Characterization of Clostridium perfringens Enterotoxin in Food Animals and Human

Nahed, H. Ghoneim, Waffaa W.M. Reda, Samah F. Darwish and Dalia A. Hamza

Department of Zoonoses, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
Biotechnology Research Unite, Animal Reproduction Research Institute, Alharm, Giza, Egypt

Abstract: Enterotoxin (CPE) - producing C. perfringens type A is considered one of the most common causes of food poisonings encoded by C. perfringens enterotoxin gene (cpe). C. perfringens is widely dispersed, but enterotoxin gene carrying (cpe-positive) isolates are rarely found in nature. So, the aim of this work was to study the occurrence and characterization of C. perfringens enterotoxin. A total of 296 rectal swabs collected from different species of food animals and 60 stool samples from human were examined in this study. The results revealed high occurrence of C. perfringens in all examined samples. Cpe-positive C. perfringens were confirmed in 3.1% of the C. perfingens isolates from animals and 3% from human. Cpe positive isolates were represented by type A, C and E. Enterotoxin was produced only from cpe-positive C. perfringens isolates of type A and C.

Key words: Clostridium perfringens * Enterotoxin * Food Animals * Man

INTRODUCTION

Clostridium perfringens is the most widely distributed pathogenic microorganism in nature. It is a diverse species, with its strains being divided into five types, A to E; according to the major toxins they produce, include alpha, beta, epsilon and iota toxins, in addition to which, most C. perfringens strains produce a range of other toxins or potential virulence factors, such as the C. perfringens enterotoxin (CPE) [1]. The ubiquitous distribution of C. perfringens has earlier been considered a logical explanation for the common occurrence of C. perfringens food poisonings. Therefore, all C. perfringens isolates were regarded as potential causative agents for C. perfringens type A food poisonings [2]. Currently it is known that only a small minority, less than 5% of global C. perfringens isolates produce C. perfringens enterotoxin (CPE) and are thus capable of causing food poisonings. The diarrheic and cramping symptoms of C. perfringens food poisoning resulted from C. perfringens enterotoxin (CPE) encoded by C. perfringens enterotoxin gene (cpe) [3]. Cpe can be detected in C. perfringens isolates representing any type (A-E) but not all isolates are, however capable of expressing the gene product, cpe-positive C. perfringens type E isolates, for example, possess mutations in an open reading frame (ORF), promoter(s) and the ribosome binding site of their cpe sequences and therefore do not produce CPE [4]. However, in CPE-associated diseases, the isolate usually represents type A, perhaps because isolates of type A account for more than 95% of global C. perfringens isolates [5]. CPE biosynthesis is associated temporally with sporulation and its synthesis begins after the induction of sporulation and increases progressively for at least 6-8 hours. Outbreaks of CPE-mediated food poisoning typically involve a large number of victims and are associated with temperature-abused meat or poultry dishes. So, the this work was planned to study the occurrence and characterization of C. perfringens enterotoxin and its role in causing food poisoning.

MATERIALS AND METHODS

Collection of Samples: Nearly 296 rectal swabs were collected from different species of food animals including cattle, buffalo, sheep and goat as shown in Table (1). In addition, stool samples were collected from 60 apparently normal persons in contact with animals.

Isolation of C. perfringens: Each sample was inoculated onto a tube of sterile freshly prepared cooked meat medium and then the tube was incubated anaerobically in
DNA Extraction: To prepare template DNA for the multiplex PCR toxin genotyping assay, pure colonies that showed double zone of haemolysis on blood and confirmed as C. perfringens by biochemical tests were grown over night in 5 ml brain heart infusion at 37°C for 24 hour under anaerobic condition.

A rapid boiling procedure was used to prepare template DNA from bacterial strains according to Sheedy et al. [8].

Multiplex PCR Assay to Determine the Toxin Genotype of C. perfringens Isolates: The multiplex PCR assay of Asten et al. [9] was used to detect the presence of genes encoding alpha-toxin (cpa), beta-toxin (cpb), epsilon-toxin (etx), iota-toxin (iap) and CPE (cpe). Primer sequences were published previously (9). Each PCR had a total volume of 25µl, which contained 5µl of DNA as template, 0.34 mM of each cpe oligo, 0.36 mM of each cph oligo, 0.44 mM of each etx oligo, 0.5 mM of each cpa oligo and 0.52 mM of each iap oligo, 1X of PCR master mix and completed to 25 µl by DNase-RNase-free water. Amplification conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. A final extension step at 72°C for 10 min was followed. Amplification products were electrophoresed in 1.5% agarose gel containing 0.5X TBE at 70 volts for 60 min. and visualized under ultraviolet light. To assure that the amplification products were of the expected size, a 50 bp DNA ladder was run simultaneously as a marker.

Extraction and Purification of C. perfringens Enterotoxin According to Waters et al. [10]: Cpe positive C. perfringens isolates that obtained in this study were cultured in CMM then transferred to modified DS sporulation medium and incubated at 37°C for 8 hours under anaerobic condition for enterotoxin production. The sporulated cells were washed once in cold distilled water then suspended in 2 ml of cold saline. The cells were disrupted by sonic treatment (6 Hertz for 20 minutes using sonicator ultra-sonic) and deprés was removed by centrifugation at 12000 xg for 20 minutes at 4°C to obtain a clear extract. The supernatant protein was precipitated by addition of ammonium sulfate (4.76g of (NH4)SO4/10 ml supernatant) and incubated overnight at 4°C [10]. The precipitated protein was then collected by centrifugation at 12000 rpm for 30 minutes at 4°C and resuspended in 25µl of sterile phosphate buffer saline. This solution was dialyzed overnight against the same buffer and any precipitate was removed by centrifugation.

Detection of Cytotoxicity of Enterotoxin on Vero Cell: The cytotoxicity of the purified enterotoxin was tested using Vero cell assay [11]. The method involved the rapid killing of Vero cells by enterotoxin produced by C. perfringens grown in Duncan and Strong sporulation medium.

RESULTS AND DISCUSSION

Although C. perfringens type A forms part of the normal intestinal flora of animals, the enterotoxin producers have been linked to enteritis in animals and food poisoning outbreaks in humans [12]. The role of food animals as the main reservoir and contamination source of cpe positive C. perfringens needs to be reconsidered [13]. Table (1) shows that the occurrence of C. perfringens recovered from rectal swabs of food animals were 58.3% in cattle, 69.6% in buffaloes, 49.4% in sheep and 34% in goat with overall occurrence 54.4%. These results indicated the presence of C. perfringens in all animal species and it is referred as the most widely distributed pathogenic microorganism in nature [14]. Therefore, spread of the infection may occur from feces of animals to the surrounding environment. Classically, typing of C. perfringens is performed with toxin neutralization test in mice. This procedure consumes a lot of antisera and experimental animals. Additionally it is time consuming. In recent years, molecular techniques such as polymerase chain reaction (PCR) have been used to type C. perfringens [15]. Regarding typing of all C. perfringens isolates recovered from food animals by using multiplex PCR, Table (1) and photo (1&2) show that the most predominant types of C. perfringens isolates were type (A) and then followed by type (C). Two isolates were found to be type D, while only one isolate was found to be type (E). None of the isolates contained type B. In addition to the major lethal toxins, a minority of...
Table 1: Incidence and typing of *C. perfringens* in the examined rectal swabs from food animals

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. of examined samples</th>
<th>No. % of <em>C. perfringens</em> positive to A</th>
<th>No. % of <em>C. perfringens</em> positive to C</th>
<th>No. % of <em>C. perfringens</em> positive to D</th>
<th>No. % of <em>C. perfringens</em> positive to E</th>
<th>No. % cpe positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>115</td>
<td>67</td>
<td>62</td>
<td>92.5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>56</td>
<td>39</td>
<td>34</td>
<td>87.2</td>
<td>5</td>
<td>12.8</td>
</tr>
<tr>
<td>Sheep</td>
<td>81</td>
<td>40</td>
<td>40</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Goat</td>
<td>44</td>
<td>15</td>
<td>14</td>
<td>93.3</td>
<td>1</td>
<td>6.7</td>
</tr>
<tr>
<td>Total</td>
<td>296</td>
<td>161</td>
<td>150</td>
<td>93.2</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

Calculated according to the number of positive samples %

Table 2: Occurrence of *C. perfringens* in human stool

<table>
<thead>
<tr>
<th>Source of samples</th>
<th>No. of examined samples</th>
<th>No. % positive</th>
<th>Type A</th>
<th>Enterotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool</td>
<td>60</td>
<td>33</td>
<td>55</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 3: Characterization of cpe positive *Clostridium perfringens* isolates.

<table>
<thead>
<tr>
<th>Cpe-positive isolates</th>
<th>Source of isolates</th>
<th>Typing</th>
<th>Spore formation</th>
<th>Enterotoxin Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field isolate 1</td>
<td>Feces of cattle</td>
<td>A</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Field isolate 2</td>
<td>Feces of cattle</td>
<td>E</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Field isolate 3</td>
<td>Feces of cattle</td>
<td>A</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Field isolate 4</td>
<td>Feces of cattle</td>
<td>A</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Field isolate 5</td>
<td>Feces of buffalo</td>
<td>C</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Field isolate 6</td>
<td>Human</td>
<td>A</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

*C. perfringens* strains produced an enterotoxin (CPE), which is responsible for the symptoms of common *C. perfringens* type (A) food poisoning [5]. Among the typed *C. perfringens* isolates recovered from food animals, *C. perfringens* carrying enterotoxin genes were isolated only from 5 animals (4 from cattle and one from buffalo, but not isolated from sheep and goat) with overall percentage of 3.1% and regarding to characterization of cpe positive isolates recovered from food animal, Table (3) shows that cpe positive isolates represent type A (3 strain), type C (1 strain) and type E (1 strain). Because the most prevalent type of *C. perfringens* in food animals were type (A) and *C. perfringens* type C isolates, so the zoonotic characteristic of *C. perfringens* type A and C should be taken in account since some strains have the ability to synthesize enterotoxins.

*C. perfringens* enterotoxin (CPE) is responsible for the diarrhea and cramping symptoms of human [16]. In the present study (Table 2) the occurrence of *C. perfringens* among stool of apparently healthy people who are in contact with animal was 55% and related to typing of isolates by using multiplex PCR, the isolated strains were type A and only one isolate contained enterotoxin gene. The occurrence of cpe-positive *C. perfringens* type A in the feces of healthy persons and the full capacity of these strains to produce CPE regarded as a risk factor for spread of cpe-positive *C. perfringens* type A food contamination. From all previous results, it was found that *C. perfringens* occur at high level in all types of examined samples and this is of high significance because some strains have the ability to synthesize enterotoxins which are responsible for causing the symptoms of *C. perfringens* food poisoning. So, the present study focused on study the occurrence and characterization of *C. perfringens* enterotoxin as well the in vitro expression of this gene to produce *C. perfringens* enterotoxin. In relation to that, the occurrence of *C. perfringens* enterotoxin genes were detected in five isolates from animals (3.6%) and one isolate from human (3%).
CPE is synthesized during sporulation and thus, sporulation in vitro is essential to measure the production of CPE of an isolate [17]. In the present study, cpe-positive *Clostridium perfringens* isolates were sporulated in modified Duncan Strong medium and the resultant purified supernatant was examined for production of enterotoxin. Vero cell assay is rapid assay that measures the biological activity of *C. perfringens* enterotoxin. This method involves the rapid killing of Vero cells by enterotoxin produced by *C. perfringens* grown in Duncan and Strong sporulation medium [11]. The cytotoxicity of Vero cell in the present study, revealed that 5 isolates of 6 show cytotoxicity and only one isolate does not show any cytopathic effect, enterotoxin production was negative, in spite of the presence of the enterotoxin gene. Sporulation is essential prior to detection of CPE from a cpe-positive *C. perfringens* isolates, since CPE is formed in the cell during sporulation. Successful sporulation was confirmed by phase-contrast microscopy and the culture was sonicated until more than 95% of the spores were free, determined by phase-contrast microscopy [11]. In the present study, the six *C. perfringens* isolates carrying enterotoxin gene were studied for their ability of sporulation on Duncan and Strong (DS) sporulation medium and enterotoxin production. It was noted from the results that *C. perfringens* enterotoxin is directly related to the ability of the microorganism to sporulate as 5 out of 6 *C. perfringens* strains grown on DS sporulation medium were spore formers and enterotoxin positive. It was noted that one cpe positive *C. perfringens* isolates was sporulated well on modified DS sporulation medium while enterotoxin production was negative and this isolate concerning to type E. From these results, it was found that *Clostridium perfringens* plays a significant role in food poisoning since it was isolated at high incidence from all types of examined samples. Only cpe positive *C. perfringens* type A and C were able to express enterotoxin which is responsible for causing the symptoms of *C. perfringens* food poisoning.

Agarose gel electrophoresis of PCR product of some *C. perfringens* isolates

Lane 1: 50bp DNA ladder.
Lane 2: *C. perfringens* type C
Lane 3: *C. perfringens* type D.
Lane 6: *C. perfringens* type E, enterotoxin.
Lane 4, 5 and 7 to 12: *C. perfringens* type A

Lane 1: 50bp DNA ladder.
Lane 2: *C. perfringens* type D.
Lane 6: *C. perfringens* type A, enterotoxin.
Lane 3 to 5 and 7 to 12: *C. perfringens* type A
REFERENCES


