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The Level of Fecal Carriage and the Toxic Potential of *Clostridium perfringens* in the Feces of a Taiwan Subpopulation

I-CHEN YANG, JAN-YI WANG AND DANIEL YANG-CHIH SHIH*

Department of Food Microbiology, Bureau of Food and Drug Analysis, 161-2 Kunyang St., Taipei City 115, Taiwan, R.O.C.

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ABSTRACT

Clostridium perfringens ranks among the most common agents of food poisoning in industrialized nations. Little is known about the level of fecal carriage and the toxic potential of this microorganism in Taiwan. One of the criteria to confirm that *C. perfringens* was the causative agent of food poisoning is to isolate 10^5 organisms/g from stools of food poisoning patients. The aim of the present study was to determine the carriage of *C. perfringens* in the feces of a Taiwan subpopulation. The percentage of strains that have toxic potential was also determined by using polymerase chain reaction (PCR) assay and reverse passive latex agglutination (RPLA). As a result, 30 (60%) of the 50 fecal samples carried *C. perfringens*. The spore counts were between 50 and 2.5×10^8 CFU/g. The average spore count of all the test samples was 4.3×10^2 CFU/g. Eleven (22%) of the samples carried more than 10^5 spores per gram. Only three (1.8%) isolates from fecal samples were *cpe* gene positive. The RPLA results were in accordance with the genotypic results. Our findings emphasize that the culture of *C. perfringens* from fecal samples needs to be supplemented with presence of *cpe* gene or enterotoxin production in the outbreak situation.

Key words: *Clostridium perfringens*, fecal, enterotoxin, PCR

INTRODUCTION

Clostridium perfringens is a Gram-positive, anaerobic, and sporulating bacterium. This bacterium is considered to be ubiquitous and has been isolated from soil, plants, and animals including cattle, poultry, and humans. Animal and human sources appear to be the most common reservoirs[1,4,9,10,17,21]. Of the populations studied, 1~52% of the individuals possessed spores in their feces[21]. *C. perfringens* spore counts in normal feces usually ranged from $<10^3$ to 10^4 CFU/g[8,9,20]. However, Vela *et al*. reported that a significant percentage of healthy individuals from northern Mexico carried higher levels of *C. perfringens* spores[20]. In order to confirm that *C. perfringens* was the causative agent of food poisoning, one of the criteria is to isolate 10^5 organisms/g from stools of food poisoning patients[6].

C. perfringens ranks among the most common agents of food poisoning in industrialized nations, such as Norway, USA, Japan, and England[4]. The predominant symptoms include acute diarrhea and cramping[4,10,19,21]. Linkage between clinical significant properties and the enterotoxin (ENT) of this pathogen, human volunteer studies, and gene deletion studies showed that the ENT was the major virulence factor of *C. perfringens* food poisoning[18]. The disease is due to the production of ENT during sporulation of the organism in the small intestine following ingestion of large numbers of vegetative cells of ENT-positive *C. perfringens*[13,18,21]. With its production regulated by sporulation, ENT is produced in large amounts only during sporulation and is released upon lysis of the mother cell[7,12,22]. The demonstrations of the enterotoxigenity of *C. perfringens* with phenotypic methods include reverse passive latex agglutination (RPLA), enzyme-linked immunosorbent assay (ELISA) or Vero cell assay[4,10,16]. ENT is encoded by *cpe* gene which seems to be quite conserved and favorable for genotypic diagnosis[4,16].

To our knowledge, little is known about the level of fecal carriage and the toxic potential of this microorganism in the feces of people in Taiwan. The aim of the present study was to determine the carriage of *C. perfringens* in a Taiwan subpopulation. The percentage of strains that have toxic potential was also determined with PCR assay and RPLA.

MATERIALS AND METHODS

I. *Samples*

Clostridium perfringens ATCC 12917 (ENT+) and BCRC 10913 (ENT–) were used as reference strains. A total of 50 fecal samples were randomly collected from individuals in the metropolitan area of Taipei, Taiwan. Test subjects were relatives, neighbors, and friends of colleagues from Bureau of Food and Drug Analysis (Taipei, Taiwan), and did not exhibit symptoms of gastrointestinal disorders at the time of sampling. Nine samples were collected

* Author for correspondence. Tel: +886-2-26531265; Fax: +886-2-26531268; E-mail: shihdyc@nlfd.gov.tw

from children and adolescents (ages 1 to 19), 29 from adults (ages 20 to 49), and 12 from elderly (ages 50 and older). Among them, 23 were from male and 27 were from female. According to a previous report, the ability of *C. perfringens* spores to remain viable before and after long-term storage was almost the same. This ability is a very important advantage of fecal spore enumeration method for confirming outbreaks[8]. Samples were frozen at -20°C and transported with ice bag to the laboratory, where they were examined immediately or held at -20°C until analyzed (usually within 1 week).

II. *C. perfringens Enumeration*

One gram of fecal sample was weighed into screw-capped tube containing 9 mL of sterile saline water. The tubes were then placed in a water bath of 75°C for 15 min. Appropriate decimal dilutions of the samples were made with saline water. Each dilution was mixed thoroughly by vortex before each transfer. For plate count, 0.1 mL of the dilutions was placed on Tryptose-sulfite-cycloserine (TSC) agar (Merck KGaA, Darmstadt, Germany) containing egg yolk emulsion (HiMedia Laboratories, Ltd., Mumbai, India) and spread aseptically. After the aliquots were absorbed in the agar, additional 10 mL of TSC agar without egg yolk emulsion was poured into dishes. Plates were incubated anaerobically at 35°C for 24 hr using Oxoid anaerobic jars equipped with Oxoid AnaeroGen and catalyst (Oxoid, Ogdensburg, NY, USA). Plates containing 20~200 black colonies with a 2~4 mm opaque white zone surrounding the colonies were selected for counting. Ten typical *C. perfringens* colonies were selected for iron-milk presumptive confirmation test. Colonies exhibiting "stormy fermentation" in iron-milk presumptive confirmation test were inoculated into confirmation media for complete confirmation, including motility-nitrate and lactose-gelatin media. The media were incubated anaerobically at 35°C for 24 hr. Cultures that exhibited typical microscopic morphology and were lactose positive, nitrate reduction positive, gelatin positive, and motility negative, were considered to be *C. perfringens*. Organisms suspected to be *C. perfringens* which did not meet the criteria described above were also tested by salicin and raffinose fermentation. The number of *C. perfringens* cells in sample was calculated on the basis of percent of colonies tested that were confirmed as *C. perfringens*. The formulation of above media, including iron-milk, motility-nitrate, lactose-gelatin, salicin, and raffinose fermentation media and the testing procedure could be referred to the official method of analysis of AOAC and the bacterialogical analytical manual of USFDA[2,15].

III. *Detection of the cpe Gene by PCR*

Primers ET1: 5'-TGTAGAATATGGATTTGGAAT-3' and ET2: 5'-AGCTGGATTTGAGTTTAATG-3' were used according to the method of Ridell *et al.*[16]. The primers amplify a fragment of 363 bp. For DNA extraction, bacteria were placed in BHI (Merck KGaA, Darmstadt, Germany) and incubated overnight at 35°C. One milliliter of culture was removed and centrifuged at 10,000 ×*g* for 2 min. The pellet was resuspended in 50 μL of sterile water, boiled for 10 min and centrifuged again for 5 min at 10,000 ×*g*. The supernatant was transferred to a new microfuge tube and stored at -20°C before use.

PCR was performed by using a PCR thermal cycler (PCR cycler 9700, Applied Biosystems, CA, USA). The final reaction mixture (25 μL) contained 300 μM concentrations of each dNTP, 1× PCR buffer, 2 U of Taq DNA polymerase (Protech Technology Enterprise Co. Ltd., Taipei, Taiwan), 200 nM of each primer, and 1 μL (approximately 20 ng) of template DNA. Amplification consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 51°C for 30 sec, and 72°C for 30 sec. A final 7-min 72°C extension was followed. PCR products were analyzed on a 2% (wt/vol) agarose gel and electrophoresis was carried out for 30 min at 100 V.

IV. *DNA Sequencing and Sequence Analysis*

PCR amplicons obtained were directly sequenced using ABI 3730 XL DNA Analyzer (Applied Biosystems, CA, USA). The resulting sequences were compared with other nucleotide sequences in GenBank by using Nucleotide-nucleotide BLAST (blastn).

V. *Enterotoxin Immunoassay*

Test bacterium is cultured with Difco thioglycollate medium (BD, Franklin Lakes, USA) at 35°C for 4 hr. Four-hour thioglycollate culture (0.75 mL) was used to inoculate 15 mL of modified Duncan-Strong sporulation medium[15] prepared for promoting enterotoxin production. The inoculated broth was incubated at 35°C for 18~24 hr. After cultivation, the culture fluid was centrifuged at 10,000 ×*g* for 15 min and the supernatant was tested for *C. perfringens* ENT by using a reversed passive latex agglutination test kit, PET-RPLA (Denka Seiken, Tokyo, Japan). The assay was performed according to the manufacturer's instructions. The formulation of modified Duncan-Strong sporulation medium could be referred to the bacterialogical analytical manual of USFDA[15].

RESULTS AND DISCUSSION

One of the criteria to confirm that *C. perfringens* was the causative agent of food poisoning is to isolate 10^5 viable counts or spores per gram of feces[6]. In case of infectious diarrhea, fecal spore counts may range from 10^6 to 10^8 spores per gram[8]. In this study, 30 (60%) of the 50 feces carried *C. perfringens* and the spore counts were between 50 and 2.5×10^8 spores/g. The average spore count of all test samples was 4.3×10^2 spores/g, agreeing with previous

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reports that the *C. perfringens* spore counts in normal feces usually ranged from < 10^3 to 10^4 CFU/g[8,20] and fewer than the criterion to confirm this pathogen as the causative agent of food poisoning. Moreover, the spore counts of 32 (64%) samples were less than 10^4 spores/g. However, 11 (22%) of the samples carried more than 10^5 spores/g in this study. It showed that a significant percentage of the healthy population in Northern Taiwan carried high levels of *C. perfringens* spores. This result was consistent with that reported by Vela *et al.*, in which 27% of the fecal samples from healthy individuals carried 10^5 or more spores per gram of feces[20]. Hence, the criterion, isolating 10^5 viable counts or spores per gram from feces to confirm *C. perfringens* as the causative agent of food poisoning, alone might not be sufficient to confirm *C. perfringens* food poisoning.

It was known that ENT is the major virulence factor of *C. perfringens* food poisoning[13,18,21]. Ridell *et al*. reported that the prevalence of the enterotoxin gene of the food and clinical isolates associated with food-poisoning outbreaks were 86% and 88%, respectively[16]. It was interesting to study the prevalence of *cpe* gene and ENT of this pathogen in the feces of healthy population in Taiwan to compare the prevalence of *cpe* gene and ENT of the feces between food-poisoning cases and healthy population, as well as clarify the need to detect *cpe* gene or ENT to confirm *C. perfringens* food poisoning. In this study, 165 *C. perfringens* strains were isolated and tested for the presence of the *cpe* gene. A *cpe*-positive (ATCC 12917) and a *cpe*-negative (BCRC 10913) *C. perfringens* strain were used as controls. The predicted size was 363 bp (Figure 1). The PCR products were sequenced and the resulting sequences were compared with other nucleotide sequences by using blastn tool on NCBI homepage. The similarities of the resulting sequences and corresponding sequences in GenBank were > 99.0%. This result further confirmed that the PCR product was as predicted and the sequence of *cpe* gene was also conserved in *C. perfringens* used in this study. The result that only 3 (1.8%) among 165 isolates were *cpe* positive showed that only a small percentage of *C. perfringens* from feces of the healthy population carried *cpe* gene. The prevalence of *cpe* gene in the healthy population was obviously lower

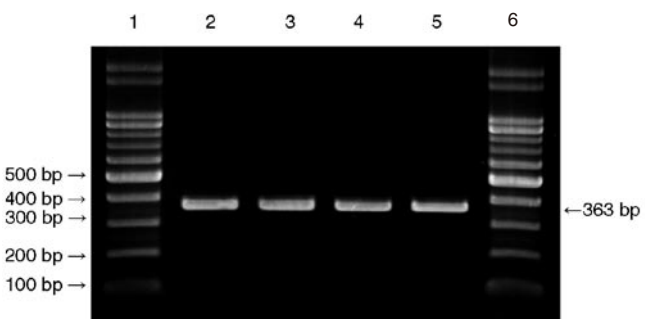

Figure 1. PCR detection for *cpe* gene of *C. perfringens*. Lanes 1 and 6, 100 bp ladder as molecular weight marker; lane 2, *C. perfringens* ATCC 12917; lane 3 to 5, *C. perfringens* BFDA 20-5, 22-1, and 22-10 collected from the feces of healthy individuals.

than that in the food poisoning cases. Hence, both the existence of *cpe* gene in the isolates from feces of infected individuals as well as the confirmation that the strains from infected individuals may be required to confirm in outbreak situations. The low percentage of *cpe* positive isolates is in accordance to previous reports that the *cpe* gene is restricted to only minority of all isolated *C. perfringens*[13,16,17]. The explanation for the *cpe*-negative isolates could be that the *cpe* gene is easily acquired and lost[5,16]. Considerable experimental evidence supported that the involvement of mobile genetic elements in the mobilization and/or transfer of the *cpe* gene and the low level of *cpe*-positive strains can serve as reservoir strains for the enterotoxin[4,5,17].

All of the three *cpe*-positive isolates also gave positive results in PET-RPLA test in this study. The genotypic detection agreed with the phenotypic detection. Result of this study and previous reports showed that all *C. perfringens* strains with *cpe* gene tested to date were able to produce enterotoxin[4,16] and made the genotypic method a cheaper and faster alternative for the enterotoxin assay. Several other studies indicated that some *cpe*-positive *C. perfringens* strains failed to sporulate in common media and did not produce a detectable amount of ENT or the gene was a silent, unexpressed *cpe* gene[3,10]. These results conflicted with our result. However, the limitation of *in vitro* detection of ENT might be overcome by improving or modifying the sporulation media, cultural conditions or the immunoassay itself. That means the *in vitro cpe*-positive/ENT-negative strains might be enterotoxic in the *in vivo* condition and bear the ability to cause food poisoning. Therefore, the *cpe* detection assay is recommended instead of the ENT assay when more information is acquired with further *in vivo* study of *cpe*-positive strains.

In this study, all of the three *cpe*-positive strains co-existed with the *cpe*-negative strains in the same samples. Lukinmaa *et al*. indicated that in the fecal samples from the infected persons of food poisoning outbreaks, a colony of normal flora might have been picked up for further testing instead of enterotoxigenic *C. perfringens*[10]. Similarly, the isolation of enterotoxigenic *C. perfringens* strains from meat may be difficult because of the presence of large amount of nonenterotoxigenic cells[14]. According to our experience, there were no obvious phenotypic differences between *cpe*-positive and *cpe*-negative colonies. Thus, it is very important to pick up more than one isolate from the fecal cultures to ensure that the *cpe*-positive strain will not be missed.

When analyzing, a total of 313 colonies on TSC were chosen for further identification. Among them, 263 strains exhibited "stormy fermentation" in iron-milk presumptive test. This reaction is characterized by rapid coagulation of milk followed by fracturing of curd into spongy mass which usually rises above medium surface[15]. After complete confirmation tests, 165 (62.2%) among 263 strains were confirmed as *C. perfringens*. No commercial products were available as confirmation media. The confirmation tests for *C. perfringens* are both labor and time-consuming.

In contrast, the result of stormy fermentation can be read within 6 hr. Therefore, iron-milk presumptive test could be used as a screening test especially when numerous samples need to be analyzed.

In conclusion, considerable portion of fecal samples from healthy individuals carried more than 10^5 spores per gram. Only three (1.8%) isolates from fecal samples were *cpe* gene positive. The PET-RPLA results agreed with the genotypic results. Our finding emphasize that the culture of *C. perfringens* from fecal samples needs to be supplemented with confirmation of *cpe* gene presence or ENT in the outbreak situation.

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