Animal Health Research Institute,
Assiut Regional Laboratory.

# PREVELANCE OF CLOSTRIDIUM PERFRINGENS AND ITS ENTEROTOXINS IN RAW MEAT AND POULTRY IN ASSIUT CITY

(With 2 Tables and One Figure)

By

***GHADA M. MOHAMED and LUBNA M. EBRAHEEM***

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## مدى تواجد ميكروب الكلوستريديم بيرفرنجنز وسمومه فى اللحوم الطازجة والدجاج الطازج فى مدينة أسيوط

*غادة محمد محمد ، لبنى محمد ابراهيم*

تم جمع ١٢٠ عينة من اللحوم والدجاج بواقع ٦٠عينة من اللحوم من محلات الجزارة المختلفة و ٦٠ عينة من الدجاج من مختلف الرياشات في مدينة أسيوط لدراسة مدى تواجد ميكروب الكلوستريديم بيرفرنجنز وسمومه حيث تم عزل الميكروب من ٢٠ عينة لحوم بواقع ٣٣,٣% بمتوسط ٩,٣٨ ± ٠,١٥ جرام وكان العدد الأصغر والأكبر لهذه العينات يتراوح ما بين ٣,٥- ٣,٦×١٠ $^{١}$ بينما تم عزل الميكروب من ٢٥ عينة دجاج بواقع ٤١,٧% بمتوسط ٦,٣٥ ± ٠,١ وكان العدد الأصغر والأكبر لهذه العينات يتراوح ما بين ٢,٧- ٣,٦ × ١٠ $^{١}$. وقد تم تحديد الجين المسئول عن سموم الكلوستريديم بيرفرنجنز بواسطة اختبار انزيم البلمرة المتسلسل.

## SUMMARY

A total of 120 samples of raw buffalo meat and poultry carcases were collected from different butchers shops and poulteres processing shops in Assiut City to study the prevalence of *Clostridium perfringens* and their toxins. *Cl.perfringens* was isolated from 20 (33.3%) of the meat samples with a mean value of 9.38±0.15 MPN/g, the minimal and maximal numbers in these samples varied from 3.6-3.5×10$^{1}$. While 25 (41.7) of poultry samples were positive for *Cl.perfringens* where the mean value was 6.35±0.1 MPN/g, with a minimum of 3.6 and a maximum of 2.7×10$^{1}$. The presence of the *Cl.perfringens* enterotoxin (CPE) was determined by PCR using previously published primer sequences.

**Key words:** *Meat, poultry, C.perfringens, PCR.*

# INTRODUCTION

*Clostridium perfringens* is a leading cause of bacterial food-borne illness in countries where consumption of meat and poultry is high (Lin and Labbe, 2003).

*Cl.perfringens* is an anaerobic sporefoming and ubiquitous pathogen bacterium widely distributed in the environment and frequently occurs in soil, water and the intestinal tract of certain animals and humans (Omer *et al.*, 2005).

Illness occurs due to consumption of large numbers ($>10^6$) of viable vegetative cells of *Cl.perfringens* per gram of implicated food, followed by sporulation and enterotoxin (CPE) formation in the small intestine (Saito, 1990).

This toxin is both necessary and sufficient for the enteric virulence of *Cl.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 (Stringer *et al.*, 1982).

Numerous epidemiological investigations have revealed that the majority of food borne outbreaks are associated with the consumption of meat and poultry products (Brynestad and Granum, 2002).

A recent study suggests that the strong association between type A isolates carrying a chromosomal CPE gene and *Cl.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 (Nasr *et al.*, 2007)

Death from *Cl.perfringens* type A food poisoning is not common but do occur in the elderly and debilitated. Only a small fraction (~ 1 to 5%) of all *Cl.perfringens* isolates, mainly belonging to type A, carry the CPE gene (Daube *et al.*, 1996).

The purpose of the present study was to determine the occurrence of *Cl.perfringens* in meat and poultry sold in Assiut markets and to determine the enterotoxin of some strains using PCR technique.

# MATERIALS and METHODS

**1- Collection of samples:**

A total of 120 samples of raw buffalo meat and poultry carcases (60 of each) were collected from different butcher and poulteres shops in Assiut city to be examined for the presence of *Clostridium perfringens* and their toxins. The samples were transferred separately and aseptically in an ice box without delay to the laboratory of Animal Health Research Institute in Assiut where they were examined.

**2- Bacteriological analysis:**

**A- Isolation:**

One gram of each sample was aseptically transferred to sterile Cooked Meat Broth (CMB) tubes. Inoculated tubes were anaerobically incubated at 37°C for 24h. From positive tubes which showed turbidity and gas production a loopfull was streaked onto Sulphite Polymyxin Sulfadiazine Agar (S.P.S. agar) plates. Inoculcated plates were incubated anaerobically at 37°C for 24h. Suspected *Cl.perfringens* colonies, black surrounded by an opaque zone, were picked up and purified for further identification according to the methods outlined by Angelotli *et al.* (1962).

**B- Enumeration (MPN/g): (A.P.H.A. 1992).**

Ten gram portions of each sample were diluted in 90ml of sterile 0.1% buffered peptone water and homogenized using a blender at high speed (8000 r.p.m) for 5 minutes to obtain a dilution of $10^{-1}$, then decimal dilutions were prepared using buffer peptone water. One ml. of the previously prepared $10^{-1}$, $10^{-2}$ and $10^{-3}$ of the samples was inoculated separately into 3 replicate tubes of Lactose Sulphite (L.S) broth supplied with inverted durham's tubes, and incubated anaerobically at 46°C for 24-48h. Lactose Sulphite tubes showing blacking with gas production were recorded and considered positive for *Cl.perfringens*. Numbers of *Cl.perfrings*/g were calculated from MPN Table for 3 by using MPN (Most Probable Number) tubes as recommended by AOAC (1990).

**C- Identification:**

Clonies thought to be *Cl.perfringens* were picked up for further confirmation according to Krleg and Holt (1984); Holt *et al.* (1994).

**D- DNA extraction:**

Template DNA was obtained from cultures of the confirmed isolates from chicken and meat (3 for each). Cells were grown for 24h. at 37°C in cooked meat media.

One milliliter of culture was centrifuged at 5.000xg for 15min, and the cell pellet was washed twice with sterile saline and resuspended in 200ML of high-pressure liquid chromatography – grade water and then placed in a boiling water bath for 20min. After centrifugation, 10μL of supernatant fluid was used as the template for PCR. (Tong and Labbe, 2003)

**Oligonucleotides:**

Previously described enterotoxin gene (CPE) oligonucleotide primers for the *Cl.perfringens* that allow the amplification of a 233 bp DNA fragment were used in this work (Meen and Songer, 1997). The forward primer sequence was:

5'-GGA GAT GGT TGG ATA TTA GG and the reverse primer sequence was:

5'- GGA CCA GCA GTT GTA GAT A

**PCR reaction conditions:**

For amplification, reaction mixtures (total volume 50μl) included 5μl of Taq DNA polymerase assay buffer (SIGMA), 10μl of template DNA, 1mM concentrations of each primer, 0.2mM concentrations of daoxynucleoside triphosphates, 1.5mM $MgCl_2$, and 2U of Taq DNA polymerase (Fermentas).

Amplification was carried out in a Bio-RAD thermal cycles with 30 cycles of 1min at 94°C, 2 min. at 55°C, 3 min. at 72°C, and a final extention time of 4min. at 72°C. The results were determined by electrophoresis of 20ML of PCR products in a 1.5% agarose gel for 30min. at 80V and staining with ethidium bromide. The 233-bp PCR products of CPE was observed. PCR marker, 100bp ladder (Promega) was used as standard. Amplified bands were visualized by UV illumination and photographed (Tong and Labbe, 2003).

## RESULTS

**Table 1:** Incidence of Clostridium perfringens in meat and poultry samples (n=60 of each).

| Tested samples | Positive samples | |
|---|---|---|
| | No. | % |
| Meat | 20 | 33.3 |
| Poultry | 25 | 41.7 |

**Table 2:** Statistical analytical results of *Cl.perfringens* count/g of meat and poultry samples using MPN Technique (n=60 of each).

| Tested samples | Positive samples | | Count /g | | | |
|---|---|---|---|---|---|---|
| | No. | % | Min. | Max. | Mean | ± S.E |
| Meat 60 | 28 | 46.7 | 3.6 | $3.5 \times 10^1$ | 9.38 | 0.15 |
| Poultry 60 | 32 | 53.3 | 3.6 | $2.7 \times 10^1$ | 6.35 | 0.1 |

**CPe gene detection (FV1)**

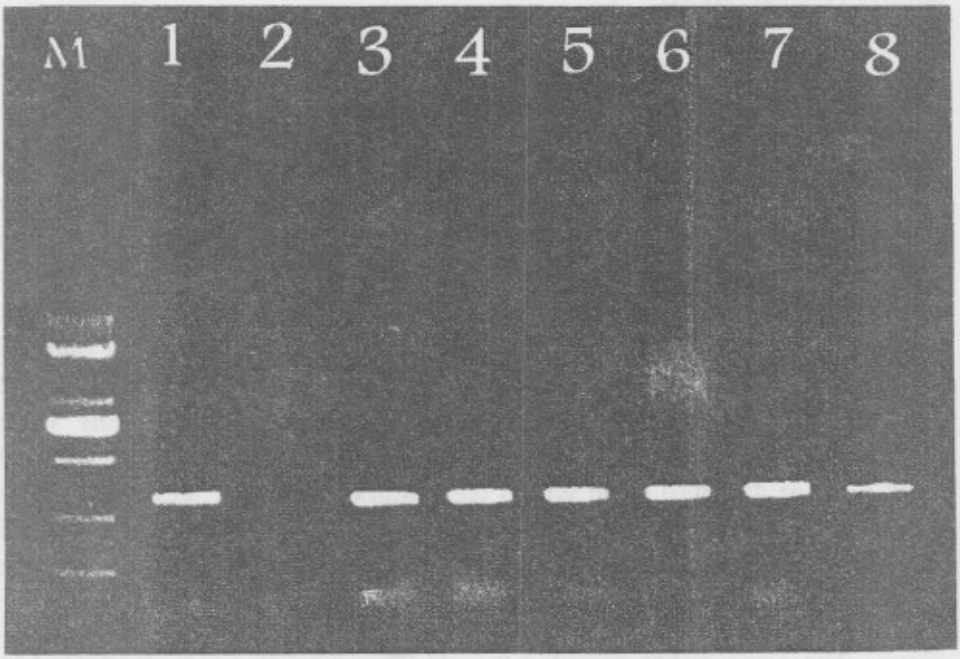


**Fig. 1:** Agarose gel electrophoresis of amplication products obtained from genomic DNA of *Cl.perfringens* enterotoxin gene (233bp PCR product).

Lane M: Molecular weight standard (100bp ladder).

Lane 1 : Positive control.

Lane 2 : Negative control.

Lane 3 to 8: Representative *Cl.perfringens* isolates from meat and poultry samples.

All six reference strains were positive for *Cl.perfringens* enterotoxin gene in the PCR test with primer pairs.

# DISCUSSION

Our study purposely surveyed both raw meat and chicken which are commonly implicated as vehicles for *Cl.perfringens* type A food poisoning outbreaks.

In recent decades many surveys have been conducted on the incidence of *Cl.perfringens* in raw and processed meat and poultry, these reports indicated widespread occurrence of the organism in meat and poultry (Labbe, 2000).

According to numerous surveys, about 50% (range 30 to 80%) of raw or frozen meat and poultry contains *Cl.perfringens* (Labbe, 1988).

As shown in Table 1, 33.3% of the meat samples tested in the present study were found to be contaminated with *Cl.perfringens* and these results are nearly in agreement with those published by El-Mahrouk (2007) who reported an incidence of the organism in meat samples as 39%. The results also in agreement with Wen and McClane (2004) who detected the contamination rates of the organism ranging between 20-40% from all the examined meat samples. Rodriguez *et al.* (2002) revealed that *Cl.perfringens* were isolated from 42 (55%) out of 76 samples of slaughtered meat and 30 (61%) out of 49 retail meat which were higher than that recorded in this study. On the other hand, Miwa *et al.* (1984) reported the isolation of *Cl.perfringens* from 2% of the examined meats by using PCR technique which seemed to be lower than our results.

The same Table revealed that 41.7% of poultry samples tested in the present study were found to be contaminated with *Cl.perfringens* and these results agreed with El-Khateib *et al.* (1988) who found the organism in percentage of 40% of raw chicken meat products. However Nasr *et al.* (2007) could isolate the organism from 46.6% of chicken fillet.

The prevalence of *Cl.perfringens* in poultry samples tested in the present study was higher than that reported by Lin and Labbe (2003) where they found this bacterium in 39 (29.5%) of the 132 samples including chicken meat, chicken leg and chicken neck. Similarly Saito (1990) and Miwa (1984) isolated the organism from 24% and 12% of chicken meat samples.

In contrast to this and previous other studies high incidence was reported by other researchers: Omer *et al.* (2005) found this bacterium in 28 (70%) of 40 ground poultry samples. Miwa *et al.* (1998) detected *Cl.perfringens* in 42 (84%) of 50 chicken samples examined and Nasr et al., (2007) reported the incidence of this organism in chicken quarter as 57.9%. On the other hand, Ternstrom and Molin (1987) Fail to detect the organism in chicken samples.

By using the MPN technique, the results outlined in Table 2 indicated that the incidence of *Cl.perfringens* in meat samples was 46.5% and had MPN/g values ranging from $3.6–3.5\times10^{1}$ with a mean of 9.38±0.15 MPN/g. This observation is in agreement with El-Mahrouk (2007) who reported a range of 0-35 MPN/g. Moreover, Lin and Labbe (2003) and Wen and McClane (2004) tested the MPN/gram in retail foods and found that its values ranged from 0-32. Similary Miwa *et al.* (1984) recorded $<10^{2}$ MPN/100g of the organism in meat samples. While Rodriguez (2002) recorded an average of $2.2\times10^{4}$ MPN/g of the organism in slaughtered meat and 8 × 10 MPN/g of retail meat which are considered higher than that recorded in our study. At the same Table the incidence of *Cl.Perfringens* in poultry samples was 53.3% and had MPN/g values ranging from 3.6-2.7 × $10^{1}$ with a mean of 6.35 ±0.1 MPN/g. These results are considered lower than that obtained by Miwa *et al.* (1984) who recorded $<10^{2}$-$4.3\times10^{2}$ MPN/100g of chicken samples. Also our results considered highest than that obtained by Omer *et al.* (2005) who recorded a mean number 2-6 MPN/g of ground poultry samples with minimal and maximal numbers varied from 0.3-9.3 MPN/g. Miwa *et al.* (1998) detected *Cl.perfringens* in chicken samples at the level of $< 10^{2}$ and $10^{4}$ MPN/100g. which seem to be higher than that recorded in the present investigation.

*Clostridium perfringens* isolates are commonly classified into five types (A to E) based on the production of four typing toxins (alpha, beta, epsilon and iota toxins) (Songer, 1996). Type A isolates, the most abundant toxinotype, produce alpha toxin, but not beta, epsilon, or iota toxin (Immerseel *et al.*, 2004). Some type A isolates also produce another toxin, *Cl.perfringens* enterotoxin (CPE). These enterotoxigenic type A strain cause several human enteric diseases, including *Cl.perfringens* type A food poisoning, which is among the three most common food borne illnesses in the United States, and some cases of

non-food-borne human gastrointestinal disease, including antibiotic – associated diarrhea and sporadic diarrhea (Carman, 1997).

Concerning the detection of cpe gene of *Clostridium perfringens,* several investigators have reported it (Shalaby and Elmabrouk 2006; Naser *et al.*, 2007).

Interpretation of results given in Fig. 1 revealed that 6(13.3%) out of 45 positive isolates confirmed as *Clostridium perfringens* strains choosen for PCR identification were positive for cpe gene.

Shalaby and Elmahrouk (2006) used PCR assays to detect cpe production gene as a method for determining the enterotoxigenicity of *Cl.perfringens* isolates. They stated that PCR is suitable for detection of *Cl.perfringens* enterotoxin gene from raw meat. Our results were in agreement with Nasr *et al.* (2007) who used PCR for confirmation of the presence of enterotoxin (CPE) in the predicted 233 bp fragment.

In conclusion, most meat and poultry meat exposed for sale in Assiut markets proved to contain *Cl.perfringens.* Determining the toxin genotypes of *Cl.perfringens* gens is epidemiologically significant, since *Cl.perfringens* type A food poisoning is nearly caused by cpe positive type A isolates. PCR assay is suitable for detection of *Cl.perfringens* enterotoxin gene. More studies are needed to overcome the presence of enterotoxigenic *Cl.perfringens* in meat and poultry.

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