Haryana Vet. (June, 2014) **53 (1)**, 39-42

Research Article

PREVALENCE OF α, β AND NETB TOXIN PRODUCING STRAINS OF *CLOSTRIDIUM PERFRINGENS* IN BROILER CHICKENS IN HARYANA

S. DATTA[1], N.K. RAKHA[1]*, G. NARANG[2], D. ARORA[2] and N.K. MAHAJAN[2]
[1]Department of Veterinary Medicine, [2]Department of Veterinary Public Health and Epidemiology
College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar- 125 004

Received: 16.12.2013; Accepted: 23.05.2014

ABSTRACT

A total of 87 samples (40 samples from enteritis-affected birds and 47 samples were from healthy birds) of intestinal contents from commercial broiler chickens were processed for isolation of *Clostridium perfringens*. Of these, 32 (68.08%) samples from healthy birds and 34 (85%) samples from diseased birds yielded *C. perfringens*. Thirty *C. perfringens* isolates each from healthy birds and enteritis-affected birds were subjected to the PCR for the detection of α toxin (*cpa*), β-2 toxin (*cpb2*) and NetB (NetB) toxin gene using gene specific primers. Of the 30 isolates from healthy birds; 10, 2 and 7 isolates were positive for α toxin alone, β-2 toxin alone and both α and β toxins, respectively. In case of enteritis-affected birds, this number was 16, 2 and 10, respectively. None of the isolates was found positive for NetB toxin. The study indicates that α and β-2 toxin producing strains of *C. perfringens* are prevalent in broiler chickens in Haryana.

Key words: C. *perfringens*, α toxin, β toxin, NetB toxin, broiler chickens

Enteritis is a major syndrome in poultry with large number of predisposing factors, which are routinely involved in its etiology and epidemics. Necrotic enteritis is an acute form of enteritis caused by *Clostridium perfringens* in broiler chickens, turkeys and other farm animals (Ficken and Wages, 1997). Clinically, necrotic enteritis is a very common intestinal disorder causing necrosis of the intestinal mucosa associated with cholangio-hepatitis and multifocal to massive necrotic enteritis (Lovland and Kaldhusdal, 1999) and causes economic losses to the poultry farmers. Sub-clinical form may be the most important manifestation of enteritis as it is likely to go undetected and hence untreated (Kaldhusdal and Hofshagen, 1992).

C. perfringens is also a normal inhabitant of the intestinal tract of the healthy human being, poultry and other farm animals (Hathaway and Johnson, 1998; Jay, 2000). *C. perfringens* commonly occurs in soil, water, segments of dead and decaying vegetable matter and also in the poultry houses and is readily isolated from the intestine of the various birds and mammals (Hofshagen and Stenwig, 1992). Species belonging to *Clostridium* can be subtyped into five toxinotypes based on the production of four major toxins namely α, β, epsilon and iota toxin (Songer,1996; Olkowski *et al.*, 2008). The major infection in poultry is caused by type 'A' and to a lesser extent by type 'C' *strains of C. perfringens*. Type A strains produce the chromosomal encoded á toxin while type C produce α toxin along with β toxin. Álpha toxin is a phospholipase C and is considered to be the major contributory factor towards the development of the intestinal mucosal necrosis, a characteristic lesion of necrotic enteritis in poultry (Al-Sheikhly and Truscott, 1976). Álpha toxin hydrolyses lecithin a major component of the cell membrane and thus destroys the red blood cells, platelets and a muscle leading to the myonecrosis. Beta toxin induces haemorrhagic necrosis of the intestinal mucosa. NetB was discovered in an Australian strain of *C. perfringens* type A that was isolated from necrotic enteritis (NE) affected broiler chicken (Keyburn *et al*., 2010). NetB toxin has also amino acid sequence identity to *C. perfringens* (38–40% identity).

The recent observations in our laboratory indicate that the incidence of necrotic enteritis in poultry particularly in broiler populations is increasing rapidly (Agrawal *et al.*, 2009). Therefore, a study was planned to type various isolates of *C. perfringens* based on the prevalence of α, β and NetB toxin genes.

MATERIALS AND METHODS

A total of 87 intestinal samples (40 samples from enteritis-affected birds and 47 from healthy birds) were

*Corresponding author: drrakha@gmail.com

collected from broiler birds of 4 to 6 weeks of age which were brought to the DI Lab., Hisar for disease investigation. Intestinal contents were collected from these birds. Apparently healthy broiler chickens were procured from a local slaughter house in Hisar. Intestinal contents of 4-5 birds were pooled to make one pooled sample. Such 87 pooled samples were processed for the isolation of *C. perfringens*. The total numbers of positive samples from healthy and diseased birds were 32 and 34, respectively. Thirty isolates each from healthy and diseased birds were subjected to polymerase chain reaction (PCR) using specific primers for α, β and NetB toxin genes (Sigma Co).

Isolation and Confirmation of *C. perfringens*: Isolation of *C. perfringens* from intestinal contents was done using one step enrichment and selective plating as described earlier (Agrawal *et al.,* 2009). Alternative thioglycollate media (Hi media) was used as an enrichment medium. Briefly, 10gm of pooled sample was inoculated in 90ml of enrichment media in a sterile flask and homogenized for 2-3 min. The homogenized sample was heated at 75^{0}C for 20 min followed by incubation at 37^{0}C for 24 h. For isolation of *C. perfringens*, an agar overlay technique in sterile test tubes was employed using Tryptose Sulphite Cycloserine (TSC) agar. Thioglycollate enriched inoculum (0.1ml) was taken in a sterile test tube and 5 ml of sterile TSC agar was added with thorough mixing. It was allowed to solidify for 5-10 min and again 2-3 ml of TSC agar was overlayed. The test tube was then incubated at 44-45^{0}C for 18-24 h. The black cotton wool like colonies of 2-3 mm in size suspected for *C. perfringens* were taken out with the help of a loop and incubated at 37^{0}C in thioglycollate broth for 24 h and the organisms were subjected to further biochemical tests for confirmation.

Polymerase Chain Reaction: DNA was extracted from biochemically-confirmed C. *perfringens* isolates using Bacterial\Fungal DNA Kit (Zymo Research Pvt. Ltd.) as per the manufacturer's protocol. Primers for *cpa* (α toxin) and *cpb2* (β-2 toxin) gene of *C. perfringens* as reported by Baums *et al.* (2004) and NetB toxin gene as reported by Keyburn *et al.* (2008) were procured from Sigma, Aldrich. The details of primers used are given in Table 1.

The PCR was standardized separately for *cpa* and cpb2 gene as per the method described by Baums *et al*. (2004) with certain modifications. Reference strains of *C. perfringens* Type A and Type C were procured from the Division of Biological Standardization, IVRI, Izatnagar. Both the strains were maintained as stock culture in alternative Thioglycollate broth medium and were regularly tested and periodically checked for purity, morphology and biochemical characterization. The PCR was setup in 25 µl reaction volume. The reaction mix found optimum for specific amplification was: DNA 5.0µl, 2X PCR master mix 12.5 µl, forward and reverse primers 10 pmol each and nuclease free water to make total volume of 25.0 µl. The reaction was carried out in a thermalcycler using the following conditions for alpha and beta-2 toxin genes: denaturation at 94^{0}C for 3 min, 35 cycles of denaturation at 94^{0}C for 1 min, annealing at 58^{0}C for 1.30 min and extension at 72^{0}C for 1.30 min, and a step of final extension at 72^{0}C for 5 min.

The reaction mix used for amplification of NetB gene included: 5.0µl DNA, 12.5µl of 2X PCR master mix, 1.0µl each (10 pmol) forward and reverse primer and nuclease free water to make total volume of 25.0 µl. The reaction was carried out in a thermalcycler using following conditions: denaturation at 94^{0}C for 2 min, 35 cycles of denaturation at 94^{0}C for 0.5 min, annealing at 58^{0}C for 0.5 min and extension at 72^{0}C for 1 min,

Table 1
Details of primers used in this study

Gene	Primers	Primer sequence (5'-3')	Product size
cpa (α toxin)	Cpa_for	AGTCTACGCTTGGGATGGAA	900 bp
	Cpa_rev	TTCCCTGGGTTGTCCATTTC	
cpb2 (β-2 toxin)	Cpb2_for	CAAGCAATTGGGGGAGTTTA	200 bp
	Cpb2_rev	GCAGAATCAGGATTTTGACCA	
NetB (Net B toxin)	AKP78_for	GCTGGTGCTGGAATAAATGC	560 bp
	AKP79_rev	TCGCCATTGAGTAGTTTCCC	

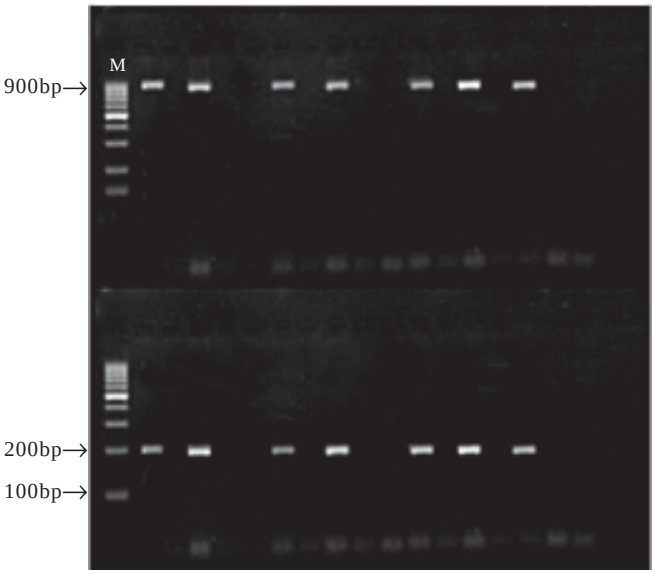

Fig 1. PCR product of 900 base pair *cpa* gene (alpha toxin) and 200 bp *cpb2* gene (beta toxin) of field isolates of *C. perfringens* in 1.5% agarose gel (Lane 2 onward), Lane M: 100 bp DNA molecular size marker, Lane 1: +ve control *C. perfringens* type C "positive for both alpha (*cpa*) and beta-2 toxin (*cpb2*) gene.

and a step of final extension at 72^0C for 12 min. The PCR products of *cpa* gene (900bp), *cpb2* gene (200bp) and NetB gene (560bp) were subjected to agarose gel electrophoresis.

RESULTS AND DISCUSSION

Eighty seven intestinal contents samples were processed for isolation of *C. perfringens* from both healthy and enteritis-affected broilers. Of these, 40 samples were from diseased birds while 47 were from healthy birds. *C. perfringens* could be isolated from 32 healthy (68.08%) and 34 diseased (85%) birds. Thirty isolates each from healthy and diseased birds were subjected to PCR using gene specific primers for determining the presence of α, β and NetB toxins.

The standard isolates of *C. perfringens* from IVRI revealed the presence of both alpha (900 bp) and beta-2 (200 bp) genes as shown in Fig. 1. A band of 900bp (cpa gene) for α toxin and a band of 200 bp for β-2 toxin was also observed in positive field cases (Fig. 1). Out of 30 *C. perfringens* isolates from healthy birds, 10 (33.3%) were positive for α toxin alone and 2 (6.7%) for β-2 toxin alone. In addition, seven (23.3%) isolates were positive for both α and β-2 toxins. In case of the diseased birds, 16 (53.3%) isolates were positive for á toxin alone and 2 (6.7%) for β-2 toxin alone. Ten (33.3%) isolates were positive for both α and β-2 toxins (Table 2). Thus, 19 and 28 isolates from healthy and diseased birds, respectively were toxin producing. Similar observations were made by Agrawal *et al*. (2009) who reported 11 (39.2%) isolates of *C. perfringens* to be positive for α toxin alone and nine (32.10%) isolates to be positive for both α and β toxins. The study indicates the presence of α and β-2 toxin producing strains of *C. perfringens* in healthy as well as enteritis-affected broiler chickens of Haryana. There was an increase in number of the toxin producing isolates from diseased birds. Higher percentage of α and beta-2 toxin gene producing *C. perfringens* strains in diseased broiler birds indicates its possible role in pathogenesis of enteritis.

None of the isolates was positive for NetB toxin gene even when they were repeatedly analyzed by PCR using already standardized technique and also by making minor modifications in the PCR reactions. NetB is a pore forming toxin with structure equal to 3.9 Angstrom (Savva *et al.*, 2013). Sergio *et al*. (2014) studied site-directed mutagenesis which was used to identify various amino acids that play a role in NetB oligomerisation and pore-formation. This toxin forms pores that damage the phospholipid membrane bilayer of both human and animal cells, causing an influx of ions (i.e., Na+, Cl", Ca2+, etc.) that leads to osmotic cell lysis. Many of these toxins have been believed to contribute to the virulence of bacteria and they play an important role in the pathogenesis of human and animal infections (Keyburn *et al.*, 2010). Further studies are required to

Table 2

Prevalence of toxin genes in *C. perfringens* isolated from healthy and enteritis affected commercial broiler chickens

No. of samples subjected to PCR	Positive for *a* toxin alone	Positive for β-2 toxin alone	Positive for both α and β-2 toxin	Negative for both α and β-2 toxin	Positive for NetB toxin alone
Healthy birds (30)	10 (33.3%)	2 (6.7%)	7 (23.3%)	11 (36.7%)	Nil
Diseased birds (30)	16 (53.3%)	2 (6.7%)	10 (33.3%)	2 (6.7%)	Nil

determine whether *C. perfringens* strains circulating in poultry in this region have the ability to produce NetB toxin or not.

REFERENCES

Agrawal, A., Narang, G., Rakha, N.K. Mahajan N.K. and Sharma, A. (2009). *In vitro* lecithinase activity and antibiogram of *Clostridium perfringens* isolated from broiler chickens. *Haryana Vet.* **48**: 81-84.

Al-Sheikhly, F. and Truscott, R.B. (1976). The pathology of necrotic enteritis of chickens following infusion of crude toxins of *Clostridium perfringens* into the duodenum. *Avian Dis.* **21**: 241-255.

Baums, C.G., Schotte, U., Amtsberg, G., Goethe, R. (2004). Diagnostic multiplex PCR for toxin genotyping of *Clostridium perfringens* isolates. *Vet. Microbiol*. **100**: 11-16.

Ficken, M.D. and Wages, D.P. (1997). Necrotic Enteritis In: Diseases of Poultry. Calnek, B.W. (ed.). (10th edn.), Mosby-Wolfe. pp. 261-264.

Hathaway, C.L. and Johnson, E.A. (1998). *Clostridium* : The Spore-bearing Anaerobes. In: Systematic Bacteriology. Balows, A. and Duerden, B.I. (edts.). Topley and Wilson's Microbiology and Microbial Infections, London, Arnold.

Hofshagen, M. and Stenwig, H. (1992). Toxin production by *Clostridium perfringens* isolated from broiler chickens and capercaillies (*Tetrao urogallus*) with and without necrotizing enteritis. *Avian Dis*. **36**: 837-843.

Jay, J.M. (2000). Modern Food Microbiology. (4th edn.), Chapman & Hall, Inc., New York.

Kaldhusdal, M. and Hofshagen, M. (1992). Barley inclusion and avoparcin supplementation in broiler diets. 2. Clinical, pathological, and bacteriological findings in a mild form of necrotic enteritis. *Poult. Sci.* **71**: 1145-1153.

Keyburn, A.L., Bannam, T.L., Moore, R.J. and Rood, J.I. (2010). Net B, a pore-forming toxin from necrotic enteritis strains of *Clostridium perfringens*. *Toxins (Basels)* **2**: 1913-1927.

Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E., Parker, D., Rubbo, A., Rood, J.I. and Moore R.J. (2008). NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. *PLoS Pathog.* **4**: 26.

Lovland, A. and Kaldhusdal, M. (1999). Liver lesions seen at slaughter as an indicator of necrotic enteritis in broiler flocks. *FEMS Immunol. Med. Microbiol.* **24**: 345-351.

Olkowski, A.A., Wojnarowicz, C., Chirino-Trejo, M., Larveld, B. and Sawivki, G. (2008). Subclinical necrotic enteritis in broiler chickens: Novel etiological consideration based on ultra-structural and molecular changes in the intestinal tract. *Res. Vet. Sci.* **85:** 543-553.

Savva, C.G., Fernandes da Costa, Bokori-Brown, M., Naylor, C.E., Cole, A.R., Moss, D.S., Titball, R.W. and Basak, A.K. (2013). Molecular architecture and functional analysis of NetB, a pore-forming toxin from *Clostridium perfringens*. *J. Biol. Chem.* **288(5):** 3512-3522.

Sergio, P., Fernandes da Costa, Savva, C.G., Bokori-Brown, M., Claire, E., Naylor, Moss, D.S., Basak, A.K., and Titball, R.W. (2014). Identification of a key residue for oligomerisation and pore-formation of *Clostridium perfringens* NetB. *Toxins (Basel)* **6(3)**: 1049-1061.

Songer, J.G. (1996). Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Rev.* **9**: 216-234.