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INCIDENCE OF *CLOSTRIDIUM PERFRINGENS* IN SOME MEAT PRODUCTS WITH ENTEROTOXIC GENES DETECTION

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ABSTRACT

The aim of the present study was to determine the presence of *Clostridium perfringens,* including toxic genes in some meat products. A total of 80 samples of minced meat, beef burger, sausage and canned beef luncheon (20 of each) were collected from different supermarkets in Kafrelsheikh city. *C.perfringes* was isolated with incidence of 15, 15, 20 and 30%, respectively. Mean values of *C.perfringes* were 6.33x10^2, 1.25x10^3, 2.09x10^3 and 4.98x10^3 cfu/g, respectively. The tested meat product samples confirmed the presence of positive *C.perfringes* for toxic genes as cpa, cpb, etx, iap and cpe. The obtained results revealed that 16 positive *C.perfringens* isolates were classified into 5(31.25%) isolates were *C.perfringens* type A; 3(18.75%) *C.perfringens* type B; 2 (12.5%) *C.perfringens* type D; 1 (6.25%) *C.perfringens* type E, 5 (31.25%) were non-toxigenic *C.perfringens* and *C.perfringens* type C not detected in any of meat product samples. The result revealed that type A was the most predominant type. The percentage of toxigenic and non-toxigenic strains was 68.75% and 31.25%. The public health importance of the isolated organism was discussed.

Key words: *Clostridium perfringens, Enterotoxin gene, meat products, PCR.*

INTRODUCTION

The microbiological quality and safety of commercially processed meat is major area of concern for producers, consumers and public health officials worldwide (Okolocha and Ellerbroek, 2005). Products excessively contaminated with microorganisms are undesirable from the stand point of public health, storage, quality and general aesthetics (Mead, 1989). Processed meat products constitute a good media for bacterial growth and multiplication, depending on many factors such as pH, temperature biosafety measures and personal hygiene, which may lead to food intoxication and affect on the public health (FAO/WHO, 1983).

The quality of meat product depends on the quality of the used meat, additives, sanitary condition of the equipments and the processing procedures (Teufel *et al.,* 1982).

Anaerobic bacteria constitute an important group of microorganisms which are responsible for many public health hazards as well as spoilage due to lack of oxygen. Clostridia are the most anaerobic organisms which contaminate food, due to production of their resistant spores (Barnes, 1985).

C.perfringens is Gram-positive, spore forming, rod-shaped anaerobic bacteria and more widely spread than other pathogenic bacteria, its principal habitats are in the soil and the intestinal contents of man and animals (Songer, 2010). As well as, it has a great effect on the human health causing food poisoning, also *C.perfringens* causes a number of diseases for example necrotic enteritis in broiler chicken, enteritis in piglets, abomastitis and haemorrhagic enteritis in calves, and gas gangrene, food-poisoning, and gastrointestinal illness in humans (Golden *et al.,* 2009). This pathogenicity is associated with lethal exteracellular toxins which have been defined as enzyme activity as collagenase, hyaluronidase and deoxyribonuclease (Norris and Pettipher, 1987). All *C.perfringens* food poisoning outbreaks have been caused by strains type (A) in which meat is an excellent medium for the bacterial growth. *C.perfringens* type A produces an enterotoxin (CPE) and can cause food poisoning outbreaks with diarrhea

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and severe abdominal pain related to consumption of food (Stevens and Bryant, 1997). Meat products may also be contaminated by different types of such microbes.

Food outbreaks caused by *C.perfringens* are usually those presenting with high counts in the meat products which have been exposed to insufficient cooking. Contamination of meat and meat products with *C.perfringens* may be through different sources, mainly internally from animal after slaughtering as postmortem invasion or externally from contaminated hands, skin of animals, soil, water and processing equipments (Satio, 1990).

So, the aim of the present study was to determine the incidence of *C.perfringens* in some meat products collected from different supermarkets in Kafrelsheikh city and screening the presence of enterotoxin genes.

MATERIALS AND METHODS

1. Collection of samples:
A total of 80 random samples of meat products represented by mined beef, beef burger, sausage and canned beef luncheon (20 of each) were collected under septic condition from different supermarkets in Kafrelsheikh city. The collected samples were transferred to the laboratory with minimum of delay and subjected to the bacteriological examination.

2. Isolation and Identification of *C. perfringens*:
The technique recommended by Koneman *et al.* (1992) and Collee *et al.* (1996) was applied to detect *C. perfringens* in such examined samples. Accurately, 25 grams of each sample were diluted in 225 ml of sterile 0.1% peptone water and homogenized for 2 minutes. Thus, 1ml of each homogenized food suspension was added to each of two tubes containing 10 ml of sterile cooked meat broth (CMB). One of the two inoculated tubes was heat shocked at 72°C for 20 minutes before anaerobic incubation at 37°C for 24 hours to enrich for *C. perfringens* spores. The other tube was directly incubated anaerobically at 37°C for 24 hours for *C. perfringens* vegetative. However, the positive tube was streaked onto one plate of nutrient agar containing 10% sheep blood and 40ug/ml neomycin and incubated for 24 hours at 37°C in an anaerobic jar. The suspected colonies were picked up and subcultured for further identification (morphologically and biochemically).

3. Total anaerobic count of *C.perfringens* in meat products:
It was carried out according the technique adapted by Cruickshank *et al*. (1975) and APHA (2001). The selective media plates of *C.perfringens* (TSC Agar, Trypton Sulphate Cycloserine) were streaked with 0.1 ml of the first and second dilution prepared from the collected samples diluted in sterile pepton water, incubated anaerobically at 37°C for 18-24 hrs in the Gas Pack anaerobic jar and the average counts calculated.

4. Detection of *C.perfringens* genes by multiplex PCR:
4.1. Primer sequences of *C. perfringens* used for PCR system:
Application of PCR for identification and characterization of cpa (400 bp), cpb (196 bp), etx (655 bp), iap (446 bp) and cpe (233 bp) virulent genes for characterization of *Clostridium perfringens* was performed essentially by using primers (Pharmacia Biotech) as shown in the following Table 1:

Primer	Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	Reference
CPA (F)	cpa	5′ TGCATGAGCTTCAATTAGGT ′3	400	Heikinheimo and Korkeala (2005)
CPA (R)		5′ TTAGTTTTGCAACCTGCTGT ′3		
CPB (F)	cpb	5′ GCGAATATGCTGAATCATCTA ′3	196	
CPB (R)		5′ GCAGGAACATTAGTATATCTTC ′3		
ETX (F)	etx	5′ GCGGTGATATCCATCTATTC ′3	655	
ETX (R)		5′ CCACTTACTTGTCCTACTAAC ′3		
IA (F)	iap	5′ ACTACTCTCAGACAAGACAG ′3	446	
IA (R)		5′ CTTTCCTTCTATTACTATACG ′3		
CPE (F)	cpe	5′ GGAGATGGTTGGATATTAGG ′3	233	
CPE (R)		5′GGACCAGCAGTTGTAGATA′3		

4.2. DNA extraction (Shah *et al.,* 2009):
All strains were streaked on blood agar plates and incubated under anaerobic conditions at 37 ºC for 20–22 hours. After incubation, one or two typical colonies were picked and suspended in 100 µl of distilled water. The tubes containing the suspensions were heated to 99ºC for 10 minutes and centrifuged for 5 min at 10000 rpm. All bacterial DNA was stored at -70ºC prior to use. A total volume of 10 µl was used as a template in the PCR.

4.3. Amplification reaction of *C. perfringens* (Meer and Songer, 1997):
The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The multiplex PCR reaction mixture contained 50 ng *C. perfringens* template DNA, 62.5 pmol each cpa primer, 40 pmol each cpb primer, 55 pmol each etx primer, 70 pmol each iap primer and 45 pmol each cpe primer, dNTPs to a final concentration of 0.1 mM PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM $Mgcl_2$), 0.1 Triton X-100, 2 units of Taq DNA polymerase and sterile dH_2O. Accordingly, the DNA was initially denaturated at 95°C for 2 min and amplified for 35 cycles (1min at 94°C, 1min at 55°C, 1min at 72°C for denaturation, annealing and extension phases, respectively) and followed by an additional period of extension for 10 min at 72°C. Amplified PCR products were separated by 2% of agarose gel electrophoresis (Applichem, Germany, GmbH) with 0.5 µg ethidium bromide/ ml. Thus, 20 µl PCR products were subjected to electrophoresis for 45-60 min at 80 volts. Finally, the amplified products were visualized on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

5. Statistical analysis:
The obtained results were statistically evaluated by using analysis of variance according to Feldmen *et al.* (2003).

RESULTS

Table (2): Incidence and statistical analytical results of *C.perfringens* cfu/g in the examined meat products (n=20 each):

Meat Products	+ve samples		Min	Max	Mean ± S.E*
	No.	%			
Minced meat	3	15	1.0×10^2	1.2×10^3	$6.33\times10^2 \pm 1.47\times10^2$
Beef burger	3	15	1.0×10^2	2.9×10^3	$1.25\times10^3 \pm 0.29\times10^3$
Sausage	4	20	2.0×10^2	6.5×10^3	$2.09\times10^3 \pm 0.53\times10^3$
Canned beef luncheon	6	30	5.0×10^2	1.1×10^4	$4.98\times10^3 \pm 1.06\times10^3$

*High significant differences between meat products (P>0.01)

Table 3: Distribution of the toxin genes and types of *C.perfringens* isolated from meat products:

C. perfringens type	Positive genes	Meat products				Total
		Minced meat	Beef burger	Sausage	Canned beef luncheon	
A	cpa	1	1	1	2	5
B	cpa, cpb, etx	-	1	1	1	3
C	cpa, cpb	-	-	-	-	-
D	cpa, etx	-	-	1	1	2
E	cpa, iap	-	-	-	1	1
Non toxigenic	Negative	2	1	1	1	5
Total		3	3	4	6	16

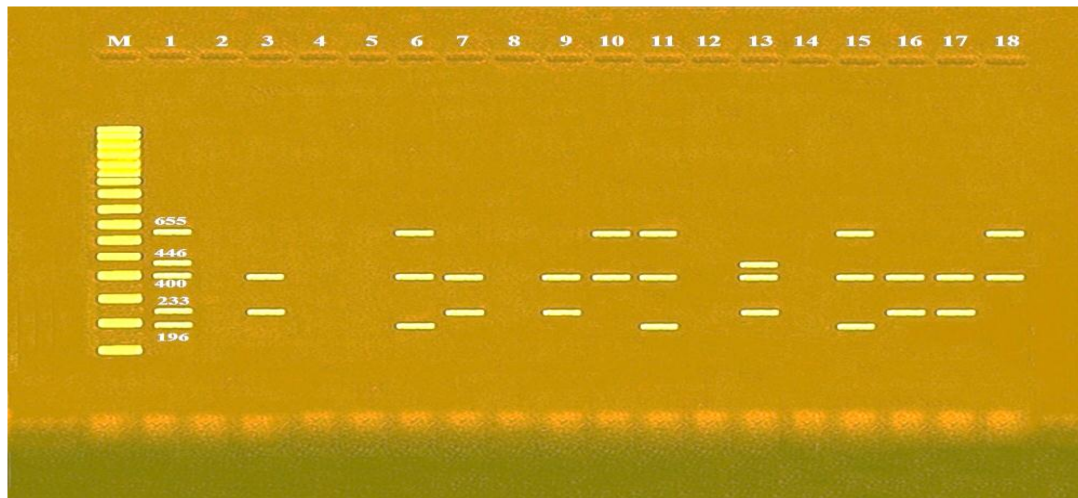

Figure (1): Agarose gel electrophoresis of multiplex PCR of cpa (400 bp), cpb (196 bp), etx (655 bp), iap (446) and cpe (233 bp) virulent genes for characterization of *Clostridium perfringens*.

Lane M: 100 bp ladder as molecular size DNA marker.
Lane 1: Control positive *C. perfringens* for cpa, cpb, etx, iap and cpe genes.
Lane 2: Control negative.
Lanes 3, 7, 9, 16 & 17: Positive *C. perfringens* strains for cpa and cpe genes.
Lanes 6, 11 & 15: Positive *C. perfringens* strains for cpa, cpb and etx genes.
Lanes 10 & 18: Positive *C. perfringens* strains for cpa and etx genes.
Lanes 13: Positive *C. perfringens* strain for cpa, iap and cpe genes.
Lanes 4, 5, 8, 12 &14: Negative *C.perfringens* strains cpa, cpb, etx, iap and cpe genes.

DISCUSSION

C.perfringens produce at least 17 toxins, including the *C.perfringens* enterotoxin (CPE) (Johansson *et al.,* 2006) which is known to cause human food poisoning. *C.perfringens* intoxication can be due to ingestion of food containing an enterotoxigenic strain in a concentration $\geq 10^5$ cfu/g (Stagnitta *et al.,* 2002). In vivo, enterotoxin production is associated to sporulation in the intestine (Dela *et al.,* 2006), while an adequate culture medium is needed for in vitro production (Stagnitta *et al.,* 2002). Vegetative cells that reach the intestine and undergo sporulation produce CPE, which in turn is responsible for clinical symptoms. This toxin-infection is characterized by nausea, diarrhea, abdominal pain and gases, 6 to 12 hrs after intake of contaminated food. Recovery is fast, usually within 12 hrs (Miyamoto *et al.,* 2004). There is a correlation between CPE synthesis and spore formation, and this is the basis for enterotoxigenic and non-enterotoxigenic strain differentiation (Hathway, 1990).

C.perfringens has been classified to five toxotypes (A, B, C, D and E) based on production of four main toxins (α Alpha, β Beta, ε Epsilon and ι Iota toxins) (Johansson *et al.,* 2006). This bacterium also produces ten other toxins such as CPE, beta2 toxins, and theta toxin (Effat *et al.,* 2007).

Data shown in Table (2) revealed that *C.perfringens* was isolated from minced meat, beef burger, sausage and canned beef luncheon by percentage of 15%, 15%, 20% and 30%, respectively. Meanwhile, the mean values of *C.perfringens* counts were $6.33x10^2 \pm 1.47x10^2$, $1.25x10^3 \pm 0.29x10^3$, $2.09x10^3 \pm 0.53x10^3$, and $4.98x10^3 \pm 1.06x10^3$ cfu/g, respectively and there is high significance differences (P>0.01) between *C.perfringens* counts in the examined meat products. The results of incidence were lower than results of Sharma *et al.* (1993); El-Lawendy (1996); Torky (2004); Elham and Nahla (2011), while nearly agreement with Wen and McClane (2004). The results of counts were lower than Yossef (1984); Hassan (2001) and Eleiwa (2003). Higher results in canned beef luncheon due to unhygienic excessive handling, additives and spices, contamination during processing and preservation (Miki, 2008).

It is obvious from Table (3) and figure (1) that 16 positive *C.perfringens* isolates were classified into 5(31.25%) isolates were *C.perfringens* type A; 3(18.75%) *C.perfringens* type B; 2 (12.5%) *C.perfringens* type D; 1 (6.25%) *C.perfringens* type E, 5 (31.25%) were non-toxigenic *C.perfringens* and *C.perfringens* type C not detected in any of meat product samples. The result revealed that type A was the most predominant type. The percentage of toxigenic and non-toxigenic strains was 68.75% and 31.25%.

All type A strains produce α toxin, type B produce α, β and ε toxins, type C produce α and β toxins, type D produce α and ε toxins, and type E produce α and ι

toxins (Layana *et al.,* 2006). *C.perfringens* type B and E are recognized as frank pathogens for demostic animals and human, while type A are commensals in the intestinal tract of vertebrates, and the ability of higher expression of α toxin decides about lethal properties of these strains (Songer, 1996).

The pathogenicity of the organism is associated with several toxins which are also used for toxin typing of the bacteria, within them all strains of the bacterium produce α toxin encoded by (cpa gene). The other major lethal toxins produced by the organism are β (cpb gene), ε (etx gene) and ι (iap gene) that are closely related with the virulence of the bacteria (Titball *et al.,* 1999). In addition to these major lethal toxins, some strains, with a ratio of 0 to 5% have a capability of producing *C.perfringens* enterotoxin encoded by cpe gene that is the main cause of common *C.perfringens* type A food poisoning (Juneja *et al.,* 2010). Strains type A carry the cpe gene in 5-8% of the global population (Miyamoto *et al.,* 2004). C and D strains can also carry cpe gene and produce CPE (Czeczulin *et al.,* 1996).

CPE induces clinical symptoms in vivo within 15-30 min of delivery of purified toxin (Smith, 1979). In man, symptoms develop when $>5x10^8$ viable enterotoxigenic vegetative cells of *C.perfringens* reach the digestive system (Hatheway, 1990). The expression of cpe mRNA and CPE protein synthesis was strongly blocked in cells in the vegetative stage of growth, increasing up to 1500 times after starting the sporulation process (Melville *et al.,* 1994). The classical approach of *C.perfringes* food poisoning involves the detection of $> 10^6$ bacterial cells/g in fecal samples or serological detection of CPE after inducing sporulation of an isolate, or both (Smith, 1979). Characterization of enterotoxigenic *C.perfringens* isolates can be difficult as the ability to sporulate in vitro can vary with different media (Van Damme-Jongston *et al.,* 1990). Moreover, CPE synthesis can occure in nonsporulating culture of *C.perfringens* (Petit *et al.,* 1999).

Food poisoning caused by *C. perfringens* may occur when meat cooked and held without maintaining adequate heating before serving. In such cases the spores of some strains are resistant to temperature even at 100°C for more than 1 hr, their presence in food may be unavoidable and the oxygen level may be sufficiently reduced during cooking to permit growth of clostridia spores that survive cooking may germinate and grow rapidly in food that inadequately refrigerated after cooking (Asha *et al.,* 2006). *C.perfringens* may be present in vegetables, spices and sea-sonings that were used as additives (Rodriguez *et al.,* 2002).

C.perfringens is responsible for two different foodborne diseases, Type A and C, and gas gangrene in humans as well as necrotic enteritis and enterotoxemia in poultry (Immerseel *et al.,* 2004).

CONCLUSION

This study showed that the isolation rate of *C.perfringens* was high in meat products which play an important role in food poisoning. The anaerobic counts of the examined samples were not enough to induce food poisoning in human, since millions of viable *C.perfringes* are required to induce food poisoning in human (10^6 microorganisms/g). So, careful inspection of raw materials, production lines and storing conditions should be intensified to eliminate serious contamination and produce safe and high quality products as well as ensuring compliance with legislation. Also, the use of PCR proved that it is rapid accurate test for detection of pathogenic food poisoning bacteria.

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الهدف من هذه الدراسة هو تحديد مدى تواجد الكلوستريديا بيرفيرنجنسن والجينات المسؤولة عن افراز السموم فى بعض منتجات اللحوم. تم تجميع ٨٠ عينة من منتجات اللحوم (اللحم المفروم والبرجر البقرى والسجق واللانشون البقرى المعلب (٢٠ عينة من كل نوع) من بعض الاسواق بمدينة كفر الشيخ. وتم عزل الكلوستريديا بيرفيرنجنسن بنسبة ١٥و١٥و٢٠و٣٠% من اللحم المفروم والبرجر البقري والسجق واللانشون البقري المعلب على التوالى. وقد أظهرت النتائج أن متوسط العد الكلى للكلوستريديا بيرفيرنجنسن ٦.٣٣x١٠^{٢} و ١.٢٥x١٠^{٣} و ٢.٠٩x١٠^{٣} و ٤.٩٨x ١٠^{٣} خلية/جم علي التوالى. وقد تم التأكد من وجود الجينات المسؤولة عن افراز السموم فى معزولات الكلوستريديا بيرفيرنجنسن. وأظهرت النتائج عزل ١٦ عترة من الكلوستريديا بيرفيرنجنسن وكان منهم ٥ عترات كلوستريديا بيرفيرنجنسن نوع (A) بنسبة ٣١.٢٥ %وكلوستريديا بيرفيرنجنسن (B) (٣، ١٨.٧٥%) وكلوستريديا بيرفيرنجنسن (D) (٢، ١٢.٥%) وكلوستريديا بيرفيرنجنسن (E) (١،٦.٢٥%) وكلوستريديا بيرفيرنجنسن غير مسببة للتسمم (٥، ٣١.٢٥%) و لم يتم عزل كلوستريديا بيرفيرنجنسن نوع (C) من عينات منتجات اللحوم. هذا وقد أظهرت النتائج ان كلوستريديا بيرفيرنجنسن نوع (A) هى الأكثر تواجداً وكانت نسبة عترات الكلوستريديا بيرفيرنجنسن المسببة للتسمم والغير مسببة للتسمم ٧٨.٧٥% و٣١.٢٥%. وتم مناقشة الأهمية الصحية للميكروب المعزول ووضع التوصيات.