Characterization of enterotoxin producing \textit{Clostridium perfringens} isolated from foods of animal origin

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ABSTRACT

Screening of 105 samples comprising of milk, meat and their products for isolation of \textit{Clostridium perfringens} revealed a total of 39 (37.14%) samples to be positive for \textit{C. perfringens}, yielding an equal number of isolates. Among the total isolates, 11 (20.0%) belonged to raw cow’s milk. The remaining isolate was from paneer sample. Similarly, 28 (56.0%) \textit{C. perfringens} isolates were recovered from meat and meat products. Majority (37) of the isolates belonged to toxpin type A while two isolates recovered each from raw beef and chicken meats were of toxpin type C. Among the 39 isolates of \textit{C. perfringens}, only 5 (12.82%) isolates could reveal the presence of \textit{cpe} (enterotoxin) gene. The protein profile (SDS-PAGE) of both the crude and partially purified enterotoxins prepared from \textit{cpe} positive \textit{C. perfringens} isolates (type A and C) revealed a very little difference. Both the partially purified enterotoxins of type A and C of \textit{C. perfringens} displayed almost similar type of cytopathic effects (CPE) and death of cells in Vero cell line.

Key words: \textit{Clostridium perfringens}, Cpe gene, Enterotoxin protein profile, Toxin gene

INTRODUCTION

\textit{Clostridium perfringens} is considered to be a common cause of bacterial food-borne disease. Human beings acquire the infection through the consumption of contaminated milk, meat and their products. Although different types of \textit{C. perfringens} are present in the food and food products of animal origin, majority of them are non-enterotoxigenic. Considering the public health importance of the organism, a study was undertaken with an idea to isolate \textit{C. perfringens} from milk, meat and their products; toxin typing of the isolates based on the molecular detection of major toxin genes and to detect enterotoxin (cpe) gene in the isolates. The enterotoxins (CPE) extracted from those \textit{cpe} positive isolates were further characterized in respect to their protein profile as well as Vero-cell cytotoxicity assay.

MATERIALS AND METHODS

Isolation and identification of \textit{Clostridium perfringens}: This study was carried out in the Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati. During the present study, \textit{C. perfringens} was isolated from various food and food products of animal origin used for human consumption, viz. milk, meat and their products. Samples were collected from in and around Guwahati, Assam and Tura, Meghalaya during 2010 and 2011. Collected samples were inoculated in dehydrated Robertson’s Cooked Meat Broth (RCMB) medium and then incubated at 42 °C for 48 hours under anaerobic condition (Singh \textit{et al.,} 2005). Primary identification of the \textit{C. perfringens} colonies were based on the colony morphology, staining and biochemical characteristics as per the method described by (Singh \textit{et al.,} 2005).

Detection of major virulence genes in \textit{Clostridium perfringens} isolates by PCR: All the isolated \textit{C. perfringens} were screened for the presence of major virulence gene, either alone or in combination by simplex PCR. DNA was extracted from each \textit{C. perfringens} isolate by hot cold lysis technique, described by Titball \textit{et al.,} 1989. Extracted DNA of each isolate was tested for the presence of \textit{cpa, cpb, etx} and \textit{iA} gene by simplex PCR. Isolates were also tested for the molecular detection of enterotoxin associated \textit{cpe} gene. The PCR was carried out as per the method of (Titball \textit{et al.,} 1989) with slight modification suggested by Das \textit{et al.,} (2008), by using previously reported primers (Table 1). Amplification was carried out in 25µl reaction volume, comprising of 12.5µl 2 X PCR mastermix (Fermentas, USA), 0.5 µl each of the respective forward and reverse primers, 3 µl of template DNA and nuclease free water to make the final volume up to 25 µl. The PCR reactions were performed in a thermocycler (Applied Biosystems) with the PCR conditions mentioned in Table 2. The amplicons were confirmed by agarose gel electrophoresis using 1.5% agarose (Amresco) containing ethidium bromide in 1 X tris acetate-EDTA(TAE) buffer and the gel was visualized on a gel documentation system (Kodak, Biostep, Germany).

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**Characterization of Clostridium perfringens enterotoxin (cpe):** Two randomly selected cpe positive isolates of *C. perfringens* representing type A and C were subjected for extraction of enterotoxin as per the method of Norhan et al., (2010). Extracts were partially purified by ammonium sulphate precipitation and characterized in respect to their protein profile and their effect in Vero cell line.

Pure colonies of cpe positive *C. perfringens* isolates were grown in RCMB medium anaerobically at 37°C for 24 hours. After checking the purity, inoculums were transferred to Duncan and Strong (DS) sporation medium and incubated at 37°C for 8 hours under strict anaerobic condition and examined microscopically for the presence of central or sub-terminal oval non-bulging endospores. The sporulated cells were washed once in cold distilled water and then suspended in 200 ml of cold saline. The cells were disrupted by sonic treatment (6 Hertz for 20 minutes) and the debris was removed by centrifugation at 12000Xg for 20 minutes at 4°C to obtain a clear extract. The resultant supernatant was used as crude enterotoxin. The crude enterotoxin samples were further partially purified by ammonium sulphate precipitation technique described by Mahony et al., (1989). The protein concentrations of each of the partially purified enterotoxins were estimated by Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

**Protein profile of partially purified enterotoxin:** The crude and partially purified enterotoxins of the randomly selected cpe positive isolates were subjected to Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) to study their protein profile and to determine their molecular weights (Laemli, 1970).

**Biological assay of partially purified enterotoxin:** Enterotoxins extracted from the two randomly selected cpe positive isolates of *C. perfringens* were tested for their biological activity in Vero cell line using the methods described by (Uemura et al., 1984) and Mahony et al.,(1989) with slight modification. The Vero cell cytotoxicity was interpreted on the basis of cytopathic effects (CPE) as well as the ratio of death cell to live cell counts.

**RESULTS AND DISCUSSION**

During the present study, a total of 39 (37.14%) samples out of the 105 food samples of animal origin were found to be positive for *C. perfringens*, yielding an equal number of isolates (Table 3). The present study revealed that out of the 55 milk (cow) and milk products, 11 (20.0%) isolates could be recovered, of which 10 were isolated from raw milk samples and the remaining one from paneer sample. Similarly, out of the 50 meat and meat products, a total of 28 (56.0%) samples yielded *C. perfringens*. Among those positive samples, 18 belonged to smoked- beef, 4 belonged to raw beef, 3 belonged to chicken meat, 2 belonged to carabees, while only one sausage sample yielded *C. perfringens* (Table 3). Similar observation was also recorded by Moustafa and Martin (1993), who also reported the presence of *C. perfringens* in cow's milk. Transmission of clostridial infection via cow milk was reported by many previous workers (Barrett, 1989; Osman et al., 2009). Recovery of *C. perfringens* from raw meat and meat products during the study was in agreement with the findings of various workers (Norinaga et al., 1998; Morera et al., 1999).

All the 39 isolates were found to possess cpa gene (Fig.1) encoding for alpha toxin. Presence of cpa gene in all the five toxin types (A-E) of *C. perfringens* was also reported by various other workers (Baums et al., 2004; Piatti et al., 2004). Based on the detection of toxin gene, all the isolates recovered from milk and milk products were found to be of...
Table 3: Detection of major toxin genes in the isolated *C. perfringens* by simplex polymerase chain reaction (simplex-PCR)

<table>
<thead>
<tr>
<th>Nature of the sample</th>
<th>No. of samples processed</th>
<th>No. of samples positive for C. <em>perfringens</em></th>
<th>No. of isolates positive for major toxin genes detected by simplex PCR</th>
<th>Toxin Type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Milk and Milk Products</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>35</td>
<td>10</td>
<td>10 (20.0)</td>
<td>A</td>
</tr>
<tr>
<td>Milk powder</td>
<td>4</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Curd</td>
<td>8</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Paneer</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>55</td>
<td>11</td>
<td>11 (20.0)</td>
<td></td>
</tr>
<tr>
<td><strong>B. Meat and Meat Products</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>A</td>
</tr>
<tr>
<td>Carabeef</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>Smoked Beef</td>
<td>21</td>
<td>18</td>
<td>18</td>
<td>A</td>
</tr>
<tr>
<td>Chicken</td>
<td>13</td>
<td>3</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>Sausage</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50</td>
<td>28 (56.0)</td>
<td>28</td>
<td>26 (A)+2 (C)</td>
</tr>
<tr>
<td><strong>Grand Total</strong></td>
<td>105</td>
<td>39 (37.14)</td>
<td>39</td>
<td>37 (A)+2 (C)</td>
</tr>
</tbody>
</table>

Fig-1. Detection of *cpa* gene encoding for alpha toxin (324bp) in *C. perfringens* by simplex PCR

Lane A, B, D, E, I, J, K, L and M: Samples positive for *cpa* gene; Lane C, G & H: Samples negative for *cpa* gene; Lane F: Positive control (ATCC-13124); Lane MM: 100bp DNA Ladder and Lane N: Negative control

Fig-2. Detection of *cpb* gene encoding for beta toxin (180bp) of *C. perfringens* by simplex PCR

Lane A: Negative control; Lane B & J: 100bp DNA ladder; Lane C, E, F, G, H & I: Negative for *cpb* gene; Lane D: Positive for *cpb* gene

Five of the isolates were found to be positive for the *cpe* (Fig.3) gene encoding for enterotoxin production (Table 3). Among those *cpe* positive isolates, three belonged to type A recovered from smoked beef (2) and raw cow milk (1), while other two belonged to type C isolated from beef and chicken. Enterotoxin producing *C. perfringens* type A was reported to be responsible for the third most common food-borne illness in the United States and can also cause non-food borne human illness such as antibiotic-associated diarrhea and sporadic diarrhoea (McClane, 2007).

Four protein of molecular weight 52.5, 43.0, 35.0 and 28.8 kDa were found to be common in both crude and partially purified enterotoxins irrespective of their type. A 35.0 kDa protein was found to be the common protein in both crude and partially purified enterotoxins of type A and C (Fig.4). Presence of this protein might be a confirmatory indication of enterotoxin in the crude as well as partially purified extracts of type A and C.
The partially purified enterotoxins extracted from C. perfringens type A and C could exhibit almost similar type of cytotoxic activities causing rounding of 30 percent of Vero cells after 1 hour and complete rounding of all the cells after 4 hours of inoculation (Fig.5). The inoculated partially purified enterotoxins also caused death of the inoculated Vero cells (Fig.6). Mahony et al., 1989 described that enterotoxin produced by C. perfringens caused rapid killing of Vero cells. Morphological change to the Vero cells was reported by McClane and Wnek (1990) following inoculation of C. perfringens enterotoxin.

CONCLUSION

During the present study, 39 isolates of C. perfringens was recovered from varieties of food and food products of animal origin used for human consumption. The milk and milk products revealed presence of C. perfringens in raw cow’s milk (10) and paneer (1). Among the meat and meat products, C. perfringens was isolated from smoked beef (18), beef (4), chicken meat (3), carabeef (3) and sausage (1). All the 39 isolates were found to possess cpa gene, of which, two isolates were also positive for cpb gene. Based on the detection of toxin gene, 37 C. perfringens isolates were identified as toxin type A while the remaining 2 were of type C. Detection of cpe gene was possible from 5 of the isolated C. perfringens of which three were of type A (two isolated from smoked beef and one from raw cow’s milk) and two were of type C (each from beef and chicken). The protein profile of the crude enterotoxins prepared from two randomly selected cpe positive C. perfringens isolates revealed six and nine protein bands in type A and C, respectively sharing four proteins of 72.4, 52.5, 35.0 and 28.8 kDa in common. On the other hand, the partially purified enterotoxin of C. perfringens type A and type C exhibited four and six protein bands, respectively sharing four protein bands of 52.5, 43.0, 35.0 and 28.8 kDa in common. The protein profile of both crude and partially purified enterotoxins of C. perfringens type A and C also revealed four common proteins with
molecular weight of 52.5, 43.0, 35.0 and 28.8 kDa. Both the enterotoxins extracted from the randomly selected type A and C of *C. perfringens* were found to be cytotoxic for Vero cell line causing alteration of cell morphology and death of Vero cells.

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