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Research Article

TOXIN GENOTYPING OF CLOSTRIDIUM PERFRINGENS ISOLATED FROM ENTERITIS-AFFECTED BROILER CHICKENS

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ABSTRACT

The aim of the study was toxin genotyping of *Clostridium perfringens* isolates from enteritis-affected flocks of broiler chickens. Of the 68 intestinal content samples collected for the isolation of *C. perfringens*, 49 (72.05%) samples yielded this pathogen. Of the 49 isolates, 28 were tested for the presence of alpha toxin (cpa) and beta-2 toxin (cpb2) genes by polymerase chain reaction. Eleven isolates (39.3%) were positive for alpha toxin alone and none of the isolates was positive for beta-2 toxin alone, however nine isolates (32.1%) were positive for both alpha and beta-2 toxins. Eight isolates (28.6%) were negative for both alpha and beta-2 toxins. Nucleotide sequencing of three isolates amplified using cpa gene specific primers revealed that all three isolates had 98.2-100% similarity with previously published sequences of *C. perfringens* strains from Japan, Denmark, USA, China, U.K. and India. Our results indicate that alpha and beta-2 toxin producing *C. perfringens* are prevalent in enteritis-affected flocks of broiler chickens. Further studies are required to determine the presence of other toxins (NetB and enterotoxin) in these isolates.

Key words : *Clostridium perfringens*, enteritis, alpha toxin, beta-2 toxin, PCR, nucleotide sequencing

Clostridium perfringens is a common environmental bacterium and is readily isolated from the intestinal contents of birds and mammals. Members of the *C. perfringens* can be subtyped into five toxin types (A, B, C, D and E) based on the production of four major toxins: alpha (α), beta (β), epsilon and iota (Meer and Songer, 1997). *C. perfringens* infection in poultry is caused mainly by type A strains and to a lesser extent by type C strains. Type A strains produce the alpha toxin, while type C strains produce both alpha and beta toxins. Alpha toxin is coded by cpa/plc gene while beta toxin which is of two types (beta-1 and beta-2), is coded by cpb1 and cpb2 genes, respectively (Petit *et al.*, 1999). Alpha toxin, a phospholipase C, is considered to be a major contributory factor towards development of intestinal mucosal necrosis, a characteristics lesion of necrosis enteritis (NE) in poultry (Al-Sheikhly and Truscott, 1977a; 1977b). Beta toxin induces hemorrhagic necrosis of the intestinal mucosa. It is generally accepted that predisposing factors are required for *Clostridium* to cause disease in poultry (Riddell and Kong, 1992; Kaldhusdal and Hofshagen, 1992); mucosal damage caused by coccidiosis being the most common one (Al-Sheikhly and Al-Saieg, 1980; Williams, 2005).

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Clostridiosis, a disease produced by *C. perfringens* occurs in two forms: acute and subclinical. The acute form occurs as NE in 2-5 week old birds. Subclinical form is the most important form of NE as this form may go undetected and hence untreated (Kaldhusdal and Hofshagen, 1992). This form is characterized by focal necrosis in intestine and cholangiohepatitis in liver. Clostridiosis in broiler flocks is concealed by routine use of feed additives and by ionophorus anticoccidials. The incidence of *C. perfringens* associated NE in poultry has increased in countries that banned the use of antibiotics as growth promoters (Van Immerseel *et al.*, 2004). In India, the use of antibiotics as growth promoters is still in practice. Although the *C. perfringens* has been detected in cases of NE in broiler chickens in many countries, not much epidemiological data is available with regards to its isolation and types circulating in broiler chickens in India. However, a report from Meghalaya reveals the presence of this bacterium in cases of NE in broiler chickens (Das *et al.*, 2008). To have a better understanding about the types of *C. perfringens* in broiler chickens, we conducted this study to isolate and type *C. perfringens* from enteritis-affected broiler

flocks in Haryana, a north-western state of India.

MATERIALS AND METHODS

Bacterial Strains: Reference type A and type C strains of *C. perfringens* were procured from the Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar. The strains were maintained as stock cultures in thioglycollate broth and were tested for purity, morphology and biochemical characteristics regularly.

Sampling: Intestinal contents were collected from commercial broiler chicks brought to the Department for disease investigation from September 2008 to February 2009. Intestinal contents collected from 4-5 enteritis-affected birds in a flock were pooled to make one pooled sample (pooled sample hereinafter will be referred to as 'sample'); a total of 68 samples were collected. All samples were processed for the isolation of *C. perfringens* using one step enrichment and selective plating (Varnam and Evans, 1991) as described earlier (Agrawal *et al.*, 2009). The isolates were confirmed to be *Clostridium* based on their morphology and biochemical characters.

Primers and DNA Extraction: The primers for cpa and cpb2 genes of *C. perfringens* were procured from Sigma Chemicals; the details are given in Table 1. Total DNA was extracted from isolates using E.Z.N.A. Bacterial DNA Kit (Omega BIO-TEK) as per the manufacturer's protocol. The extracted DNA was subjected to polymerase chain reaction (PCR) using gene specific primers for the detection of alpha and beta-2 toxins.

PCR: The PCR protocols described by Drigo *et al.* (2008) were used for both genes with certain modifications PCR was performed in 25 µl reaction mixture containing 1X PCR buffer, 10 mM dNTP, 25 mM MgCl2, 1X Taq DNA polymerase, template and 10 pmol of forward and reverse cpa primers for the amplification of cpa gene. Similar reaction mixture was prepared for cpb2 gene. The thermal cycling conditions for the amplification of both genes were: initial denaturation at 94°C for 3 min followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1% agarose gel and were visualized under UV transilluminator. Bands of 900 bp and 200 bp corresponding to alpha toxin and beta-2 toxin, respectively were observed in positive cases.

Table 1
Target toxin gene and oligonucleotide primer sequence

Gene	Primers*	Primer sequence (5'-3')
cpa (α toxin)	cpa_F	AGTCTACGCTTGGGATGGAA
	cpa_R	TTCCCTGGGTTGTCCATTTC
cpb2 (β2 toxin)	cpb2_F	CAAGCAATTGGGGGAGTTTA
	cpb2_R	GCAGAATCAGGATTTTGACCA

*Primers described by Baums *et al.* (2004).

Sequencing: The PCR products were purified using QIA quick gel extraction kit (QIAGEN). Purified PCR products of both genes of three isolates were sequenced using automated DNA sequencer ABI PRISMTM 3130 Version 3.0. Sequences generated were then subjected to BLAST analysis (www.ncbi.nlm.nih.gov) to confirm their identity. Details of previously published sequences of cpa and cpb2 (coding for alpha and beta-2 toxins, respectively) genes of *C. perfringens* used for sequence comparison, are given in Tables 2 and 3, respectively. For cpa gene, phylogenetic tree was drawn based on partial nucleotide sequences corresponding to nucleotide positions 160-910 of GenBank accession number DQ184079 and positions 172-922 of GenBank accession number AY823400 of cpa gene of *C. perfringens* from broiler chickens. For cpb2 gene, phylogenetic tree was drawn based on partial nucleotide sequences corresponding to nucleotide positions 323-450 of GenBank accession number EU085382 and positions 602-729 of GenBank accession number AJ537550 of beta-2 toxin gene of *C. perfringens* from broiler chickens. The evolutionary tree for each toxin was scaled using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood

Table 2
Details of previously published sequences of cpa/plc gene (α toxin) of *C. perfringens* used for sequence comparison

C. perfringens strain	Geographic origin	GenBank accession number
PB6KN5L7 (plc)	Japan	D32123
S41 (plc)	Denmark	EU839805
NRRL B-23526 (plc)	USA	DQ184154
S14 (plc)	Denmark	EU839783
C57-1 (plc)	China	AY823400
C. perfringens A fragment d	Denmark	AF477009
NRRL B-23717 (plc)	USA	DQ184079
C. perfringens A fragment c	Denmark	AF475144
CP61 (cpa)	India	DQ787188
CP54 (cpa)	India	DQ787185
CP66 (cpa)	India	DQ787190
Phospholipase C (cpa)	UK	AF204209

Table 3
Details of previously published sequences of cpb2 gene of *C. perfringens* used for sequence comparison

C. perfringens strain	Geographic origin	GenBank accession number
AN 5036/01	Sweden	DQ201571
Pcpb2W30554	USA	AY730632
AN 4361/00	Sweden	DQ201554
Cp23	The Netherlands	EU085382
AN 4362/00	Sweden	DQ201555
Strain 26664/3	Switzerland	AJ537550
ATBSi7IR	Iran	GU581176
ATBS120IR	Iran	GU581181
ATBS100tIR	Iran	GU581185

method. Phylogenetic analysis was conducted using MEGA 4.0 software. The cpa gene sequences of this study were submitted to the GenBank with the accession numbers: HR/11833/2008 (HQ332225), HR/12035/2009 (HQ332226) and HR/12069/2009 (HQ332227).

RESULTS AND DISCUSSION

Isolation of *C. perfringens*: Of the 68 samples, *C. perfringens* was isolated from 49 (72.05%) samples.

PCR: Of the 49 isolates, 28 were tested for the presence of alpha (cpa gene) and beta-2 (cpb2 gene) toxins by PCR. Of the 28 isolates, 11 (39.3%) were positive for alpha toxin alone whereas, nine (32.1%) were positive for both alpha and beta-2 toxins. None of the isolates was positive for beta-2 toxin alone. Eight isolates (28.6%) were negative for both alpha and beta-2 toxins. Thus, a total of 20 isolates were positive either for alpha toxin or alpha and beta toxins. Both types (A and C) of *C. perfringens* have been reported from cases of NE (Engstrom *et al.*, 2003; Svobodova *et al.*, 2007). In contrast, Yoo *et al*. (1997) reported all isolates of *C. perfringens* in diarrheic chickens to be of type A by molecular typing. Yoo *et al.* (1997), Engstrom *et al.* (2003) and Svobodova *et al.* (2007) reported that approximately 90% of *C. perfringens* were type A. Further studies are required over a period of time with more number of samples to determine the actual prevalence of different types of *Clostridium* producing different types of toxins. Eight isolates (28.6%) in our study were negative for both alpha and beta-2 toxins indicating that they may be positive for other toxins such as NetB and/or enterotoxin. Presence of NetB toxin or enterotoxin in *C. perfringens* has earlier been reported by Keyburn *et al.* (2008, 2010).

Sequencing and Phylogenetic Analysis: For alpha toxin, PCR purified products of cpa gene from three isolates (HR/11833/2009, HR/12035/2009 and HR/12069/2009) were sequenced. Nucleotide sequencing revealed that these three isolates had 98.2-100% similarity with previously published cpa gene sequences of *C. perfringens* strains from Japan, Denmark, USA, China, U.K and India used for comparison. Phylogenetic analysis revealed that all three isolates of this study were in a single clade and were closely placed with strains from Japan, Denmark, USA and China (Fig. 1). The previously published cpa gene sequences from India used for comparison were in a separate clade. Das *et al.* (2008) from Meghalaya (India) sequenced six field isolates of *Clostridium* and reported that the partial cpa gene of Indian field isolates (DQ787185, DQ787188, DQ787190) and reference strains from Japan, USA and Denmark had 97.6% to 100% sequence homology irrespective of source of origin and geographical distribution.

For beta-2 toxin, PCR purified products of cpb2 gene generated from three isolates (HR/11693/2008, HR/11998/2009 and HR/12069/2009) were sequenced which revealed 100% similarity with previously published cpb2 gene sequences used for comparison. Based on cpb2 gene phylogeny, the isolates were closely placed with strains from Sweden, USA and Netherlands (Fig. 2). Johansson *et al*. (2006) reported sequence similarity of 93.3% to 100% cpb2 gene of 28 isolates of *C. perfringens* obtained from different

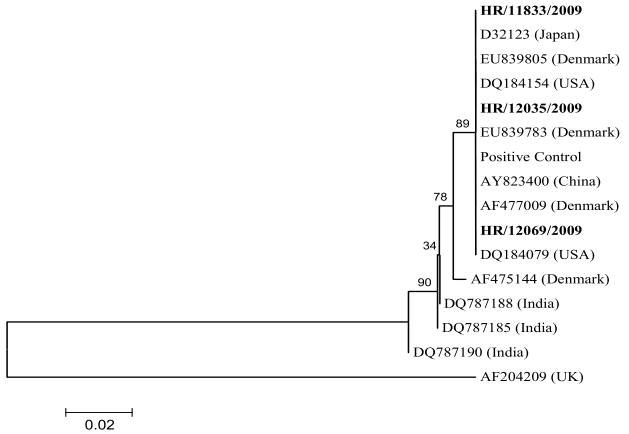

Fig 1. Phylogenetic tree based on partial nucleotide sequences of *cpa* gene of *Clostridium perfringens* from broiler chickens. The accession numbers (country) are previously published *cpa* sequences of *C. perfringens*. The strain names in bold are the sequences of this study.

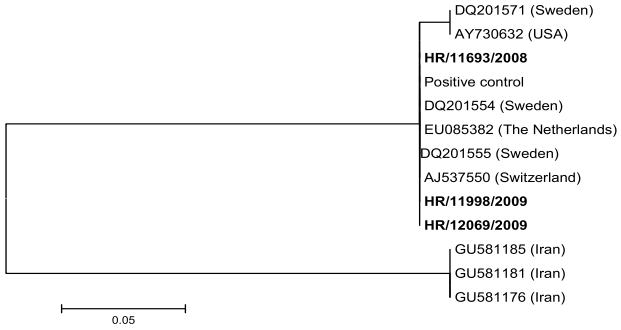

Fig 2. Phylogenetic tree based on partial nucleotide sequences of cpb2 gene of *Clostridium perfringens* from broiler chickens. The accession numbers (country) are previously published cpb2 gene sequences of *C. perfringens*. The strain names in bold are the sequences of this study.

animals including poultry in Sweden. In this study, we used primers that could amplify a part of cpb2 gene (200 bp) because our aim was only to detect the presence of beta-2 toxin; our sequencing results of cpb2 gene showed very high similarity with most of the previously published gene sequence whose gene length is about 500-600 bp. Further studies using primers that can amplify more region of cpb2 gene are required to determine the differences at genetic level.

This study reveals that alpha and beta-2 toxin producing strains of *C. perfringens* are prevalent in enteritis-affected flocks of broiler chickens in Haryana state. There is a need to study further the isolates for the presence of other toxins; the possibility of presence of NetB and/or enterotoxin cannot be ruled out.

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