Epidemiological studies on *Clostridium perfringens* food poisoning in retail foods

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Summary

Clostridium perfringens (C. perfringens) is an important anaerobic pathogen causing food-borne gastrointestinal (GI) diseases in humans and animals. Meat and meat products are the most common vehicles of *C. perfringens* type A food poisoning. Contamination of meat by the intestinal contents of slaughtered animals may serve as an important source of this pathogen to the food supply. One hundred and fifty-five non-outbreak food samples were obtained from meat and retail food and examined for the presence of *C. perfringens*. Multiplex polymerase chain reaction assay to determine the toxin genotype of *C. perfringens* isolates, and extraction and purification of *C. perfringens* enterotoxin from enterotoxin gene (*cpe*)-positive isolates were carried out. The homogeneity of the purified enterotoxin was demonstrated by polyacrylamide gel electrophoresis. In addition, stool samples were collected from 150 persons in contact with animals, and enzyme-linked immunosorbent assays were carried out for the qualitative determination of *C. perfringens* enterotoxin in the stool samples. The results demonstrated that approximately 2.6% of the tested meat and retail meat samples were contaminated with *cpe*-positive *C. perfringens*. The recommended laboratory criteria used to implicate *C. perfringens* in food-borne disease should involve the detection of *C. perfringens* enterotoxin production or the presence of the *cpe* gene in foods or faeces, or in the suspected *C. perfringens*

isolates. In the present study some isolates such as tuna contained the enterotoxin gene although they had a low count of *C. perfringens*.

Keywords

Clostridium perfringens – Enterotoxin – Food poisoning – Retail food.

Introduction

Meat and meat products are the most common vehicles of *Clostridium perfringens* type A food poisoning. Contamination of meat with the intestinal contents of slaughtered animals may serve as an important source of this pathogen to the food supply (1, 2).

The available epidemiological data reveal that *C. perfringens* is considered as one of the most commonly occurring bacterial agents of food-borne illness in developing countries, ranking behind *Salmonella* spp., *Campylobacter* spp. and *Staphylococcus aureus* (3). The ubiquitous distribution of *C. perfringens* has been considered a logical explanation for the common occurrence of *C. perfringens* food poisoning. Therefore, all *C. perfringens* isolates are regarded as potential causative agents for *C. perfringens* type A food poisoning (4). 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 poisoning. The relatively greater heat resistance of the strains with a chromosomally located enterotoxin gene (*cpe*) is a plausible explanation for these strains' survival in cooked food, thus causing instances of food poisoning (5). Diseases associated with CPE usually represent type A, perhaps because isolates of type A account for more than 95% of global *C. perfringens* isolates, and temperature abuse of food is considered the major contributing factor to food poisoning, with the most common vehicle being meat or poultry (6). Optimal conditions for food poisoning arise when contaminated food is held or served at a temperature range of 10–54°C, allowing growth of the organism. When large numbers of vegetative cells are subsequently ingested, they sporulate and release CPE into the intestinal lumen. The CPE is a single polypeptide chain with a molecular weight of 35 kDa

that binds to receptors on target epithelial cells (7). Genetic studies of *cpe* have shown that *cpe* can be either chromosomal or plasmid-borne and only a small minority of the global *C. perfringens* population is *cpe* positive. The resistance phenotype of chromosomal-*cpe* (C-*cpe*) isolates extends beyond temperature resistance to also include, for both vegetative cells and spores, enhanced resistance to osmotic stress (from sodium chloride) and nitrites (8). This broad-spectrum nature of the C-*cpe* resistance phenotype suggests that these bacteria may employ multiple mechanisms to persist and grow in foods prior to their transmission to humans.

The ingestion of contaminated food by *C. perfringens* type A isolates is followed by gastrointestinal disease, when enzyme-resistant CPE is set free during sporulation. In most cases the bacterium has to grow up to more than 10^6 colony-forming units (cfu)/g food to cause gastrointestinal disease (9, 10). The diarrhoea and cramping symptoms of *C. perfringens* food poisoning result from CPE encoded by the *C. perfringens* enterotoxin gene (*cpe*) (11).

The objectives of this study were to evaluate meat and retail food for the presence of enterotoxigenic *C. perfringens* and to investigate whether meat and retail food can be a reservoir for *C. perfringens* food poisoning in humans.

Materials and methods

Collection of samples

One hundred and fifty-five non-outbreak food samples were obtained from retail outlets in Giza governorate, Egypt. These outlets included a mix of slaughterhouses, butcher shops, grocery stores and large supermarkets. In addition, 40 samples from the intestinal contents of cattle and buffaloes at slaughterhouses were included in this study. A breakdown of these samples is shown in Table I. Stool samples from 150 persons in contact with animals (60 from apparently healthy persons, and 90 from patients with diarrhoea) were also collected.

Insert Table I

Preparation of food samples

Ten-gram portions of meat and retail food samples were diluted in 99 ml of sterile 0.1% peptone water and homogenised in a blender at 200 rpm for 1–2 min; 1 ml of each homogenised food suspension was added to a tube containing 10 ml of sterile cooked meat broth (CMB).

Isolation of *Clostridium perfringens*

Each sample was inoculated onto a tube of sterile freshly prepared cooked meat medium, and then the tube was incubated anaerobically in an anaerobic jar using an anaerobic gas generating kit at 37°C for 24–48 h. A loopful from the previously incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200 µg/ml). Each plate was incubated anaerobically at 37°C for 24–48 h. The plates were examined for the characteristic colonies of *C. perfringens*. Subcultures from the suspected colonies were identified morphologically and biochemically as described by Koneman *et al*. (12).

Total anaerobic count of *Clostridium perfringens* in meat and retail food

The method recommended by the International Organization for Standardization (ISO) (13) was used.

DNA extraction

A rapid boiling procedure was used to prepare template DNA from bacterial strains, according to the methods of Sheedy *et al.* (14).

Multiplex polymerase chain reaction assay to determine the toxin genotype of *Clostridium perfringens* isolates

A multiplex polymerase chain reaction (PCR) assay was used to detect the presence of genes encoding alpha-toxin (*cpa*), beta-toxin (*cpb*), epsilon-toxin (*etx*), iotatoxin (*iap*) and CPE (*cpe*). The primer sequences have been published previously (15). Each PCR had a total volume of 25 µl, which contained 5 µl of DNA as template,

10 picomole (pmol) of each primer and 1× PCR master mix, made up to 25 μl with DNase–RNase-free water. The 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 followed. Amplification products were electrophoresed in 1.5% agarose gels containing 0.5× TBE (Tris borate ethylenediamine tetraacetic acid) at 70 V for 60 min and visualised under ultraviolet light.

Extraction and purification of *C. perfringens* enterotoxin

The *cpe*-positive *C. perfringens* isolates obtained in this study were cultured in modified Duncan–Strong (DS) sporulation medium and incubated at 37°C for 8 h under anaerobic conditions for enterotoxin production. The sporulated cells were washed and suspended in 2 ml cold saline. The cells were disrupted by sonic treatment (6 Hz for 20 min using an ultrasonic sonicator) and debris was removed by centrifugation at 12,000 ×g for 20 min at 4°C to obtain a clear extract. The resultant cell extract was precipitated by addition of ammonium sulphate (4.76 g $(NH_4)_2SO_4$/10 ml supernatant) and incubated overnight at 4°C. The precipitated protein was then collected by centrifugation at 12,000 rpm for 30 min at 4°C and resuspended in 25 μl of sterile phosphate buffered saline. This solution was dialysed overnight against the same buffer and any precipitate was removed by centrifugation (15, 16, 17).

Polyacrylamide gel electrophoresis

The homogeneity of the purified enterotoxin was demonstrated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described by O'Connor (18).

The molecular weights of the developed protein bands were estimated from the migration of standard protein samples by using Gel-pro Analyzer software (version 4.5; Media Cybernetics, Inc., USA).

Enzyme-linked immunosorbent assay for the qualitative determination of *Clostridium perfringens* enterotoxin in diarrhoeic stool samples

The steps were carried out according to the manufacturer's instructions (RIDASCREEN® *C. perfringens* Enterotoxin, Darmstadt, Germany).

Results and discussion

Meat and meat products are the most common vehicles of *C. perfringens* type A food poisoning. Contamination of meat with the intestinal contents of slaughtered animals may serve as an important source of this pathogen to the food supply (1, 2). Table I shows that the rate of recovery of *C. perfringens* from meat at slaughterhouses was 52.9% and 43.5% in meat from cattle and buffaloes, respectively. The rate of occurrence of *C. perfringens* in meat after it had left the slaughterhouse for butcher shops was 69.2% for cattle and 88.2% for buffaloes. From this result, it was observed that the occurrence of *C. perfringens* in butcher shops was higher than that reported in slaughterhouses. This may be due to contamination of meat from equipment at butcher shops, in addition to contamination of carcasses during transportation from the slaughterhouse to butcher shops. Moreover, in slaughterhouses, the process of washing carcasses may eliminate some contaminating bacteria, as observed by Gomaa *et al.* (19), who found that washing of the carcasses at Abiss slaughterhouse in Alexandria decreased the count of bacteria.

The typing of *C. perfringens* isolates obtained from meat at slaughterhouses revealed that all isolates were of type A and none of the isolates contained the enterotoxin gene. On the other hand, typing of *C. perfringens* isolates obtained from meat at butcher shops showed that all isolates were of type A and two of them contained the enterotoxin gene, one from cattle meat (11.1%), the other from buffalo meat (6.7%). Assays for the *cpb*, *iap* and *etx* genes were negative in all tested isolates. Figure 1 shows the amplification of the alpha toxin gene at 324 bp (base pairs), representing *C. perfringens* type A; Figure 1 also shows enterotoxigenic *C. perfringens* type A with the

amplification of the enterotoxin-producing gene (*cpe*) at 233 bp. The description of *cpe*-positive isolates obtained from meat samples shows that the *cpe*-positive isolates represent type A. It is obvious that the presence of a large number of isolates of biotype A and the fact that they produce enterotoxin indicate the risk of toxic infection caused by consumption of meat from such carcasses, if treated inappropriately. Meat products are gaining popularity because they represent quick, easily prepared meals and solve the problems of a shortage of fresh meat (20). Meat products are recognised as a major source of food-borne pathogens that cause food poisoning in humans. The occurrence of *C. perfringens* in retail food was studied (Table I), and it was found that the isolation rate of *C. perfringens* was 30% in beefburgers, 10% in sausage, 40% in beef luncheon meat, 10% in frozen kofta, 5% in canned beef and 20% in tuna.

Insert Figure 1

Typing of *C. perfringens* isolates from these retail foods clarified that all the isolates were of type A and the enterotoxin gene was found only in two isolates derived from tuna. These findings indicate that some retail foods which are ready to eat (e.g. tuna) are contaminated with *C. perfringens* isolates of type A carrying the enterotoxin gene and that these are important risk factors for food intoxication involving *C. perfringens*. Regarding the total anaerobic count of *C. perfringens* in meat and retail foods (Table II), the count of *C. perfringens* ranged from 1.2×10^2 to 7×10^4 per gram; the highest level of contamination was found in cattle meat while the lowest was found in tuna.

Insert Table II

The Nordic Committee on Food Analysis (NCFA) (21) recommend that the laboratory criteria used in association with clinical presentation and epidemiological evidence to implicate *C. perfringens* in food-borne disease should be as follows: high numbers of viable cells (≥105/g) in suspect foods, and the presence of elevated faecal spore counts (>106/g). These widely used laboratory criteria are a point of weakness because they do not involve the detection of CPE

production or the presence of the *cpe* gene in foods or faeces, or in the suspected *C. perfringens* isolates. In the present study some isolates, such as tuna, contained the enterotoxin gene despite having a low count of *C. perfringens*. The *C. perfringens* enterotoxin (CPE) is responsible for diarrhoea and cramping symptoms in humans. In this study the prevalence of *C. perfringens* in the faeces of apparently healthy people who are in contact with animals and meat (food handlers) was 55%. Typing of the isolates by multiplex PCR showed that the isolated strains were of type A (Table I), and only one isolate contained enterotoxin (Table III).

Insert Table III

Detection of CPE in stool samples has been suggested to be the definitive method of implicating this organism as the cause of illness, therefore stool samples from 90 persons with diarrhoea who are in contact with animals and meat were examined to detect the presence of CPE using ELISA. The results of the ELISA showed that only 3 out of the 90 diarrhoeic stool samples were CPE positive (Table III). The occurrence of *cpe*-positive *C. perfringens* type A in the faeces of healthy persons (15), and the full capacity of these strains to produce CPE in patients with diarrhoea, indicates that humans handling food should be regarded as a risk factor for the spread of *cpe*-positive *C. perfringens* type A food contamination.

Taken together, the results of this study showed that *C. perfringens* occurred at a high level in all the types of sample examined. This is of high significance because some strains have the ability to synthesise enterotoxins that are responsible for causing the symptoms of *C. perfringens* food poisoning. In this regard, *C. perfringens* enterotoxin genes were detected in five samples (four from retail and meat samples and one from a human faecal sample). Table IV shows that all *cpe*-positive isolates were of type A. The CPE is produced during sporulation and, thus, sporulation *in vitro* is essential to measure the production of CPE by an isolate. In the present study, *cpe*-positive *C. perfringens* isolates were sporulated in modified DS medium and the resultant purified supernatant was examined for

production of enterotoxin. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has become an important tool for protein profiling which reflects the genetic identity and non-identity of microorganisms (22). In the current study, the purified enterotoxins of some *cpe*-positive *C. perfringens* isolates were studied by SDS-PAGE (Table V), which showed that there were three to six protein bands of molecular weights ranging from 35 to 122.46 kDa. There was a degree of homogeneity among all tested field isolates, as they shared common antigenic bands at 35 and 78.38 kDa. These findings are similar to those of other authors (23, 24), who found a higher percentage of conservation (exceeding 90%) among *C. perfringens* isolates.

Insert Tables IV and V

From this study, it was concluded that *C. perfringens* plays a significant role in food poisoning because it was isolated at high frequency from all types of sample examined and because some strains isolated had the ability to synthesise enterotoxins that are responsible for causing the symptoms of *C. perfringens* food poisoning. The recommended laboratory criteria used to implicate *C. perfringens* in food-borne disease should involve molecular typing methods to determine the enterotoxigenicity of *C. perfringens* isolates related to the food poisoning outbreak. The enterotoxigenic isolates need further investigation regarding the chromosomal or plasmid localisation of *cpe*.

Competing interests

There are no competing interests.

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Table I

Incidence and typing of *Clostridium perfringens* in the examined food

Source of samples	No. of samples examined	Samples positive for *C. perfringens*		Typing of *C. perfringens* isolated from food animals using multiplex PCR			
		No.	%	Type A		*cpe*	
				No.	%*	No.	%
Cattle meat at slaughter house	17	9	52.9	9	100	0	0
Buffalo meat at slaughter house	23	10	43.5	10	100	0	0
Cattle meat at butcher shops	13	9	69.2	9	100	1	11.1
Buffalo meat at butcher shops	17	15	88.2	15	100	1	6.7
Beefburger	10	3	30	3	100	0	0
Sausage	10	1	10	1	100	0	0
Beef luncheon meat	10	4	40	4	100	0	0
Frozen kofta	10	1	10	1	100	0	0
Canned beef	20	1	5	1	100	0	0
Tuna	25	5	20	5	100	2	40
Total	155	58	37.4	58	100	4	2.6

cpe: *C. perfringens* enterotoxin gene

PCR: polymerase chain reaction

%* calculated according to the number of positive samples

The results for the alpha-toxin (*cpa*), beta-toxin (*cpb*), epsilon-toxin (*etx*) and iotatoxin (*iap*) genes were negative in all tested isolates

Table II

Total anaerobic count of *Clostridium perfringens* in meat and retail foods

Samples	Average count of *C. perfringens* per gram
Cattle meat	7×10^4
Buffalo meat	1.7×10^4
Beefburger	2×10^2
Sausage	2.3×10^3
Beef luncheon meat	4×10^2
Frozen kofta	4.6×10^3
Canned beef	9×10^2
Tuna	1.2×10^2

Table III

Occurrence of *Clostridium perfringens* in human faeces

Total no. of faecal samples examined	Samples positive for *C. perfringens* by culture and PCR						Samples positive for *C. perfringens* enterotoxin by ELISA		
150	No. of samples examined	Positive for *C. perfringens*		Type A positive isolates		*cpe* positive isolates	No. of samples examined	Positive for enterotoxin	
		No.	%	No.	%*	*No.*	%*	No.	%
	60	33	55	33	100	1	3	90	3	3.3

cpe: *C. perfringens* enterotoxin gene

ELISA: enzyme-linked immunosorbent assay

PCR: polymerase chain reaction

%* calculated according to the number of positive samples

Table IV

Description of *cpe*-positive *Clostridium perfringens* isolates

cpe-positive isolates	Source of isolate	Typing	Spore formation	Enterotoxin production
Field isolate 1	Cattle meat	A	Positive	Positive
Field isolate 2	Buffalo meat	A	Positive	Positive
Field isolate 3	Tuna	A	Positive	Positive
Field isolate 4	Tuna	A	Positive	Positive
Field isolate 5	Human faeces	A	Positive	Positive

cpe: *C. perfringens* enterotoxin gene

Table V

Different protein fractions detected in purified enterotoxin of *cpe*-positive *Clostridium perfringens* isolates using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

	Fractionated samples (kDaltons)			
	Sample (1)	Sample (2)	Sample (3)	Sample (4)
1	122.46	78.38	78.38	78.38
2	108.02	45.00	45.00	45.00
3	78.38	35.00	35.00	35.00
4	48.00			
5	35.00			
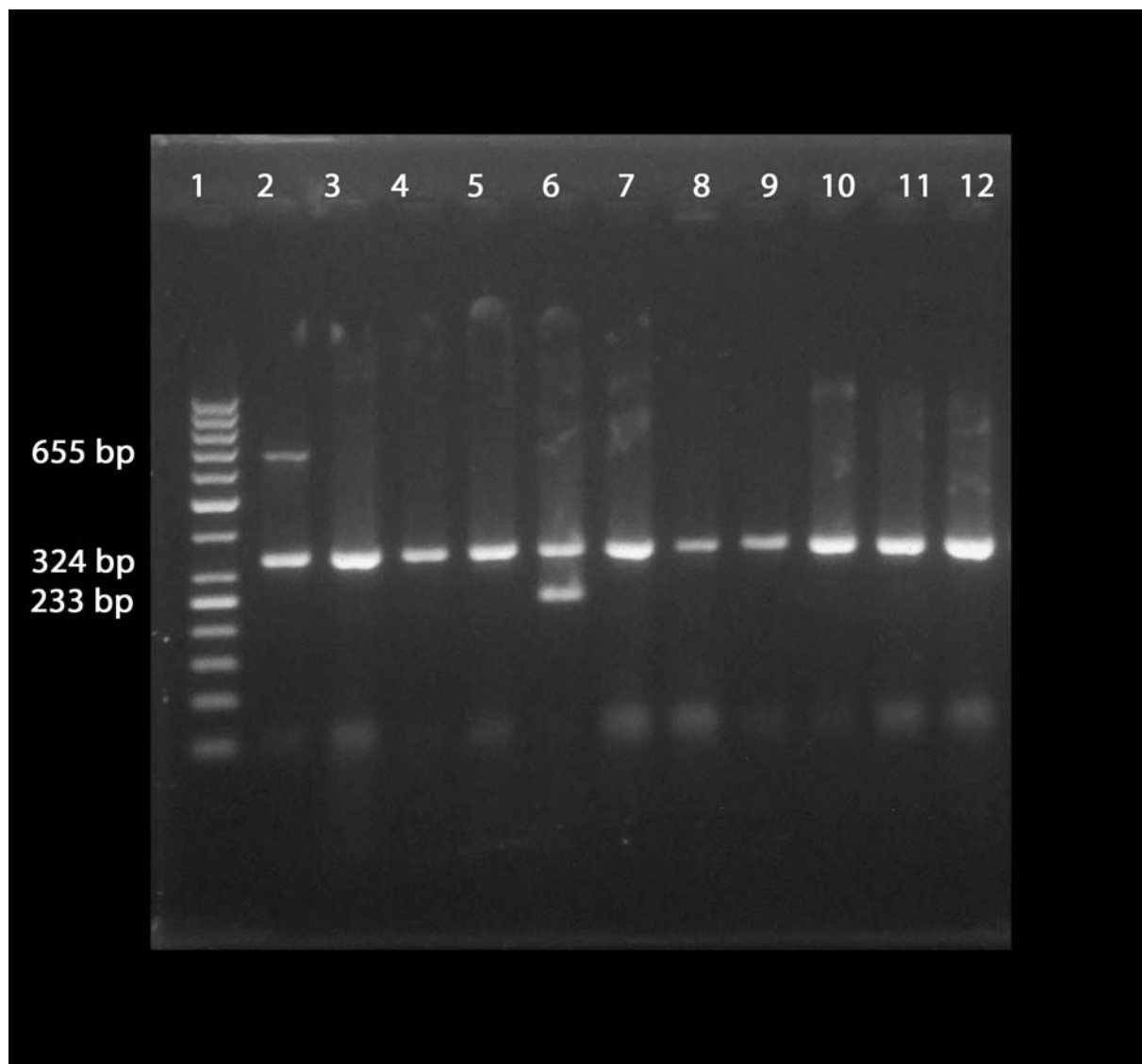
Fig. 1

Agarose gel electrophoresis of polymerase chain reaction products of *Clostridium perfringens* isolates

Lane 1: 50 base pair DNA ladder

Lane 2: *C. perfringens* type D positive control

Lane 6: *C. perfringens* type A, enterotoxin

Lanes 3, 4, 5 and from 7 to 12: *C. perfringens* type A