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OCCURRENCE AND MOLECULAR TYPING OF *CLOSTRIDIUM PERFRINGENS* USING VIRULENCE GENE IN SOME COOKED READY- TO-EAT MEAT AND POULTRY PRODUCTS IN ASSIUT CITY, EGYPT

(With 3 Tables and 2 Figures)

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مدى تواجد والتصنيف الجزيئى للكلوستريديم برفرنجنز بإستخدام جين الضراوة فى منتجات اللحوم والدواجن المطهية والجاهزة فى مدينة أسيوط – مصر

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ميكروب الكلوستريديم برفرنجنز المسبب للأمراض المعوية، هذا الميكروب ينتج بويغات بعضها مقاوم للحرارة، لذلك تم تحليل بعض منتجات اللحوم والدواجن المطهية الجاهزة للتعرف على تواجد الميكروب من عدمه. تم عد الخلايا فى منتجات اللحوم والدواجن الجاهزة ٨٠ عينة بواقع ٢٠ عينة من كل من الكفتة، شاورمة اللحمة، الفراخ المشوية وشاورمة الفراخ. وجد أن العدد فى معظم العينات كان أقل من ١٠ مستعمرة / جرام (٨٨,٧ %). أيضا تم تحليل العينات بإستخدام إختبار البلمرة المتعدد والثنائى للتعرف على جينات التسمم وأوضحت الفراخ المشوية أنها الأعلى فى معدل تواجد هذا الميكروب بنسبة ٤٠%، كما أن الجين المسبب للتسمم الغذائى وجد فيها بنسبة ٢٠%. وكان نسبة العزل فى الكفتة وشاورمة الفراخ للميكروب هى ٢٠% و٥% بالترتيب ولم يوجد فى شاورمة اللحمة بعد التأكد بإستخدام إختبار البلمرة.

SUMMARY

Clostridium perfringens is spore former microorganism and some spores are heat-resist, so some cooked ready-to-eat meat and poultry products were analyzed for its presence. *C.perfringens* vegetative cells were counted

in ready-to-eat (RTE) meat and poultry products (80 samples; 20 of each kofta, meat shawrma, grilled poultry and poultry shawrma) and found that most (88.7%) of the examined samples indicated < 10 CFU/ gm. The organism was isolated on Tryptose sulphite cycloserine agar with egg yolk (TSC with egg yolk), then the examined samples were analyzed by using multiplex and duplex PCR for detection and genotyping of *C.perfringens* toxin genes. Grilled poultry showed the highest incidence of *C.perfringens* (40%) by using PCR and Type A producing enterotoxin (cpe gene) which is responsible for food poisoning could be detected at rate of 20%. The incidence in kofta and poultry shawrma was 20% and 5%, respectively, but in meat shawrma after confirmation with PCR the organism failed to be detected.

Key words: *C. perfringens, Toxin genes, Ready-To-Eat.*

INTRODUCTION

C. perfringens grows well on RTE meat and poultry products due to absence of oxygen. *C. perfringens* is ubiquitous in the environment, also in raw meat either vegetative or spore form. RTE meat and poultry products contaminated with high levels of certain strains of *C. perfringens* vegetative cells may lead to food poisoning. Vegetative form is killed by heating, but spore form not. The acidic conditions encountered upon passage through the gastrointestinal tract trigger the sporulation of vegetative cells (Wrigley *et al.*, 1995).

C. perfringens is a gram-positive, spore-forming, nonmotile, obligately anaerobic, encapsulated rod. The heat sensitivity of *C. perfringens* spores being defined as not surviving 100°C for 60 min. Four toxins, alpha, beta, epsilon, and iota, are used for classifying all strains into five types (A–E) depending on which combination of these four toxins a given strain produces. Most cases of food poisoning due to *C. perfringens* are due to type A that produces only the alpha toxin, which is distinct from CPE (*cpe*) that is also produced. The toxin is released when cells lyse following spore formation and acts on epithelial cells causing diarrhea and loss of water and ions. The alpha toxin (*cpa*) is considered the main lethal toxin of *C. perfringens* and is a multifunctional phospholipase causing hydrolysis of membrane phospholipids resulting in cell lysis. Food poisoning due to *C. perfringens* usually occurs 8–24 hr after ingestion of food containing large numbers of vegetative cells. Diarrhea and severe abdominal pain usually occur (Levin, 2010).

Berry and Gilbert (1991) stated that production of enterotoxin occurred during sporulation in intestine and McClane (1997) reported that the organism can grow and multiply rapidly after germination, so that cooked meat and poultry products must be cooled rapidly to restrict germination, outgrowth, and vegetation. Enterotoxin production (CPE) is associated with sporulating cells. Pre-formed CPE in foods is often not implicated in foodborne illness, as heating for 5 min at 60°C will inactivate the enterotoxin. Petit *et al.* (1999) cleared that the toxinogenic typing A, B, C, D or E of *C. perfringens* is not based upon the serologic specificity of CPE-related food borne illness, but many other exotoxins produced by the microorganism and designated α, β, ε, and ι. Type A strains produce only α -toxin, type B α -, β - and ε-toxin, type C α - and β -toxin, type D α - and ε-toxin, and type E α - and ι -toxin. The different toxin types cause different forms of enteritis and enterotoxaemia in various hosts (Songer, 1996). Various PCR protocols including multiplex PCR assays have been established to genotype *C. perfringens* isolates with respect to the genes *cpa*, *cpb*, *etx*, *iap*, *cpe* and *cpb2*, encoding the α -, β -, ε-, ι -, entero- and β_2 -toxin, respectively (Meer and Songer, 1997 and Kadra *et al.*, 1999).

The type A food poisoning is the major virulence factor in the common form of food poisoning due to the production of the enterotoxin (CPE). CPE is produced in the small intestine after ingestion of at least 10^7 *C. perfringens* cells. *C. perfringens* type C food poisoning is rare. If not treated, the disease is often fatal and has a mortality rate of 15–25% even with treatment. These toxins are all produced during the vegetative growth of *C. perfringens* type C (Brynestad and Granum, 2002).

C. perfringens spores that germinate after heat treatment, and ultimately contaminate the RTE product, depends on such factors as the time-temperature factor (Crouch and Golden, 2005).

The aim of the work was to detect density and amount of *C. perfringens* in RTE meat and poultry products, also to genotyping the isolated strains by multiplex and duplex PCR because there is few data about studies on incidence of *C. perfringens* in RTE meat and poultry products.

MATERIALS and METHODS

Samples:

Eighty samples, 20 each of kofta, meat shawrma, grilled poultry and poultry shawrma collected from several restaurants in Assiut city were used for isolation of *C. perfringens*. The samples were transmitted rapidly to the laboratory under sterile condition.

Counting of *C. perfringens:*

Preparation of the samples:

Ten grams of each sample were weighted and thoroughly mixed with 90 ml of 0.1% peptone water.

Decimal dilutions were prepared from the previous dilution. 1 ml from each dilution was placed and mixed with 15 ml of melted Tryptose sulphite cycloserine agar with egg yolk (TSCE) into sterile Petri dishes. After solidification, the solidified agar was overlaid with further 10 ml from of Tryptose sulphite cycloserine agar without egg yolk (TSC) agar and incubated anaerobically at 37°C for 20±2h. All blackish colonies were counted and recorded (Robert and Greenwood, 2003).

Isolation of *C. perfringens*

Preparation of the sample:

10 g of the sample were homogenized with 10 ml of Thioglycollate medium as enrichment and incubated anaerobically at 37°C for 20±2h.

Loopfuls were streaked on Tryptose sulphite cycloserine agar with egg yolk (TSC with egg yolk), and incubated anaerobically at 37°C for 20±2h. Colonies thought to be *C. perfringens* appear black due to reduction of sulfite and with halo zone due to lethicinase enzyme. Colonies were confirmed by Gram stain, streaking on blood agar (β-haemolytic colonies with double zone of haemolysis), motility, nitrate and lactose-gelatin medium (Wen and McClane, 2004).

PCR assay for detection and genotyping of *C. perfringens* isolates.

1- DNA extraction:

Two or three colonies from each isolate grown on reinforced clostridial agar were suspended in 300 µl distilled water, and the mixture was then incubated at 56°C for 30 minutes. The samples were treated with 300 µl of TNES buffer (20 mM Tris pH 8.0, 150mM NaCl, 10mM EDTA, 0.2% SDS) and proteinase K (20 mg/ml). After incubation at 37°C for 2 h the mixture was boiled for 10 minutes. To that suspension, same volume of phenol (saturated with Tris-HCL) was added; the suspension was shaken vigorously by hand and centrifuged at 1000 rpm for 10 minutes. The upper phase was transferred into another tube and sodium acetate (0.1 volumes) and ethanol (2.5 volumes) were added. The suspension was kept at –20°C for 1.5 h and than centrifuged at 11000 rpm for 10 minutes. The pellet was washed with 95 and 70% ethanol, each step followed by 5 min centrifugation. Finally the pellet was dried and resuspended in 50 µl distilled water.

2- PCR assay:

Polymerase chain reaction analysis was performed on two reactions: the first is multiplex PCR for detection of genes encoding alpha-toxin (cpa), beta-toxin (*cpb*), epsilon-toxin (*etx*), and the second is duplex PCR for detection of iota-toxin (*cpi*) and enterotoxin (*cpe*) genes. PCR primers and fragment length are listed in Table 1.

PCRs were performed in 50 µl mixture containing 25 µl GoTaq Green Master Mix (Promega, Madison, WI), 0.5 µl of each primer (20 mM), 4µl whole-cell DNA as template and molecular DNase-RNase free water (Promega, Madison, WI) prepared to complete a final reaction volume of 50 µl. The thermal cycling conditions were as follows: initial denaturation for 4 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 60°C for the first multiplex and 56°C for the second, 1 min 20 sec at 72°C and a final step of 2 min at 72°C.

PCR products were separated by electrophoresis on 2% agarose gel (Sigma) added with 0.5 µg/ml ethidium bromide (Sigma). Gels were visualized under UV transilluminator and the image was captured using GelLogic 100 imaging System (Kodak).

Table 1: Toxin genes, oligonucleotide primer sequences and length of amplification products of the *C. perfringens* multiplex and duplex PCR (Drigo *et al.*, 2008):

Multiplex PCR		
Gene (toxin)	Primers Sequence (5'-3')	Amplicon size
cpa(α toxin)	AGT CTA CGC TTG GGA TGG AA TTC CCT GGG TTG TCC ATT TC	900 bp
cpb (β toxin)	TCC TTT CTT GAG GGA GGA TAAT TGA ACC TCC TAT TTT GTA TCC CA	611 bp
cpetx (ε toxin)	ACT GCA ACT ACT ACT CAT ACT GTG CTG GTG CCT TAA TAG AAA GAC TCC	541 bp
Duplex PCR		
cpi (ι toxin)	AAA CGC ATT AAA GCT CAC ACC GTG CAT AAC CTG GAA TGG CT	293 bp
cpe (enterotoxin)	GGG GAA CCC TCA GTA GTT TCA ACC AGC TGG ATT TGA GTT TAA TG	506 bp

RESULTS

Table 2: Frequency distribution of the examined ready-to-eat meat and poultry products based on their *C. perfringens* count.

Products	No.	<10	10- <10^2	10^2- <10^3	10^3- <10^4	10^4- <10^5
Kofta	20	17(85%)	-	-	2(10%)	1(5%)
Meat Shawrma	20	17(85%)	-	1(5%)	1(5%)	1(5%)
Grilled poultry	20	18(90%)	-	2(10%)	-	-
Poultry shawrma	20	19(95%)	-	1(5%)	-	-
Total	80	71(88.7%)	-	4(5%)	3(3.7%)	2(2.5%)

Table 3: Incidence of *C. perfringens* in the examined meat products (20 of each) by using multiplex and duplex PCR.

product	Type A	Type A and cpe positive	Type C	Total
Kofta	3(15%)	1(5%)	-	4(20%)
Grilled poultry	3(15%)	4(20%)	1(5%)	8(40%)
Poultry shawrma	-	1(5%)	-	1(5%)
Meat shawrma	-	-	-	-
Total	6(46%)	6(46%)	1(8%)	13(16, 25%)

Type A: cpa gene
Type A and cpe positive i.e producing enterotoxin(cpe): cpa + cpe genes
Type C: cpa + cpb

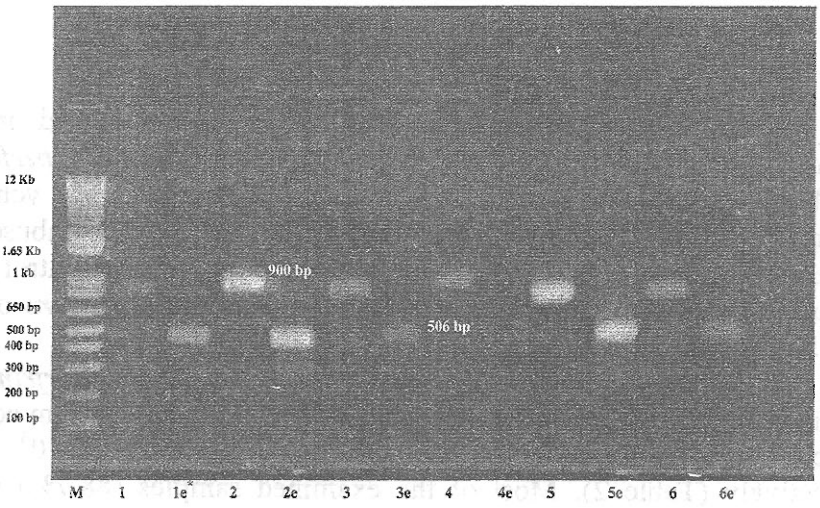

Figure 1: Amplification of *C. perfringens* toxin genes by multiplex and duplex PCR. Lane 1,2,3,4,5 and 6 *C. perfringens* typeA (α toxin gene (cpa) amplification); lane 1e, 2e, 3e, 5e and 6e *C. perfringens* type A producing enterotoxin (cpe amplification).

N.B. the same DNA is used in two lanes; one in multiplex and the other in duplex PCR reaction as shown in Table 1.

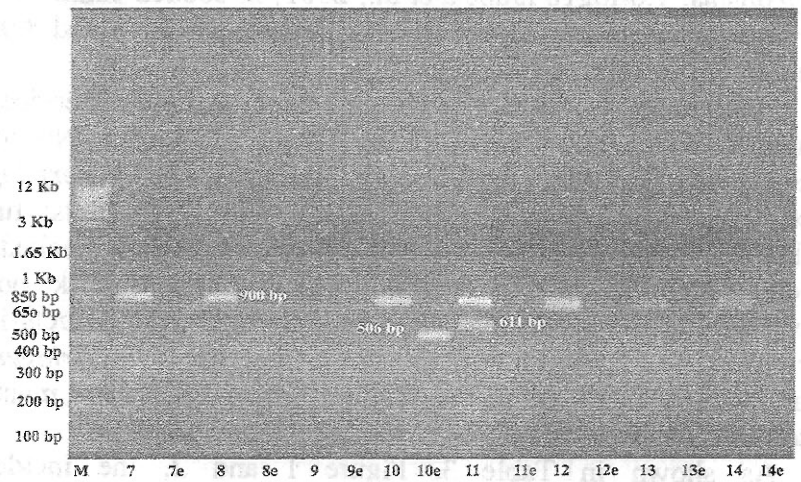

Figure 2: Amplification of *C. perfringens* toxin genes by multiplex and duplex PCR. Lane 7, 8, 10,12, 13 and 14 cpa gene amplification; lane 10e cpe gene amplification; lane 11 *C. perfringens* type C (cpa and cpb gene amplification).

DISCUSSION

Meat and poultry products are generally implicated in most outbreaks, with beef products responsible for about 40% of *C. perfringens* foodborne outbreaks (Bhunia, 2008). Roast beef is a major vehicle of outbreaks because of improper handling, temperature abuse, and inadequate cooling after cooking. The Centers for Disease Control (CDC) estimated that 248,520 foodborne illnesses due to *C. perfringens* occur in the USA annually (Mead *et al.*, 1999).

The maximum counts of colonies suspected to be C. *perfringens* in kofta, meat shawrma ranged from 10^4- $<10^5$, while the maximum counts in grilled poultry and poultry shawrma ranged from 10^2- $<10^3$ CFU/g, respectively (Table 2). Most of the examined samples (88.7%) showed count of <10/g. Kalinowski *et al.* (2003) found that the majority of the examined cooked ready-to-eat meat and poultry samples (79%) indicated < 10 CFU/ g. However, 20% of the samples showed > 10 CFU/g and 6% had >100 CFU/g. This indicated that the samples contained heat resistant spores of the pathogen. Another study reported that all the tested meat and poultry samples contained very low number (<10) of *C. perfringens* (Skariyachan *et al.*, 2010). Various studies indicated higher counts of *C. perfringens*; 7.6-log10 (Juneja *et al.*, 2001) in cooked cured beef; 8.07-log10 (Juneja and Marks, 2002) in cooked cured chicken; and 8.03-log10 (Huang, 2003) in cooked ground beef.

On January 6, 1999, Food Safety and Inspection Service (FSIS) published a final rule in the Federal Register (FSIS Docket No. 95-033F; 64 FR 732) establishing performance standards for *C. perfringens* in cooked beef, roast beef, and cooked corned beef products; fully and partially cooked meat patties; and certain fully and partially cooked poultry products, in an effort to address the public health risk posed by *C. perfringens*. The production requirements for these products included performance standards limiting multiplication of *C. perfringens* to a maximum of 1-log10 within the product during RTE food manufacture (Crouch and Golden, 2005).

As shown in Table 3, Figure 1 and 2, the incidence of *C. perfringens* in kofta was 20%, 15% of the samples were type A positive and 5% of the examined kofta samples were type A producing enterotoxin. The prevelance of the pathogen in grilled poultry was 40%, 15% of them were type A positive, 20% were type A producing enterotoxin and 5% were type C. Poultry shawrma showed incidence of 5% which producing enterotoxin. All the examined meat shawrma showed negative result for *C. perfringens*. Cpetx and cpi toxin genes are not indicated in all the

examined samples. Based on the above results grilled poultry showed the highest incidence of *C. perfringens* which may be due to contamination of the processing plant and processed carcasses of broiler chickens with *C. perfringens*, primarily from intestinal contents of poultry during the slaughter process via cross contamination (Akmak *et al.*, 2006).

The above results were nearly in agreement with Wen and McClane (2004) who found that 56 (38%) of 147 retail chicken samples in America, 24 strain were type A positive and one strain type A producing enterotoxin (cpe). 25 (23%) out of 108 samples of retail ground beef were contaminated with *C. perfringens,* 23 strain were type A positive, but non cpe positive. Skariyachan *et al.* (2010) found *C. perfringens* at a rate of 20% (2/10) in cooked meat samples and 8% (1/12) in cooked chicken samples. Also *C. perfringens* was isolated at incidence of 23% from cooked beef samples in a study performed by Kouassi *et al.* (2011).

In conclusion *C. perfringens* may cause food poisoning through consumption of RTE products especially poultry products. Its presence due to post heat treatment contamination of commodities is possible and may occur through handling, slicing and air transmission. (Ray, 1996). Improper cold storage of RTE meat and poultry products accounts for approximately 90% of the predicted *C. perfringens* foodborne illness. Outbreak observations suggest that heavily spiced foods, such as some Mexican style foods, may be a contributing factor to *C. perfringens* outbreaks (Crouch and Golden, 2005).

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