *et al.,* 2009). Additionally, a separate PCR was conducted to detect *net*B gene using primers reported in previous study (Keyburn *et al.,* 2010).

Results and Discussion

Clostridium perfringens is a major pathogen affecting livestock, poultry and humans, causing a range of enteric infections and fatal intoxications in various animal species (Markey *et al.,* 2013). Necrotic enteritis in poultry is a significant disease caused by *C. perfringens*. In this study, we isolated *C. perfringens* from a four to six week old broiler flock exhibiting symptoms consistent with necrotic enteritis. Previous studies have also reported the occurrence of this condition in both broiler (Mwangi *et al.,* 2013; Thomas *et al.,* 2014) and layer (Kurkure *et al*., 2017) flocks.

Clinical examination of the affected flock exhibited symptoms such as diarrhoea, anorexia, listlessness and ruffled feathers. Necropsy revealed predominant lesions in the GI tract. Intestines were thick, dark with bluish discolouration. Mucosal surfaces resembled turkish towel like appearance with blackish discolouration, consistent with findings of Park *et al.* (2015). Other changes observed were echymotic haemorrhages in lungs, necrotic foci and congestion in liver and mild swelling and congestion in kidneys (Fig. 1).

Coccidial oocysts were identified in the intestinal scrapings collected during necropsy. Impression smears of these scrapings, stained with Leishman's stain, revealed numerous thick bacilli (Fig. 2). Histopathological examination showed thickening of the serosal and mucosal layers, leukocytic infiltration, villous disruption, and haemorrhages in the lamina propria and submucosa (Fig. 3). Coccidial infection damages the intestinal lining, compromising its integrity and increasing permeability, which facilitates rise in the population of *C. perfringens* and establishment of infection (Shojadoost *et al.,* 2012). The outbreak occurred in May, coinciding with high environmental temperatures and humidity levels. In the present investigation, disease incidence was recorded in birds raised under a deep litter system that might have contributed to coccidial infections due to accumulation of moisture and organic material in the litter. Although *C. perfringens* is a commensal in the GIT of poultry, animals and humans, an increase in the bacterial counts was recorded in the present study, as evidenced by the abundant bacilli in the impression smears of intestinal scrappings. This aligns with the findings of McDevitt *et al.* (2006), who reported an increase in the population of *C. perfringens* from approximately 10^4 CFU in healthy birds to 10^7-10^9 CFU in chicken with NE.

Microscopically, the gastrointestinal tract exhibited thickening of the serosal and mucosal layers, along with villous disruption in the submucosal layers and lamina propria-specific lesions of necrotic enteritis (Kurkure *et al*., 2017). Bacterial culture findings showing black colonies with opalescence on Perfringens agar base and partial haemolysis in 5% sheep blood agar were in accordance with Alsaab *et al.,* (2021).

Microbiological investigations aligned with the clinical findings and confirmed the presence of *C. perfringens* in birds suspected for NE. TSC agar supplemented with egg yolk emulsion showed black colonies with a surrounding zone of opalescence. Gram staining of the colonies showed Gram positive straight bacilli (Fig. 4). Culturing on 5% sheep blood agar produced grey round, smooth colonies exhibiting partial haemolysis.

Molecular characterization established based on 16s rRNA amplified the required DNA product of 481 bp confirming the presence of *C. perfringens* in the samples collected from affected birds. Multiplex PCR for *C. perfringens* toxinotyping detected only the α-toxin (*cpa*), while all other toxins (*cpb, etx* and *iap*) were absent. Notably, this isolate was also positive for *net*B toxin gene (Fig. 5).

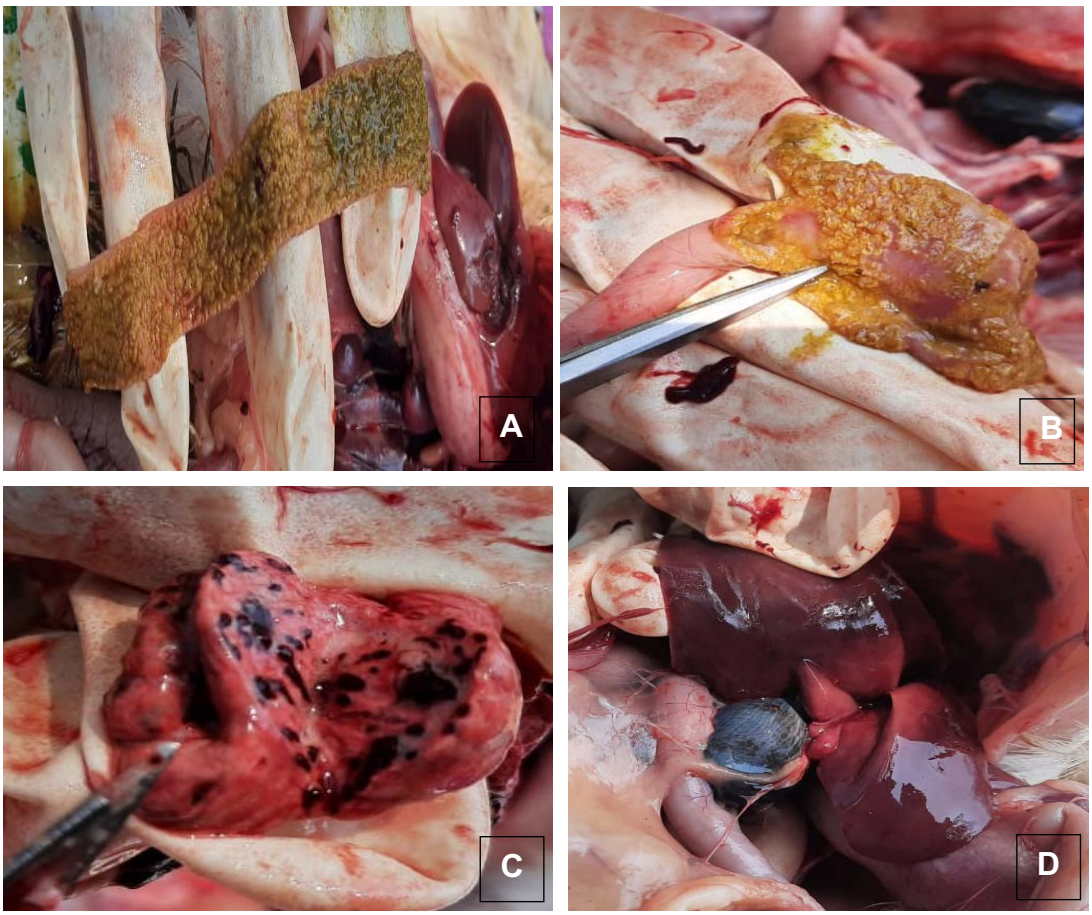

Fig.1: A & B Turkish towel like appearance of intestines with blackish discolouration. **C.** Echymotic haemorrhages on lungs. **D.** Necrotic foci and congestion in liver

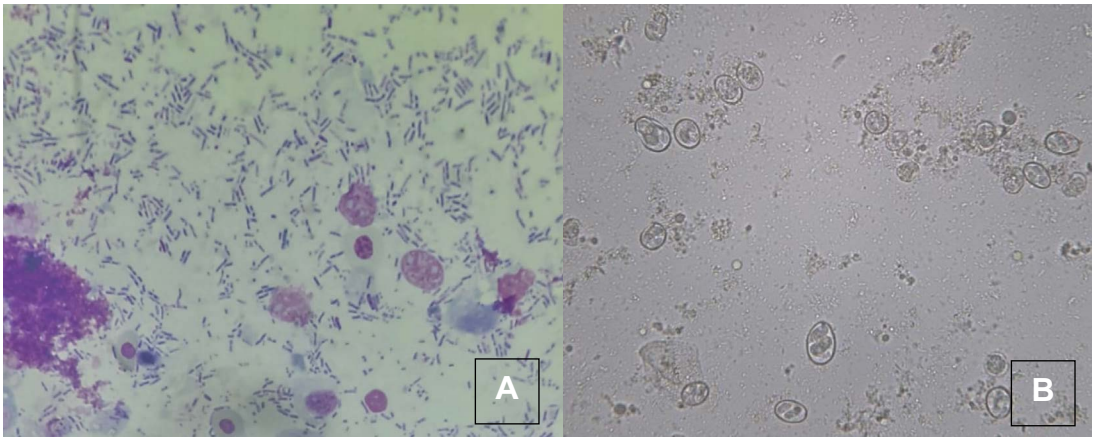

Fig. 2: A. Large number of *C. perfringens* bacilli in the impression smears of intestinal scrappings stained by Leishman's staining (1000X). **B.** Eimeria oocysts in the intestinal scrappings (400X).

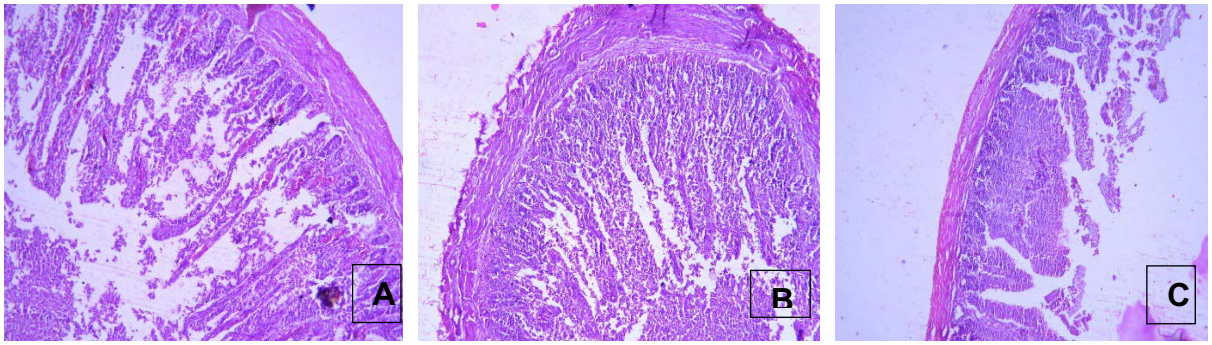

Fig. 3: Intestine showing: A. disruption of villi and congestion, B. Thickening of serosal layer, C. Necrosis

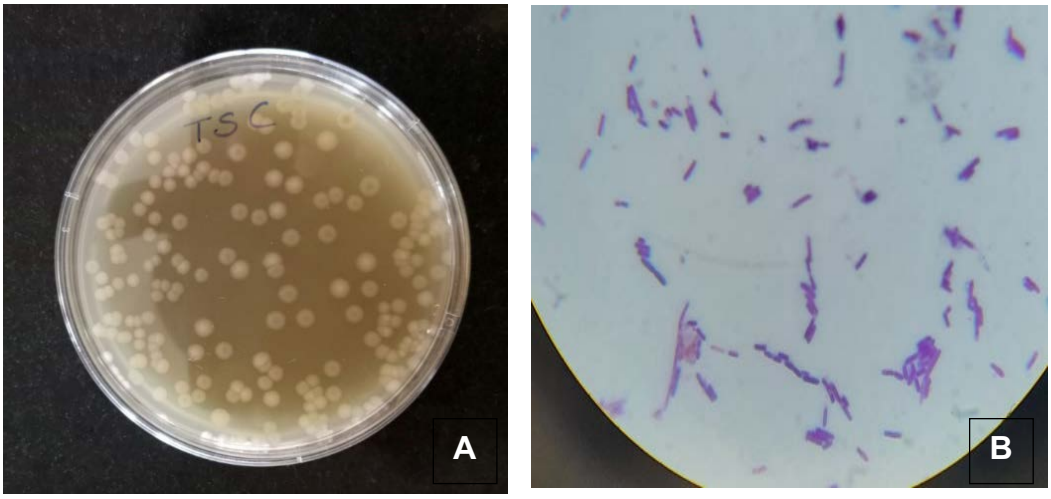

Fig. 4: A. Black colonies surrounded by zone of opalescence on TSC agar, **B.** Gram positive straight bacilli (1000X)

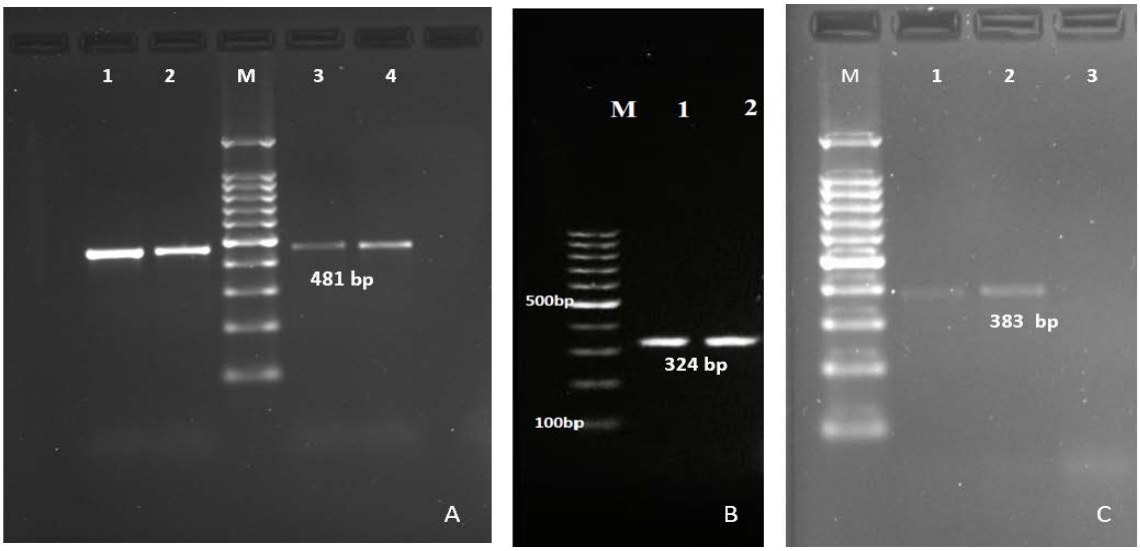

Fig. 5: A. PCR gel pictures showing amplification of 16s rRNA gene of *C.perfringens.* **B.** Multiplex PCR gel pictures showing amplification of *cpa* (Alpha toxin) gene. **C.** PCR gel pictures showing amplification of *net*B gene of *C. perfringens.*

Type A strains of *C. Perfringens* is the frequent toxin-producing type, with alpha-toxin recognized as a critical pathogenicity determinant responsible for NE in chicken and food poisoning in humans (Timbermont *et al.,* 2011). The alpha toxin is a pore-forming toxin with phospholipase, haemolytic, dermonecrotic and lecithinase activities, produced by all *C. Perfringens* subtypes. Alpha toxin gene is a part of the larger genetic element, *i.e.* bacterial chromosome (Stiles *et al.,* 2013; Simpson *et al.,* 2018).

NetB gene, first identified in 2008, has been recognized as a major pathogenicity element involved in development of NE in chicken. It is plasmid encoded and functions as a pore-forming toxin that damages the intestinal epithelial cells, leading to gut tissue necrosis (Savva *et al.,* 2013). Strains of *C. perfringens* with *netB* gene have been linked to higher disease incidence and more severe forms of NE in broiler flocks. Challenge experiments have demonstrated that strains negative for *net*B induced mild or no disease, whereas, *net*B positive strains cause severe necrotic enteritis in healthy chickens. Furthermore, chicken vaccinated with recombinant *net*B toxin showed high immunogenicity against challenge with virulent *C. perfringens*, highlighting the immunogenic potential of *net*B toxin (Keyburn *et al.,* 2013).

Conclusion

This investigation details the isolation and characterization of *C. perfringens* in broilers suffering from necrotic enteritis. Toxin typing identified the presence of genes encoding α-toxin and *net*B toxin. Future research is warranted to clarify the specific function of *net*B toxin in the disease process and to investigate whether a synergistic interaction between α-toxin and *net*B toxin contributes to heightened mortality in affected birds.

Acknowledgement

We express our sincere thanks to SVVU, Tirupati, India for funding the research.

References

Alsaab, F., Wahdan, A., & Saeed, E.M. (2021). Phenotypic detection and genotyping of *Clostridium perfringens* associated with enterotoxaemia in sheep in the Qassim region of Saudi Arabia. *Veterinary World, 14*(3), 578.

Ficken, M.D., & Wages, D.P. (1997). Necrotic enteritis. In: Calnek BW. *Diseases of Poultry.* 10th edn., Iowa State University Press Ames, Iowa, USA, pp 261-264.

Hatheway, C.L. (1990). Toxigenic clostridia. *Clinical Microbiology Review, 3(*1), 66-98.

Keyburn, A.L., Yan, X.X., Bannam, T.L., Van Immerseel, F., & Rood, J.I. (2010). Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing *net*B toxin. *Veterinary Research, 41*(2), 21.

Keyburn, A.L.R., Portela, W., Ford, M.E., Bannam, T.L., & Yan, X.X. (2013). Maternal immunization with vaccines containing recombinant *Net*B toxin partially protects progeny chickens from necrotic enteritis. *Veterinary Research, 44*, 108-114.

Kurkure, N.V., Kale, S.B., Kale, R.T., Awandkar, S.P., & Joat, N.N. (2017). Occurrence of necrotic enteritis in a commercial layer flock. *Indian Journal of Veterinary Pathology, 41*(1), 50-52.

Markey, B., Leonard, F., Archambault, M., Cullinane, A., & Maguire, D. (2013). *Clinical Veterinary Microbiology.* 2nd edn., Mosby, Elsevier, p. 229.

McDevitt, R.M., Brooker, J.D., Acamovic, T., & Sparks, N.H.C. (2006). Necrotic enteritis: A continuing challenge for the poultry industry. *World Poultry Science Journal, 62,* 221-247.

Moore, R.J. (2016). Necrotic enteritis predisposing factors in broiler chickens. *Avian Pathology, 45,* 275-281.

Mwangi, S., Timmons, J., Fitz-Coy, S., & Parveen, S. (2019). Characterization of *Clostridium perfringens* recovered from broiler chicken affected by necrotic enteritis. *Poultry Science, 98*(1), 128-135.

Parish, E. (1961). Necrotic enteritis in the fowl (*Gallus Gallus Domesticus*): Histopathology of the disease and isolation of a strain of *Clostridium Welchii. Journal of Comparative Pathology and Therapeutics, 71,* 377.

Park, J.Y., Kim, S., Oh, J.Y. et al. (2015). Characterization of *Clostridium perfringens* isolates obtained during 2010 to 2012 from chickens with necrotic enteritis in Korea. *Poultry Science, 94*(6), 1158-1164.

Savva, C.G., Fernandes da Costa, S.P., Bokori-Brown, M., Naylor, C.E., & Cole, A.R. (2013). Molecular architecture and functional analysis of *Net*B, a pore-forming toxin from *Clostridium perfringens*. *Journal of Biological Chemistry, 288,* 3512-3522.

Schlegel, B.J., Nowell, V.J., Parreira, V.R., Soltes, G., & Prescott. J.F. (2012). Toxin-associated and other genes in *Clostridium perfringens* type A isolates from bovine clostridial abomasitis (BCA) and jejunal haemorrhage syndrome (JHS). *Canadian Journal of Veterinary Research, 76,* 248-254.

Shojadoost, B., Vince, A., & Prescott, J. (2012). The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: A critical review. *Veterinary Research, 43,* 74-85.

Simpson, K.M., Callan, R.J., & Van-Metre, D.C. (2018). Clostridial abomasitis and enteritis in ruminants. *Veterinary Clinics of North America: Food Animal Practice, 34,* 155-184.

Skinner, J.T., Bauer, S., Young. V., Pauling, G., & Wilson, J. (2010). An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. *Avian Diseases, 54*(4), 1237-1240.

Stiles, B.G., Barth, G., Barth, H., & Popoff, M.R. (2013). *Clostridium perfringens* epsilon toxin: A malevolent molecule for animals and man. *Toxins, 5*(11), 2138-2160.

Thomas, P., Arun, T.R., Karthik, K., Berin, P.V., & Kumar, M.A. (2014). Molecular characterization and toxinotyping of a *Clostridium perfringens* isolate from a case of necrotic enteritis in Indian Kadaknath fowl. *Asian Journal of Animal and Veterinary Advances, 9*(7), 385-394.

Timbermont, L., Haesebrouck, F., Ducatelle, R., & Van Immerseel, F. (2011). Necrotic enteritis in broilers: An updated review on the pathogenesis. *Avian Pathology, 40,* 341-347.

Tonooka, T., Sakata, S., Kitahara, M., Hanai, M., & Ishizeki, S. (2005). Detection and quantification of four species of the genus Clostridium in infant feces. *Microbiology and Immunology, 49*(11), 987-992.

Van Asten, A.J., van der Weil, C.W., Nikolaou, G., Houwers, D.J., & Grone, A. (2009). A multiplex PCR for toxin typing of *Clostridium perfringens* isolates. *Veterinary Microbiology, 136*(3-4), 411-412.