

Enterotoxigenicity And Typing Of *Clostridium perfringens* Isolates From Some Poultry Products In Egypt

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Abstract: Nearly 113 non outbreak raw and processed chicken meat products were collected from grocery stores and supermarkets namely chicken quarter, chicken fillet, drum stick, chicken paneé, and luncheon and nagets. *C.perfringens* were isolated in percentage of 27.43, typing of *C.perfringens* isolates by I/D injection in guinea pig revealed types A,D and non-toxicogenic strains. The incidence of toxigenic strains were 83.9% while 16.1% were non-toxicogenic strains. 70.8% of the toxigenic isolates classified as *C.perfringens* type A while 12.9% of the isolates classified as *C.perfringens* type D. Studying the enterotoxigenicity of *C.perfringens* type A by using suckling mouse bioassay revealed that 45.5% of the isolates were enterotoxigenic (produce enterotoxin). Using PCR for confirmation the presence of α and enterotoxin gene in enterotoxigenic type A isolates yielded cpa and enterotoxin genes in the predicted 1167bp and 233bp fragments respectively.

Keyword: *Clostridium perfringens*, enterotoxin, PCR and poultry products

INTRODUCTION

Food illness caused by *Clostridium perfringens* is among the common illnesses resulting from the consumption of contaminated food, the vehicles of infection are typically meat and poultry products. It has been firmly established that an enterotoxin produced in the intestine following sporulation of ingested vegetative cells is responsible for the illness^[1]. This toxin is both necessary and sufficient for the enteric virulence of *C.perfringens* type A food poisoning isolates. The enterotoxin is a 320 amino acids protein proteolytically activated causing diarrhea and abdominal cramps, sometimes with vomiting and fever^[2].

Death from *C.perfringens* type A food poisoning are not common but do occur in the elderly and debilitated. Only a small fraction (~ 1 to 5%) of all *C.perfringens* isolates, mainly belonging to type A, carry the *C.perfringens* enterotoxin gene cpe gene^[3, 4]. The cpe gene can have either a chromosomal or plasmid borne location but is nearly always present on the chromosome of food poisoning isolates^[5,6].

A recent study suggests that the strong association between type A isolates carrying a chromosomal cpe gene and *C.perfringens* type A food poisoning is attributable (at least in part) to the exceptional heat resistance of those isolates, which should favor their survival in incompletely cooked or improperly held foods. Classically detection of cpe was performed by in vivo assay such as mouse intravenous injection^[1], the guinea pig skin test^[7] and in ligated rabbit ileal loops^[8]

Many surveys^[9,10] on the incidence of *C.perfringens* in raw and processed foods have been conducted without regard whether isolates were enterotoxigenic. Here we report on the enterotoxigenicity and presence of cpe gene encoding enterotoxin of isolates of *C.perfringens* from non outbreak raw or semi processed chicken meat using DNA amplification by polymerase chain reaction (PCR) in combination with classical methods.

MATERIAL AND METHODS

Nearly 113 non-outbreak food samples were collected from grocery stores, large supermarkets. A breakdown of these samples by food type is shown in (table 1). Ten gram portions of samples were diluted in 99 ml of sterile 0.1% peptone water and homogenized using sterilized surgical scissors. 1ml of each homogenized food suspension was added to each of two tubes containing 10ml of sterile cooked meat broth (CMB). To enrich for any *C.perfringens* spores present in the food sample, one of those two tubes was heat shocked at 72°C for 20 min before incubation at 37°C for 18 to 24 hrs. the other tube was directly incubated at 37°C for 18 to 24 hrs to enrich primarily for *C.perfringens* vegetative cells present in that food sample.

Each CMB enrichment culture showing growth was streaked onto one plate of nutrient agar containing 10% sheep blood and 40- μ g/ml neomycin (blood agar with neomycin) and incubated for ~ 18 hrs at 37°C in an anaerobic jar, when a food sample did grow

presumptive *C.perfringens*, those colonies were inoculated into 10ml of CMB medium, which was then incubated overnight at 37°C. To confirm the identify of those presumptive based on culture, CMB cultures as *C.perfringens*, morphological & biochemical characters^[11]. *C.perfringens* isolates were typed by intradermal inoculation test in Albino Guinea pigs.

Biological Assay for Detection of Enterotoxigenic *C.perfringens* according To^[12]: CMB culture of *C.perfringens* isolates were inoculated in Duncan and Strong (DS) sporulation medium^[13] and incubated anaerobically at 37°C for 8hrs. Infant albino mice (1-2 gm, 1-4 days old) were subjected to intra-gastric inoculation with 0.1ml of crude culture filtrate. After 4 hours they were necropsied for the small intestine weights and distention. The gut weight to the remaining body weight was calculated. A ratio of less than 0.083 was considered negative.

PCR Assay to Determine the cpa and cpe Genes: To prepare template DNA for the PCR assay, a loopful of a pure CMB culture from each isolate confirmed as *C.perfringens* by biochemical tests was streaked onto a neomycin sheep blood agar plate (NSBA). After incubation in an anaerobic jar at 37°C for 18 hours, four to five colonies from each NSBA plate were suspended in 200µl of sterilized phosphate – buffered saline (PBS). Those cell suspensions were microcentrifuged at 14.000 × g for 5 min, and the resultant pellet was similarly washed twice more with PBS before final resuspension in 100µl of PCR – grade H₂O (sigma) and then placed in boiling water bath for 20min for cell lyses. The resultant lysates were cleared by microcentrifugation at 14.000×g for 5min, and 10µl of each supernatant was then used directly as template DNA in PCR assay. The PCR assay used for detection the genes encoding alpha-toxin (cpa) and enterotoxin gene (cpe).

The sequences of the primers for *C.perfringens* alpha-toxin gene (cpa), were selected from the sequence published by^[14] were 5'-AAG ATT TGT AAG GCG CTT and 5'-ATT TCC TGA AAT CCA CTC

The sequences of the primers for *C.perfringens* gene CPE (cpe) were selected from the sequence published by^[15] were 5'-GGA GAT GGT TGG ATA TTA GG and 5'-GGA CCA GCA GTT GTA GAT A

The PCR was performed in a touch-down thermocycler (Hybaid) in a total reaction volume of 50 µl containing 5µl of 10× PCR buffer (10ml M tris-HCL [PH9.0] , 50 mM KCL, .005% tween , 0.1% triton ×100), 5µl of 25mM MgCl₂, 250mM each deoxynucleotide triphosphate, 2U of tag DNA polymerase , 1µM of each primer. Amplification was

obtained with 35 cycles following an initial denaturing step at 94°C for 30 sec. each cycle involved denaturation at 94°C for 1 min , annealing at 56°C for 1 min for cpa gene and 55°C for 2 min for cpe gene, then extension at 72°C for 2 min.

The results were determined by electrophoresis of 20µl of PCR products in a 1% agarose gel for 30 min at 80V and staining with ethidium bromide. The 1167 and 233 bp PCR products of cpa and cpe, respectively, were observed. PCR markers (Biotechnology Department Bio Basic Inc USA) consisting of nine DNA fragments ranging from 0.5 to 10.0 kilobases (KB) pairs were used as the standards. Amplified bands were visualized by UV illumination and photographed on high-density thermal paper film.

RESULTS AND DISCUSSIONS

Our study purposely surveyed raw and processed chicken meat products commonly implicated as vehicles for *C.perfringens* type A food poisoning outbreaks, namely chicken quarter, chicken fillet, drum stick, chicken paneé, luncheon and nagets. As shown in table (1) 27.43% of all (113) non outbreak food samples tested in the present survey were found to be contaminated with *C.perfringens* isolates, which in reasonable agreement with the food contamination frequency determined by^[16] who showed *C.perfringens* isolation percentage of 30% from retail food samples. The results also in agreement with^[17] who detected *C.perfringens* contamination rates ranging between ~ 20 to 40% from all meats and sea food.

All raw and processed chicken meat products showed *C.perfringens* contamination ranging between 8.3 to 14.3% with two notable exceptions (1) nagets. Chicken paneé, which consistently tested negative for the presence of *C.perfringens* and (2) chicken quarter and chicken fillet which had a much higher frequency 57.9% and 46.6% respectively of contamination with *C.perfringens*. These results agree with^[18] who isolated *C.perfringens* from samples of fresh sausage and luncheon in a percentage of 35% and 8.3% respectively and he explained that, the low incidence of *C.perfringens* may be attributed to the method adapted in preparing such products. And^[19] found *C.perfringens* in percentages of 40 and 10% in raw chicken meat products and chicken luncheon respectively.

While^[20] examined 20 random samples of luncheon collected from Cairo markets. They detected *C.perfringens* in 8 samples (40%). On the other hand^[21] fail to detect *C.perfringens* in chicken samples. Nine of the 113 tested samples contained *C.perfringens* at levels between 3.2×10¹ and 7.5×10¹/g^[22] concluded that *C.perfringens* was generally low in both breast and thigh meat as a result of hand deboning. ^[23]stated that

Table 1: Presence of *C.perfringens* in tested samples

Samples	no of samples tested	CFU/g range	*No (%) of samples contaminated with <i>C.perfringens</i>
Chicken fillet	30	3.2×10^1 - 5.3×10^1	14(46.6)
Luncheon	24	**—	2 (8.3)
Chicken quarter	19	4.7×10^1 - 7.5×10^1	11(57.9)
Chicken paneé	15	**—	2 (13.3)
Drum stick	14	**—	2 (14.3)
Nagets	11	**—	0 (0)
Total	113	—	31 (27.4)

* According to the number of samples tested.

** No *C.perfringens* count was detected.

Table 2: Typing of *C.perfringens* isolates recovered from the samples tested.

Samples	No of <i>C.perfringens</i> isolates	<i>C.perfringens</i> types			Type A cpe No (%)**
		(A) No (%)*	(D) No (%)*	Non toxigenic No (%)	
Chicken fillet	14	9(29)	3(9.7)	2 (6.54)	4(18.2)
Luncheon	2	2(6.45)			2(9.1)
Chicken quarter	11	9(29)		2(6.45)	3(13.6)
Chicken paneé	2	1(3.2)		1(3.2)	
Drum stick	2	1(3.2)	1(3.2)		1(4.5)
Nagets	0				
Total	31	22(70.8)	4(12.9)	5(16.12)	10(45.5)

No: number of examined samples

Cpe: enterotoxigenic *C.perfringens* type A

*According to the total number of sample tested

**According to number of *C.perfringens* type A

meat become contaminated by excrement born organisms of animal origin during processing operations. [24] reported that, the clostridia present initially on the feet and breast surface just before bakaging were 1-3/10 cm² and there were 31-467/g in samples of neck skin at the same stage.

Table (2) showed that *C.perfringens* isolates were classified by I/D inoculation in guinea pig as types A, D and non toxigenic strains. From results recorded in table (2) showed that 26 samples revealed toxigenic isolates in incidence of 83.9% while the rest of the samples (5) revealed non-toxigenic isolates in incidence of 16.1%. Out of 31 *C.perfringens* isolates 22(70.8%) isolates proved *C.perfringens* type A and 4 (12.9%) isolates proved *C.perfringens* type D. [25] examined 115 market chicken meat product samples he reported that 22 samples were positive for *C.perfringens*, out of which 17strains proved enterotoxigenic type A. he noticed that all *C.perfringens* recovered from cooked products proved toxigenic and the non-toxigenic strains were only allocated to the frozen raw products

Table (2) illustrated also that 10 (45.5%) out of 22 type A *C.perfringens* isolates were enterotoxigenic by using the suckling mouse bioassay. [26] reported that the type of *C.perfringens* involved in food poisoning

outbreaks is usually type A. They further noted that symptoms provoked by that type results from the release of enterotoxin by cells undergoing sporulation in the lower gastrointestinal tract, the toxin is a component of the spore coat of the organisms[27]. This toxin is both necessary and sufficient for the enteric virulence of *C.perfringens* type A food poisoning isolates[17].

There are few reports of the prevalence of cpe positive strain in non outbreak foods. [28] found that *C.perfringens* was present in an average of 37% of meat and poultry samples. [29] studied the prevalence of *C.perfringens* in 211 meat samples of different animals and characterized the isolates through virulence factors. They isolated *C.perfringens* for 91.4, 70.4 and 65.7% from goat, poultry and buffalo meat respectively. Among the isolates of *C.perfringens* screened from the presence of enterotoxin gene by PCR 9.3, 32.4 and 15.5% isolates of buffalo, goat and poultry, respectively, possessed the enterotoxin gene and [30] collected 169 camel meat samples from butcher's shop, they identified *C.perfriongens* type A with an incidence of 33.7% and the detected enterotoxin in 5.9% of *C.perfringens* isolates. While [31] reported that only 2% of *C.perfringens* strains isolated from meat and fish

were *cpe* positive. Two representative type A *C.perfringens* isolates obtained from luncheon and chicken quarter were subjected to PCR analysis for the presence of *cpa* and *cpe* genes. Amplification conducted with destroyed culture from *C.perfringens* isolates yielded in the predicted 1167-bp fragment for α -toxin gene and 233-bp fragment for enterotoxin gene (fig 1).^[30] Used PCR assays to detect *cpe* production gene as a method for determining the enterotoxigenicity of *C.perfringens* isolates. They stated that PCR is suitable for detection of *C.perfringens* enterotoxin gene from raw meat.

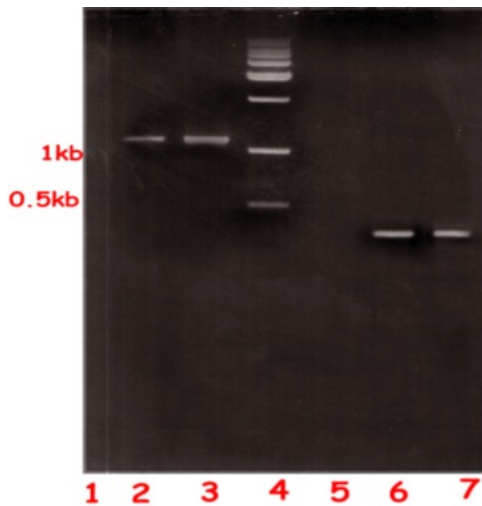


Fig. 1: Agarose gel of PCR products (*a* and enterotoxin genes)

Lane (2 and 3):- the *cpa* positive (the expected 1167-bp amplified product is indicated).

Lane (4):- the molecular size marker (DNA K B-ladder includes 9 fragments ranging from 0.5-10.0 kilobases)

Lane (6 and 7):- the *cpe* positive (the expected 233bp amplified product is indicated)

Conclusion: From results and discussions, it could be concluded that *Clostridium perfringens* contamination was generally high in both chicken quarter and chicken fillet, while tested nagets was negative for the presence of *C.perfringens*. On the other hand, the anaerobic counts of the examined samples were within the permissible limits requested by the Egyptian standard specification. Determining the toxin genotypes of *C.perfringens* food isolates is epidemiologically significant, since *C.perfringens* type A food poisoning is nearly always caused by *cpe* positive type A isolates. PCR assay is suitable for detection of *C.perfringens* enterotoxin gene. More studies are needed to overcome the presence of enterotoxigenic *C.perfringes* type A in poultry products.

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