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

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Summary

Clostridium 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 who had been 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 10–54°C, allowing for 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

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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 106 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.

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 revolutions per minute (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

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			
				Type A		*cpe*	
		No.	%	No.	%*	No.	%
Cattle meat at slaughterhouse	17	9	52.9	9	100	0	0
Buffalo meat at slaughterhouse	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

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 picomoles (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 tetra-acetic 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 the 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 Costas (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., United States of America).

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. The 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 the contamination of meat from equipment at butcher shops, in addition to the 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 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%) and 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 base pairs (bp), 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 the 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.

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.

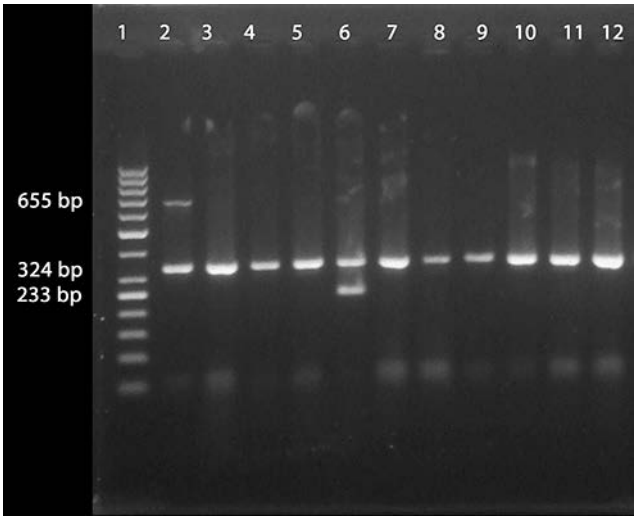

Fig. 1
Agarose gel electrophoresis of polymerase chain reaction products of *Clostridium perfringens* isolates

Lane 1: 50 base pair (bp) DNA ladder
Lane 2: *C. perfringens* type D positive control
Lanes 3, 4, 5 and from 7 to 12: *C. perfringens* type A
Lane 6: *C. perfringens* type A, enterotoxin

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

The Nordic Committee on Food Analysis (NCFA) (21) recommends 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 the isolates using multiplex PCR showed that the isolated strains were of type A (Table I), and only one isolate contained enterotoxin (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 were in contact with animals and meat were examined to detect the presence of CPE using enzime-linked immunosorbent assay (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

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		
	No. of samples examined	Positive for *C. perfringens*		Type A positive isolates		*cpe*-positive isolates		No. of samples examined	Positive for enterotoxin	
150		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

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 the production of enterotoxin. Sodium dodecyl sulphate polyacrylamide gel electrophoresis 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.

Table V
Different protein fractions detected in purified enterotoxin of *cpe*-positive *Clostridium perfringens* isolates using sodium dodecyl sulphate polyacrylamide gel electrophoresis

	Fractionated samples (kDal)			
	(1)	(2)	(3)	(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			

From this study, it was concluded that *C. perfringens* plays a significant role in food poisoning because it was isolated at a 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. ■

Études épidémiologiques sur la contamination de denrées alimentaires vendues au détail par *Clostridium perfringens*

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Résumé

Clostridium perfringens est une bactérie anaérobie majeure responsable de toxi-infections alimentaires à symptomatologie gastro-intestinale chez l'homme comme chez l'animal. La viande et les produits carnés contaminés sont les sources les plus fréquentes des toxi-infections dues aux *C. perfringens* de type A. La contamination de la viande par le contenu intestinal des animaux abattus est une voie de pénétration majeure de la bactérie dans la chaîne alimentaire. Lors d'une étude visant à déterminer la présence de *C. perfringens* en dehors des épisodes de toxi-infection, 155 échantillons de viande et de denrées alimentaires vendues au détail ont été analysés. Les échantillons ont été soumis à une amplification en chaîne par polymérase multiplex afin de caractériser les toxinotypes des *C. perfringens* isolées ; l'entérotoxine de *C. perfringens* a été extraite et purifiée à partir des isolats possédant le gène de l'entérotoxine (*cpe*). L'homogénéité de l'entérotoxine purifiée a été mise en évidence par électrophorèse sur gel de polyacrylamide. En outre, 150 échantillons de selles provenant de personnes en contact avec des animaux ont été collectés et soumis à une épreuve immuno-enzymatique en vue d'une évaluation qualitative de la présence d'entérotoxines produites par *C. perfringens* dans ces échantillons. Il ressort de l'étude qu'environ 2,6 % des échantillons de viande et de denrées alimentaires vendues au détail étaient contaminés par des *C. perfringens* possédant le gène *cpe*. Les procédures de laboratoire recommandées pour incriminer *C. perfringens* en cas de toxi-infection alimentaire devraient porter sur la mise en évidence de la production d'entérotoxines par *C. perfringens* ou sur la détection du gène *cpe* dans les aliments ou les fèces ou dans les isolats présumés de *C. perfringens*. Dans l'étude présentée par les auteurs, certains échantillons (notamment de thon) contenaient le gène de l'entérotoxine malgré un faible taux de contamination par *C. perfringens*.

Mots-clés

Clostridium perfringens – Denrées alimentaires vendues au détail – Entérotoxine – Toxi-infection alimentaire.

■

Estudios epidemiológicos de las intoxicaciones alimentarias por la presencia de *Clostridium perfringens* en alimentos vendidos al por menor

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Resumen

Clostridium perfringens es un importante patógeno anaeróbico, causante de enfermedades gastrointestinales de transmisión alimentaria en el hombre y los animales. La carne y los productos cárnicos son el vehículo más frecuente de las intoxicaciones alimentarias por *C. perfringens* de tipo A. La contaminación de la carne por el contenido intestinal de los animales sacrificados puede ser una importante vía de entrada de este patógeno en el suministro alimentario. A partir de carne y alimentos vendidos al por menor se obtuvieron 155 muestras alimentarias no relacionadas con brote alguno, que fueron analizadas para detectar la eventual presencia de *C. perfringens.* Para determinar el genotipo toxínico de los *C. perfringens* aislados se empleó una reacción en cadena de la polimerasa (PCR) múltiple. Después se procedió a extraer y purificar la enterotoxina (*cpe*) a partir de los *C. perfringens* positivos para el gen de que la codifica. Por electroforesis en gel de poliacrilamida se comprobó la homogeneidad de la enterotoxina purificada. Por otro lado, se obtuvieron muestras fecales de 150 personas que estaban en contacto con los animales y se practicaron ensayos inmunoenzimáticos para determinar, cualitativamente, la presencia de la enterotoxina de *C. perfringens* en dichas muestras. Los resultados pusieron de manifiesto que alrededor de un 2,6% de la carne analizada y de la carne vendida al por menor estaba contaminada por organismos *C. perfringens cpe*-positivos. Para discernir la intervención de *C. perfringens* en una enfermedad de transmisión alimentaria, los criterios de laboratorio recomendados pasan por detectar la producción de la enterotoxina de *C. perfringens* o la presencia del gen *cpe* en heces, alimentos o muestras sospechosas de contener *C. perfringens.* En el estudio descrito por los autores algunas muestras, como las de atún, contenían el gen de la enterotoxina, aunque presentaban un bajo recuento de organismos *C. perfringens.*

Palabras clave

Alimento vendido al por menor – *Clostridium perfringens* – Enterotoxina – Intoxicación alimentaria.

References

1. Labbé R. (2000). – *Clostridium perfringens*. *In* The microbiological safety and quality of food. Aspen Publishers, Gaithersburg, United States of America, 1110–1135.

2. Heredia N.L. & Labbé R.G. (2001). – *Clostridium perfringens*. *In* Guide to Foodborne Pathogens (R.G. Labbé, ed.). Wiley Interscience, New York, United States of America, 133–141.

3. Eisgruber H. (2004). – Typing of *Clostridium perfringens* from an epidemiological point of view. *Archiv Lebensmittelhygiene*, **55** (6), 128–130.

4. Smedley J.G., Fisher D.J., Sayeed S., Chakrabarti G. & McClane B.A. (2004). – The enteric toxins of *Clostridium perfringens*. *Rev. Physiol. Biochem. Pharm.*, **152**, 183–204. doi:10.1007/s10254-004-0036-2.

5. Sarker M.R., Shivers R.P., Sparks S.G., Juneja V.K. & McClane B.A. (2000). – Comparative experiments to examine the effects of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid genes versus chromosomal enterotoxin genes. *Appl. Environ. Microbiol.*, **66** (8), 3234–3240. doi:10.1128/AEM.66.8.3234-3240.2000.

6. McClane B.A. (2001). – *Clostridium perfringens*. *In* Food microbiology: fundamentals and frontiers, 2nd Ed. ASM Press, Washington, DC, United States of America, 351–372.

7. Brynestad S. & Granum P.E. (2002). – *Clostridium perfringens* and foodborne infections. *Int. J. Food Microbiol.*, **74** (3), 195–202. doi:10.1016/S0168-1605(01)00680-8.

8. Li J. & McClane B.A. (2006). – Comparative effects of osmotic, sodium nitrite-induced, and pH-induced stress on growth and survival of *Clostridium perfringens* type A isolates carrying chromosomal or plasmid-borne enterotoxin genes. *Appl. Environ. Microbiol.*, **72** (12), 7620–7625. doi:10.1128/AEM.01911-06.

9. Lukinmaa S., Takkunen E. & Siitonen A. (2002). – Molecular epidemiology of *Clostridium perfringens* related to food-borne outbreaks of disease in Finland from 1984 to 1999. *Appl. Environ. Microbiol.*, **68** (8), 3744–3749. doi:10.1128/AEM.68.8.3744-3749.2002.

10. Messelhausser U., Zucker R., Elmer-Englhard D., Busch U., Hormansdorfer S., Pudich U. & Holler C. (2007). – Detection and characterization of *C. perfringens* via real-time-PCR. *J. Verbraucherschutz Lebensmittelsicherheit*, **2** (2), 194–197.

11. Collie R.E. & McClane B.A. (1998). – Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *J. Clin. Microbiol.*, **36** (1), 30–36. Available at: http://jcm.asm.org/content/36/1/30.full.pdf+html (accessed on 15 March 2017).

12. Koneman E.W., Allen S.D., Dowel V.R. & Summers H.W. (1992). – Color atlas and textbook of diagnostic microbiology, 4th Ed. Lippincott Williams & Wilkins, New York, 1200 pp.

13. International Organization for Standardization (ISO) (2004). – ISO 7937:2004: Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of *Clostridium perfringens* – colony-count technique, Technical Committee: ISO/TC 34/SC 9 Microbiology, 3rd Ed. ISO, Geneva, 16 pp.

14. Sheedy S.A., Ingham A.B., Rood J.I. & Moore R.J. (2004). – Highly conserved alpha-toxin sequences of avian isolates of *Clostridium perfringens*. *J. Clin. Microbiol.*, **42** (3), 1345–1347. doi:10.1128/JCM.42.3.1345-1347.2003.

15. Ghoneim N.H., Reda W.W.M., Darwish S.F. & Hamza D.A. (2013). – Characterization of *Clostridium perfringens* enterotoxin in food animals and human. *Global Veterinaria*, **10** (2), 171–175. Available at: www.researchgate.net/publication/273760611_Characterization_of_Clostridium_perfringens_Enterotoxin_in_Food_Animals_and_Human (accessed on 15 March 2017).

16. Miwa N., Masuda T., Kwamura A., Terai K. & Akiyama M. (2002). – Survival and growth of enterotoxin-positive and enterotoxin-negative *Clostridium perfringens* in laboratory media. *Int. J. Food Microbiol.*, **72** (3), 233–238. doi:10.1016/S0168-1605(01)00702-4.

17. Waters M., Savoie A., Garmory H.S., Bueschel D., Popoff M.R., Songer J.G., Titball R.W., McClane B.A. & Sarker M.R. (2003). – Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. *J. Clin. Microbiol.*, **41** (8), 3584–3591. doi:10.1128/JCM.41.8.3584-3591.2003.

18. Costas M. (1995). – The analysis of bacterial proteins by SDS polyacrylamide gel electrophoresis. *In* Diagnostic bacteriology protocols: methods in molecular biology (J. Howard & D.M. Whitcombe, eds), **46**, Humana Press, New York, United States of America, 27–40.

19. Gomaa N.F., El-Derea H.B. & Ell-Adham E. (2003). – Hazard analysis and critical point identification at Abiss slaughter house in Alexandria. *J. Egypt Public Hlth Assoc.*, **78** (3–4), 287–303.

20. Hassan M.A. & Johan R.D. (1997). – Meat additive profiles of Egyptian meat products with relation to public health. *Alexandria J. Vet. Sci.*, **13** (7), 821–828.

21. Nordic Committee on Food Analysis (NCFA) (1997). – Detection and enumeration of *Clostridium perfringens* in foods. Method no. 95, 2nd Ed. NCFA, Espoo, Finland, 125 pp.

22. Sperner B., Schalch B., Eisgruber H. & Stolle A. (1999). – Short protocol for pulsed field gel electrophoresis of a variety of *Clostridia* species. *FEMS Immunol. Med. Microbiol.*, **24** (3), 287–292. doi:10.1111/j.1574-695X.1999.tb01296.x.

23. Abd El-Moez S.I., Hamza D.A., Dorgham S.M., Ahmed B.M., Khairy E.A. & Hedia R.H. (2014). – Molecular characterization and genetic diversity among *Clostridium perfringens* isolates. *Int. J. Curr. Microbiol. Appl. Sci.*, **3** (4), 125–134. Available at: www.ijcmas.com/vol-3-4/Sherein%20I.%20Abd%20El-Moez,%20et%20al.pdf (accessed on 15 March 2017).

24. Hamza D.A. & Ghoneim N.H. (2015). – Epidemiological studies on *Clostridium perfringens* food poisoning. Scholars' Press, OmniScriptum Marketing DEU GmbH, Germany, 124 pp.