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 el 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-Saieq, 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 el 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 el 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.

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>
<thead>
<tr>
<th>Gene</th>
<th>Primers*</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpa (α toxin)</td>
<td>cpa_F</td>
<td>AGTCTAGGCTTGGGATGGAA</td>
</tr>
<tr>
<td></td>
<td>cpa_R</td>
<td>TTCCCTGGGATGTTCCATTTC</td>
</tr>
<tr>
<td>cpb2 (β2 toxin)</td>
<td>cpb2_F</td>
<td>CAAGCAATTGGAAGATTTTA</td>
</tr>
<tr>
<td></td>
<td>cpb2_R</td>
<td>GCAGATTCAGAATTTGACCA</td>
</tr>
</tbody>
</table>

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

Table 2

<table>
<thead>
<tr>
<th>C. perfringens strain origin</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB6K5NL7 (plc) Japan</td>
<td>D32123</td>
</tr>
<tr>
<td>S41 (plc) Denmark</td>
<td>EU398605</td>
</tr>
<tr>
<td>NRRL B-23526 (plc) USA</td>
<td>DQ184154</td>
</tr>
<tr>
<td>S14 (plc) Denmark</td>
<td>EU39783</td>
</tr>
<tr>
<td>C57-1 (plc) China</td>
<td>AY823400</td>
</tr>
<tr>
<td>C. perfringens A fragment d</td>
<td>Denmark</td>
</tr>
<tr>
<td>NRRL B-23717 (plc) USA</td>
<td>DQ184079</td>
</tr>
<tr>
<td>C. perfringens A fragment e</td>
<td>Denmark</td>
</tr>
<tr>
<td>CP61 (cpa) India</td>
<td>DQ787188</td>
</tr>
<tr>
<td>CP54 (cpa) India</td>
<td>DQ787185</td>
</tr>
<tr>
<td>CP66 (cpa) India</td>
<td>DQ787190</td>
</tr>
<tr>
<td>Phospholipase C (cpa) UK</td>
<td>AF204209</td>
</tr>
</tbody>
</table>
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 (cph2 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 cph2 gene generated from three isolates (HR/11693/2008, HR/11998/2009 and HR/12069/2009) were sequenced which revealed 100% similarity with previously published cph2 gene sequences used for comparison. Based on cph2 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% cph2 gene of 28 isolates of *C. perfringens* obtained from different countries (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.
animals including poultry in Sweden. In this study, we used primers that could amplify a part of cbp2 gene (200 bp) because our aim was only to detect the presence of beta-2 toxin; our sequencing results of cbp2 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 cbp2 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.

REFERENCES


