



Case Report

DOI: 10.22067/veterinary.v9i2.56897

Received: 2016-Jun-19

Accepted after revision: 2017-Apr-18

Published online: 2018-Jan-20

NetB negative *Clostridium perfringens* infection associated with acute necrotic enteritis in mynah (*Acridotheres tristis*), grey partridge (*Perdix perdix*) and turkey (*Meleagris gallopavo*)

Jamshid Razmyar,^a Massoud Rezaee,^b Ahmad Reza Movassaghi,^b Bahram Shojadust^c

^a Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

^b Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

^c Department of Pathobiology, University of Guelph, Guelph, Ontario, Canada

Keywords

Mynah (*Acridotheres tristis*); Grey Partridge (*Perdix perdix*); Turkey (*Meleagris gallopavo*); *Clostridium perfringens*; *cpb2*; *cpa*.

Abstract

A non-enterotoxin (CPE)-producing *Clostridium perfringens* type A, associated with enteritis in a mynah (*Acridotheres tristis*), a grey partridge (*Perdix perdix*) and a turkey (*Meleagris gallopavo*) was characterized from cases with clinical symptoms from September 2010 until October 2012. Affected birds exhibited anorexia and diarrhea. Gross and histological findings were indicative of acute necrotic enteritis. *Clostridium perfringens* was isolated in bacterial cultures. Multiplex PCR for toxin profiling of the isolates revealed that the all three isolates were *Clostridium perfringens* type A, positive for *cpb2* and *cpa*.

Abbreviations

C. perfringens: *Clostridium perfringens*
PCR: Polymerase Chain Reaction
SBA: Sheep Blood Agar
TSC: Tryptose Sulfite Cycloserine agar
TSN: Tryptose Sulfite Neomycin agar
CPA: *C. perfringens* Alpha toxin
cpb2: *Clostridium perfringens* beta2 toxin
netB: Necrotic Enteritis Toxin B
cpe: *Clostridium perfringens* Entrotoxin
etx: Epsilon Toxin
itx: Iota Toxin
TcdA: *Clostridium difficile* toxin A
TcdB: *Clostridium difficile* toxin B
TcsL: *Clostridium sordellii* lethal toxin
TcnA: *Clostridium novyi* alpha-toxi

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

Tel: +98 51 3880 3742 Fax: +98 51 3876 3852
Web: ijvst.um.ac.ir Email: ijvst@um.ac.ir

Corresponding author:

Jamshid Razmyar
Department of Poultry Diseases, Faculty of Veterinary Medicine,
University of Tehran, Tehran, Iran

Telephone Number: +98 9123873258
E-mail: jrazmyar@ut.ac.ir

Introduction

Necrotic enteritis is caused by *Clostridium perfringens* (CP), a gram positive, anaerobic bacterium which is commonly an inhabitant of the gastrointestinal tract of many mammalian and avian species [1-3].

This microorganism is recognized as an important pathogen in many species. It can cause gas gangrene and food poisoning in humans, necrotic enteritis in poultry species, enterotoxemia in calves and lambs, enteritis in cattle, dogs, pigs and horses [1-3]. The role of other recently investigated toxins such as Net B, TpeL and cpb2, remains to be completely defined [4]. Researchers have clarified the role of the β , ϵ and ι toxins in the pathogenesis of enteric diseases, but the exact role of the α -toxin is still controversial [2, 5, 6]. CP has been isolated from enteritis cases of different avian species like chickens, turkeys, ostriches and rarely psittacine birds [1, 7-13]. Nevertheless there is no report of isolation of CP from enteritis in some other species such as mynahs and partridges. Diets containing high concentration of proteins, polysaccharides and fat favor the intestinal environment for the *Clostridium perfringens* growth [14, 15]. Furthermore, stressful factors can also lead to occurrence of necrotic enteritis [13].

This study describes histopathologic, microbiologic and molecular toxinotyping of three cases of NetB negative *Clostridium perfringens* infection associated with acute necrotic enteritis in a mynah (*Acridotheres tristis*), a grey partridge (*Perdix perdix*) and a turkey (*Meleagris gallopavo*).

Case description

Case 1: A one-month-old female mynah (*Acridotheres tristis*) presented with severe dysentery and anorexia. Symptomatic therapy was initiated, but clinical signs deteriorated and the animal was finally euthanized. At necropsy, small intestines were swollen, filled with gas, and thin walled. Diffused mucosal necrosis was also observed in large portions of small intestine which covered by a yellow-brown pseudomembrane.

Case 2: In a flock of 300 broiler turkeys (*Meleagris gallopavo*), 100 birds were died in a short period of time with clinical signs of diarrhea and lethargy. At necropsy, congestion and hemorrhage of the small intestine was observed in some of the recently dead birds. Tissue specimens were taken from intestines for histopathologic, microbiologic

and molecular assessments.

Case 3: Followed by mortality in a flock of partridge (*Perdix perdix*), a 40-day-old male partridge with a history of diarrhea was referred for necropsy. At necropsy, there were hemorrhagic foci and congestion in the intestines.

Pathology

Necropsy and gross pathologic examination were carried out on all three cases. Small intestine samples, were fixed in 10% neutral buffered formalin, processed and stained with Hematoxylin and Eosin [16].

Bacterial isolation and characterization

Samples for bacterial isolation were obtained aseptically with sterile swabs from the gut, and were subjected to gram staining. Subsequently, intestinal sample was streaked onto blood agar plates containing 7% defibrinated sheep blood and incubated anaerobically at 37°C for 48 hr. Colonies which showed characteristic dual hemolytic zones were picked up and sub-cultured in Tryptose Sulfite Cycloserine agar (TSC) and Tryptose Sulfite Neomycin agar (TSN) for purification. The identity of the isolates was confirmed by their colonial and microscopic morphology, hemolytic pattern and Gram staining. All culture media and additives used in this study were provided from Merck (Germany). Reference strains of *Clostridium perfringens*: ATCC 13124 (cpa); CIP 106157 (cpa, cpe); CIP 60.61 (cpa, cpb, etx, cpb2) were used as positive controls. All bird treatments were conducted according to Animal Care Guidelines of the Research Committee, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.

Multiplex PCR

A single colony of the isolate was suspended in 100 μ l distilled water, boiled for 10 min and then centrifuged at 10 000 g for 10 min. The supernatants were collected carefully and used as template DNA for PCR. Six pairs of primers were used to determine the presence of cpa, cpb, iA, etx, cpe and cpb2 genes using a multiplex PCR technique for all isolates [12, 23]. The primers and other materials used in PCR reaction were provided by Ampliqon [Odense, Denmark]. Amplification reactions were carried out in a 50 μ l reaction volume containing 5 μ l 10 x PCR buffer, 5mM dNTPs, 25 mM MgCl₂, 5 U of TaqDNA polymerase, 0.5 mM of each cpa oligo, 0.36 mM of each cpb oligo, 0.36 mM of

each cpb2 oligo, 0.52 mM of each iA oligo, 0.44 mM of each etx oligo, 0.34 mM of each cpe oligo and dH₂O. Ten µl of template DNA was added to the mixture. Amplification was programmed in a thermocycler (Techne TC-3000, England) as follows: 95°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72° C for 10 min [13].

***Tpel* single PCR**

PCR to detect TpeL was performed subsequently with 50 pM of primers [17]. PCR conditions included initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and with a final extension step at 72°C for 7 min.

Single PCR for netB:

Previously developed primers were used to detect netB gene[18]. Reactions were carried out in a 25 µL reaction volume containing: 2 µL 10 x PCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs mixture, 2.5 units of Taq DNA polymerase, 0.1 µM of each primer, dH₂O and 5 µL of DNA extraction solution. Amplification conditions were as follows: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec and a final extension at 72°C for 12 min. Reference strains of *Clostridium perfringens* ATCC 13124 (cpa); CIP 106157 (cpa, cpe); CIP 60.61 (cpa, cpb, etx, cpb2) and two isolates JRMTK01 (netb+), and JRTK01 (tpeL+) were used as positive controls. The amplification products were detected by gel electrophoresis (Padideh NojenPars, Iran) in 1.5% agarose gel in 1x TAE buffer, stained with 0.5 µg/ml EtBr. Amplified bands were visualized and photographed under UV transilluminator.

Results

Characterization of Bacterial Isolates

Bacterial isolate exhibited the characteristic features of *C. perfringens*. The colonial characters on blood agar showed dew drops, smooth, grayish, and convex colonies with a double zone of haemolysis. *Clostridium perfringens* formed black colonies in TSC and TSN agar. Microscopic characters revealed Gram positive non motile rods with box-car-shaped square cells. The Gram stain of tissue specimens from field cases of NE demonstrated

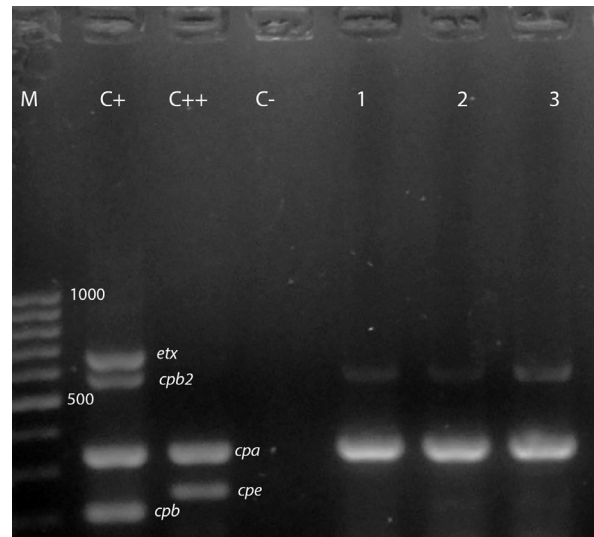


Figure 1

Agarose gel (1.5%) electrophoresis of multiplex PCR products of *C. perfringens* isolates. Lane M: size marker; Lane C+: Positive control for cpb, etx and cpb2 (CIP 60.61); lane C++: Positive control for cpa and cpe (CIP 106157); lane C-: Negative control; lane 1: isolates of *C. perfringens* obtained from case 1 (Mynah), that were positive for cpa and cpb2; lane 2: isolates of *C. perfringens* obtained from case 2 (turkey) that were positive for cpa and cpb2; lane 3: isolates of *C. perfringens* obtained from case 3 (partridge) that were positive for cpa and cpb2.

that rod-shaped bacteria with typical *C. perfringens* morphology formed large clumps primarily around the necrotic areas.

Multiplex and single PCRs

The *C. perfringens* isolates from all tested birds were characterized as type A, positive for cpb2 (Figure 1) and negative for netB and tpeL.

Histopathology

Microscopic examination of case No.1 (mynah), revealed heavy infiltration of heterophils in the intestinal mucosal layer with severe hemorrhage and congestion. Severe necrosis and desquamation of enterocytes was also observed (Figure 2). Histopathologic lesions in case No. 2 (turkey) revealed necrosis of enterocytes along with destruction of intestinal villus. Hemorrhage and congestion were also observed (Figure 3). In case No. 3 (partridge), severe destruction of intestinal villi associated with hemorrhage and congestion were evident.

Results from pathologic, microbiologic and molecular examinations were representative of necrotic enteritis and the microorganism isolated was confirmed as *Clostridium perfringens* type

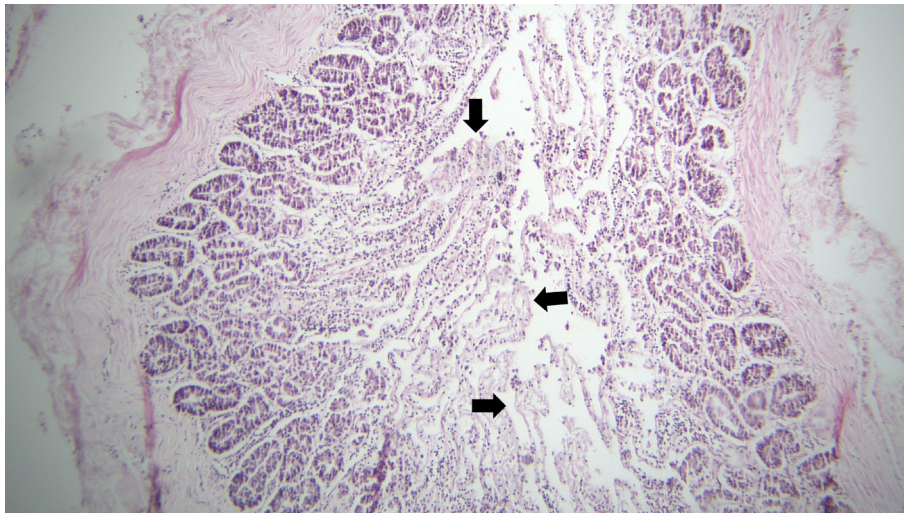


Figure 2
Necrotic enteritis in a Myna. Necrosis of the villi (arrows) are not seen at the surface of the intestinal epithelium (H&E, 100x).

A. This bacteria has been widely studied in broiler and laying chickens and is known as a cause of mortality in flocks [13]. To the authors' knowledge, there is not enough literature, about the exact prevalence of this microorganism in exotic birds and other minor avian species like partridges or even turkeys.

Discussion

Clostridium perfringens is a ubiquitous bacterium found in the environment like soils and intestinal flora. So far, there are few reports of necrotic enteritis due to *Clostridium perfringens* type A in turkey flocks [7]. Toxinotyping of *C. perfringens* isolated from diseased and healthy turkeys, revealed that 100% of the isolates were positive for α -toxin, while just 6.6% of the isolates, which were just from necrotic enteritis cases, were positive for NetB. This phenomenon shows the importance of this gene in the pathogenesis of *C. perfringens*

in turkey, as in none of the isolates from healthy turkeys, this gene was identified. Nevertheless, it should be considered that 93.4% of the necrotic enteritis cases were negative for NetB, which indicates other pathogenesis factors of CP, may be involved in the above mentioned outbreak. In this study we described isolation and identification of a *Clostridium perfringens* type A, as a cause of a huge mortality and loss in a broiler turkey flock.

To the authors' knowledge there is no report concerning enteritis caused by *Clostridium perfringens* type A in partridges. A similar report from red-legged partridges has been published in which *Clostridium perfringens* type C was isolated. Occurrence of *Clostridium perfringens* type A infection in game birds or exotic avian species is very rare, and most of the reports and studies are related to commercial flocks such as broiler chicks, ostriches and quails [7-11, 15, 19]. Our knowledge about pathogenesis of Clostridial infections in

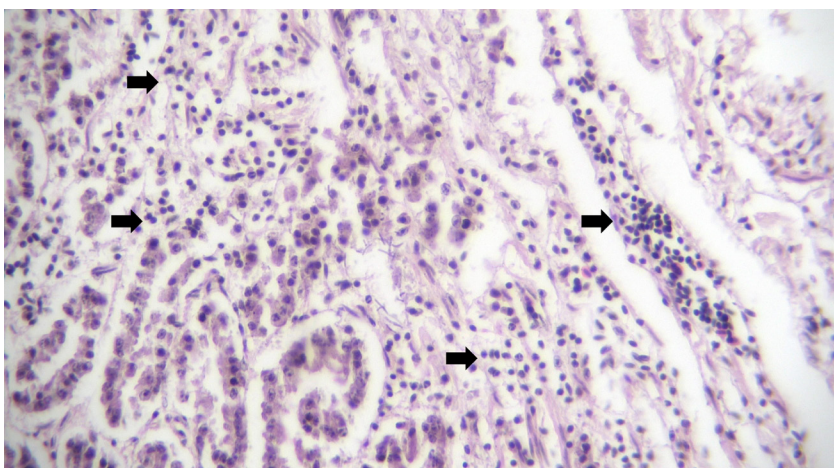


Figure 3
Necrotic enteritis in a Turkey. Arrows show the infiltration of inflammatory cells (H&E, 400x).

birds are mostly based on studies in broiler chickens which might not be the same in other bird species [1, 7, 10, 13, 15].

In the present study, all isolates were positive for *cpb2* and *cpa* and negative for *cpb*, *iA*, *etx*, *cpe*, *netB* and *tpeL* genes. These results suggest that pathogenesis of the *C. perfringens* in the species discussed in this study may be different from those in commercial poultry chickens.

NetB and *tpeL* are two key virulent factors expressed by *Clostridium perfringens* to induce clinical diseases in chickens. While importance of *netB* and *tpeL* in pathogenesis of clostridial necrotic enteritis in chickens has been shown [17, 18, 20-22], all isolates in this study were negative for *netB* and *tpeL*. These findings suggest that clinical disease may appear even in the absence of *tpeL* and *netB* genes. More studies are required to clarify the importance of different genotypes of *Clostridium perfringens* isolates, and the exact role of different toxins in the pathogenesis of necrotic enteritis in birds.

To our knowledge this is first report of occurrence and genotyping of necrotic enteritis caused by *Clostridium perfringens* type A in partridges (*Perdix perdix*) and Mynah (*Acridotheres tristis*).

Acknowledgements

We would like to thank Mr. Ali Kargar for his assistance in the Laboratory of Avian Health and Diseases, Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.

Author Contributions

Designed the study and conducted the systematic literature review: J.R. and B.Sh. Performed pathological studies: A.R.M. and M.R.

Conflict of Interest

The authors declare that they have no competing interests.

References

1. Slavic D, Boerlin P, Fabri M, Klotins KC, Zoethout JK, Weir PE, et al. Antimicrobial susceptibility of *Clostridium perfringens* isolates of bovine, chicken, porcine, and turkey origin from Ontario. Canadian journal of veterinary research = Revue canadienne de recherche veterinaire. 2011;75[2]:89-97.
2. Songer JG. Clostridial enteric diseases of domestic animals. Clinical Microbiology Reviews. 1996;9[2]:216-34.
3. Van Immerseel F, De Buck J, Pasmans F, Huyghebaert G, Haesebrouck F, Ducatelle R. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian pathology : journal of the WVPA. 2004;33[6]:537-49.
4. Nowell VJ, Poppe C, Parreira VR, Jiang YF, Reid-Smith R, Prescott JF. *Clostridium perfringens* in retail chicken. Anaerobe. 2010;16[3]:314-5.
5. Timbermont L, Lanckriet A, Gholamiandehkordi AR, Pasmans F, Martel A, Haesebrouck F, et al. Origin of *Clostridium perfringens* isolates determines the ability to induce necrotic enteritis in broilers. Comparative immunology, microbiology and infectious diseases. 2009;32[6]:503-12.

6. Van Immerseel F, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in microbiology. 2009;17[1]:32-6.
7. Crespo R, Fisher DJ, Shivaprasad HL, Fernandez-Miyakawa ME, Uzal FA. Toxinotypes of *Clostridium perfringens* isolated from sick and healthy avian species. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc. 2007;19[3]:329-33.
8. Kwon YK, Lee YJ, Mo IP. An outbreak of necrotic enteritis in the ostrich farm in Korea. The Journal of veterinary medical science. 2004;66[12]:1613-5.
9. O'Toole D, Mills K, Ellis R, Farr R, Davis M. Clostridial enteritis in red lories [*Eos bornea*]. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc. 1993;5[1]:111-3.
10. Petit L, Gibert M, Popoff MR. *Clostridium perfringens*: toxinotype and genotype. Trends in microbiology. 1999;7[3]:104-10.
11. Pizarro M, Hofle U, Rodriguez-Bertos A, Gonzalez-Huecas M, Castano M. Ulcerative enteritis [quail disease] in lories. Avian diseases. 2005;49[4]:606-8.
12. Razmyar J, Kalidari GA, Toloee A, Rad M, Movassaghi AR. Genotyping of *Clostridi-*

- um perfringens isolated from healthy and diseased ostriches [Struthio camelus]. Iranian journal of microbiology. 2014;6[1]:31-6.
13. Timbermont L, Haesebrouck F, Ducatelle R, Van Immerseel F. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian pathology : journal of the WVPA. 2011;40[4]:341-7.
 14. Engberg RM, Hedemann MS, Jensen BB. The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. British poultry science. 2002;43[4]:569-79.
 15. Gholamiandehkordi AR, Timbermont L, Lanckriet A, Van Den Broeck W, Pedersen K, Dewulf J, et al. Quantification of gut lesions in a subclinical necrotic enteritis model. Avian pathology : journal of the WVPA. 2007;36[5]:375-82.
 16. Luna LG, Pathology AFlo. Manual of Histologic Staining Methods ; of the Armed Forces Institute of Pathology. Edited by Lee G. Luna: Blakiston Division, McGraw-Hill; 1968.
 17. Coursodon CF, Glock RD, Moore KL, Cooper KK, Songer JG. TpeL-producing strains of Clostridium perfringens type A are highly virulent for broiler chicks. Anaerobe. 2012;18[1]:117-21.
 18. Keyburn AL. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. 2008;4[2].
 19. Shivaprasad HL, Uzal F, Kokka R, Fisher DJ, McClane BA, Songer AG. Ulcerative enteritis-like disease associated with Clostridium perfringens type A in bobwhite quail [Colinus virginianus]. Avian diseases. 2008;52[4]:635-40.
 20. Chalmers G, Bruce HL, Hunter DB, Parreira VR, Kulkarni RR, Jiang YF, et al. Multilocus sequence typing analysis of Clostridium perfringens isolates from necrotic enteritis outbreaks in broiler chicken populations. Journal of clinical microbiology. 2008;46[12]:3957-64.
 21. Keyburn AL, Yan XX, Bannam TL, Van Immerseel F, Rood JJ, Moore RJ. Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin. Veterinary research. 2010;41[2]:21.
 22. Martin TG, Smyth JA. Prevalence of netB among some clinical isolates of Clostridium perfringens from animals in the United States. Veterinary microbiology. 2009;136[1-2]:202-5.
 23. Salari Sedigh H, Razmyar J and Ghavidel GH. Isolation and genotyping of Clostridium perfringens from healthy and diarrheic dogs. Iranian Journal of Veterinary Science and Technology. 2016; 8[2]: 48-54