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PREVALENCE OF ENTEROTOXIGENIC CLOSTRIDIUM *PERFRINGENS* IN RAW AND MINCED BEEF

By

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ABSTRACT

Clostridium *perfringens* is among the most common causative agents of foodborne illness in humans worldwide. The current study was performed to determine the prevalence of enterotoxigenic Clostridium *perfringens* in raw and minced beef marketed in Damietta governorate by using standard culture method **(FDA, 2001)** for the isolation and confirmation of Clostridium *perfringens* in meat samples. Whereas, identification of enterotoxigenic Clostridium *perfringens* was performed by polymerase chain reaction (PCR) procedure to detect the presence of enterotoxin gene (cpe) encoding CPE in Clostridium *perfringens* isolates. Therefore, a total of 100 random meat samples, the obtained results revealed that out of 50 examined samples each of raw beef and raw minced beef, Clostridium *perfringens* was detected in 11 (22%) and 14 (28%), respectively. On the other hand, 2 (4%) and 3 (6%) of isolated Clostridium *perfringens* strains from raw and minced beef samples were positive for the presence of cpe gene by using PCR, respectively. Moreover, PCR proved to be a rapid, sensitive and reliable technique which can be used to discriminate the enterotoxigenic strains of isolated Clostridium *perfringens* from meat and meat products. In addition, the findings of this study demonstrated that some meat samples are contaminated, at the time of retail purchase, with Clostridium *perfringens* isolates having full potential to cause food poisoning which represent a health risk to consumers.

Keywords:

Clostridium *perfringens* **-** enterotoxin - raw and minced beef - PCR.

INTRODUCTION

Clostridium *perfringens* is Gram-positive, anaerobic, spore-forming, rod-shaped bacterium. Its principal habitats are in the soil, sewage and the normal intestinal microflora of humans and animals **(García and Heredia, 2011 and McClane *et al.*, 2013)**. The virulence of

C. *perfringens* is largely attributable to its ability to produce at least 17 different types of toxins. Based on the production of one or more of the four major lethal toxins α, β, ε and ι (alpha, beta, epsilon and iota), this organism is commonly classified into five types (A to E). Some C. *perfringens* strains produce another important toxin named C. *perfringens* enterotoxin (CPE), which is responsible for human foodborne gastrointestinal disease. The gastrointestinal symptoms of the foodborne disease are caused by Clostridium *perfringens* enterotoxin (CPE) produced only during sporulation of the vegetative cells of the organism in the human intestine. Among, the different types of Clostridium *perfringens*, certain strains of Clostridium *perfringens* type A are produced to CPE and contains the cpe gene which encodes CPE. Cooked meat and poultry are the most foods commonly involved in Clostridium *perfringens* food poisoning outbreaks. Among the many isotypes of C. *perfringens*, type A almost always contains the cpe gene, which encodes C. *perfringens* enterotoxin **(Smedley *et al.*, 2004; Uzal *et al.*, 2014 and Freedman *et al.*, 2016)**. C. *perfringens* foodborne infection is one of the most commonly reported foodborne enteric diseases worldwide. The World Health Organization estimated that C. *perfringens* foodborne disease caused 3,998,164 illnesses and 120,000 deaths globally in 2010 **(Kirk *et al.*, 2015)**. In the United States, C. *perfringens* was estimated to be the second most common bacterial cause of foodborne disease after *Salmonella*, causing one million illnesses each year **(Scallan *et al.*, 2011)**. C. *perfringens* was identified as the cause of 10% of foodborne disease outbreaks in England and Wales, between 1992 and 2008 **(Tam *et al.*, 2012)**. Moreover, C. *perfringens* caused 238 foodborne outbreaks in Finland from 1984 to 1999, which constituted 20% of all outbreaks, thus making C. *perfringens* one of the most important causes of foodborne infections **(Lukinmaa *et al.*, 2002)**. In addition, enterotoxigenic C. *perfringens* is responsible for several recent severe foodborne outbreaks worldwide **(CDC, 2012; Wahl *et al.*, 2013; Simone *et al.*, 2014 and Fafangel *et al.*, 2015)**. Meats (especially beef and poultry) and meat-containing foods are the major vehicles of C. *perfringens* foodborne infection in humans **(EFSA, 2005 and Grass *et al.*, 2013)**. In most instances, the actual cause of intoxication by this organism is temperature abuse of cooked foods. Spores of enterotoxigenic C. *perfringens* strains are extremely heat-resistant and can survive normal cooking temperatures. When contaminated foods are prepared in large quantities, usually in institutionalized settings such as restaurants, cafeterias, hospitals and schools

and kept warm after cooking for a long time before serving. Spores that survive cooking may germinate and multiply rapidly in foods and can reach levels that cause food intoxication much more quickly than can other bacteria **(Shandera *et al.*, 1983; Doyle, 2002 and APHA, 2008)**.C. *perfringens* enterotoxin (CPE) which is responsible for the clinical presentation in humans is sporulation associated. Ingested C. *perfringens* vegetative cells sporulated in the intestinal tract and produce an enterotoxin. CPE usually released into the intestine when the vegetative cells lyse on completion of sporulation **(Duncan, 1973; McClane, 2005 and Harry *et al.*, 2009)**. Foodborne illness caused by enterotoxigenic C. *perfringens* can take two forms.The gastroenteritis form characterized by sudden onset of intense abdominal cramps followed by watery diarrhea. It is often mild and self-limiting but it may also result in more severe gastroenteritis that leads to damage of the small intestine. The second form is necrotic enteritis which is more severe form and often fatal. Common characteristics include abdominal pain, swollen bellies, vomiting, fever and diarrhea (sometimes bloody). The more severe form of the disease may cause patchy necrosis of the small intestine, peritonitis and septicemia **(FDA, 2012 and CDC, 2015)**. Detection and confirmation of C. *perfringens* by using conventional culture methods do not distinguish enterotoxigenic from non-enterotoxigenic C. *perfringens* strains. Testing the ability of strain to produce CPE or the presence of cpe gene which encodes enterotoxin in C. *perfringens* isolate had been used for the differentiation between enterotoxin-positive and enterotoxin-negative C. *perfringens* strains **(Miyamoto *et al.*, 2012)**. CPE is synthesized only during sporulation, and thus, sporulation in vitro is essential to measure the production of CPE of an isolate. Various immunoassay methods can be used for the detection of CPE in culture filtrate of sporulated C. *perfringens* isolates such as enzyme-linked immunosorbent assay (ELISA), reversed passive latex agglutination and Western immunoblot **(Piyankarage *et al.*, 1999; Lin and Labbe, 2003 and Wen and McClane, 2004)**. However, inducing sporulation and enterotoxin production of C. *perfringens* isolates on laboratory culture media is challenging and it is often difficult to achieve. Several cpe-positive C. *perfringens* isolates did not sporulate in vitro under commonly used sporulation inducing conditions and consequently tested CPE negative in serologic assays **(Kokai-Kun *et al.*, 1994; Fach and Popoff, 1997 and Augustynowicz *et al.*, 2002**). Molecular methods such as PCR-based assays can be used for the detection of the presence of cpe gene responsible for enterotoxin production in

C. *perfringens* isolates. These methods have the advantages of being much faster, highly sensitive and more reliable than serologic assays and they do not require isolates to sporulate in vitro **(Kokai-Kun *et al.*, 1994 and Lukinmaa *et al.*, 2002)**. Therefore, it is preferable to use cpe gene detection assays for evaluating C. *perfringens* isolates enterotoxigenicity and thereby avoid potential false-negative conclusions which may occur with serological analysis. Several PCR methods for the detection of enterotoxigenic C. *perfringens* in food by targeting cpe gene encoding CPE in C. *perfringens* isolate have been described **(Wen and McClane, 2004; Stagnitta *et al.*, 2006; Miki *et al.*, 2008; Atwa and Abou EI-Roos, 2011; Gurmu *et al.*, 2013 and Shakerian *et al.*, 2016)**. Therefore, the present study was carried out to evaluate the prevalence of enterotoxigenic C. *perfringens* in raw and minced beef marketed in Damietta governorate by using standard culture method **(FDA, 2001)** for the isolation and confirmation of C. *perfringens* in meat samples. Whereas, identification of enterotoxigenic C. *perfringens* was performed by polymerase chain reaction (PCR) procedure to detect the presence of enterotoxin gene cpe encoding CPE in C. *perfringens* isolates.

MATERIAL AND METHODS

I. Sampling (FDA, 2001):

One hundred random meat samples (50 each of raw beef and raw minced beef) were collected from various local slaughterhouses and small butcher shops distributed in Damietta governorate. The samples were collected aseptically in separate sterile plastic bags, packed into an ice box and transported directly to food inspection Laboratory-Damietta sea port for analysis of the presence of C. *perfringens* immediately after their arrival to the laboratory or held for a maximum of 24 h before analysis. While, the enterotoxigenic C. *perfringens* identification was performed by polymerase chain reaction (PCR) procedure at the national research center (NRC).

II. Bacteriological analysis:

1. Isolation of C. *perfringens*:

Isolation and confirmation of C. *perfringens* was performed according to the standard method recommended by **(FDA, 2001)**. Briefly, 25 g of each meat sample was added to 225ml of sterile buffered peptone water (BPW) into a sterile stomacher bag and homogenized for 2 min at low speed using a stomacher (Stomacher 400 Circulator-Seward, UK) to obtain uniform sample homogenate with as little aeration as possible. 2ml of each 1:10 sample homogenate

was inoculated into prepared cooked meat medium broth tube. Inoculated tubes were incubated anaerobically for 48 h at 37°C. From each of cooked meat medium broth tube, one loopful was streaked onto tryptose sulfite cycloserine (TSC) agar containing 10% egg yolk and incubated in upright position in an anaerobic jar 24 h at 37°C.

2.Confirmation of C. *perfringens*:

Presumptive C. *perfringens* colonies on tryptose sulfite cycloserine (TSC) agar containing egg yolk are (black with a 2-4 mm opaque white zone surrounding the colony as a result of lecithinase activity) were confirmed by Gram-staining morphology, biochemical tests for modified iron-milk medium, motility-nitrate and lactose-gelatin. C. *perfringens* is a short, thick, Gram positive bacilli, produces a stormy fermentation in modified iron-milk medium, non-motile, reduces nitrates to nitrites, liquefy gelatin and ferments lactose with production of acid and gas.

IV. PCR assay for the detection of

V. The cpe gene of C. *perfringens* (Applied Biosystems of Thermo Fisher Scientific):

1.DNA extraction of C. *perfringens*:

DNA was extracted from each isolate confirmed as C. *perfringens* by biochemical tests using bacterial DNA extraction kit (Magnetic particles-proteinase K protocol) (Prepseq™ nucleic acid extraction kit, Applied Biosystems) according to the manufacturer guidelines.

2.Detection of cpe gene of C. *perfringens* using PCR technique:

a.Primers of C. *perfringens* enterotoxin (cpe) used in PCR:

The sequences of the primers for C. *perfringens* enterotoxin (cpe) were selected from the sequences published by **(Meer and Songer, 1997)**, a forward primer (5'-GGA GAT GGT TGG ATA TTA GG-3`) and a reverse primer (5'-GGA CCA GCA GTT GTA GAT A-3`), with amplified fragment: 233 bp were used.

b.PCR amplification of C. *perfringens*:

The PCR amplification was performed according to **(Lin and Labbe, 2003)**, in a touch-down thermocycler (Hybaid), in a total reaction volume of 50µl containing 5µl of Taq DNA polymerase assay buffer (Fisher), 10µl of template DNA, 1µM concentrations of each primer, 0.2mM concentrations of deoxynucleosides triphosphates, 1.5mM MgCl2, and 2U of Taq DNA polymerase (Fisher). Amplification was obtained with 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, and a final dwell time of 4 min at 72°C. The results were

determined by electrophoresis of 20µl of PCR products in a 1.5% agarose gel for 30 min at 80V and staining with ethidium bromide. The 233 bp PCR products of cpe were observed. PCR markers (Biotechnology Department, BioBasic Inc. USA) consisting of nine DNA fragments ranging from 0.5 to 10 kilobase (KB) pairs were used as the standards. Amplified bands were visualized by UV illumination and photographed on high-density thermal paper film (Mitsubishi Electronics America, Inc.).

RESULTS

Table (1): Prevalence of C. *perfringens* and enterotoxigenic C. *perfringens* in meat samples.

Type of samples	No. of examined samples	Positive samples			
		Contaminated with C. *perfringens*		Enterotoxigenic C. *perfringens* isolates (carrying the cpe gene)	
		No.	%	No.	%
Raw beef	50	11	22	2	4
Raw minced beef	50	14	28	3	6

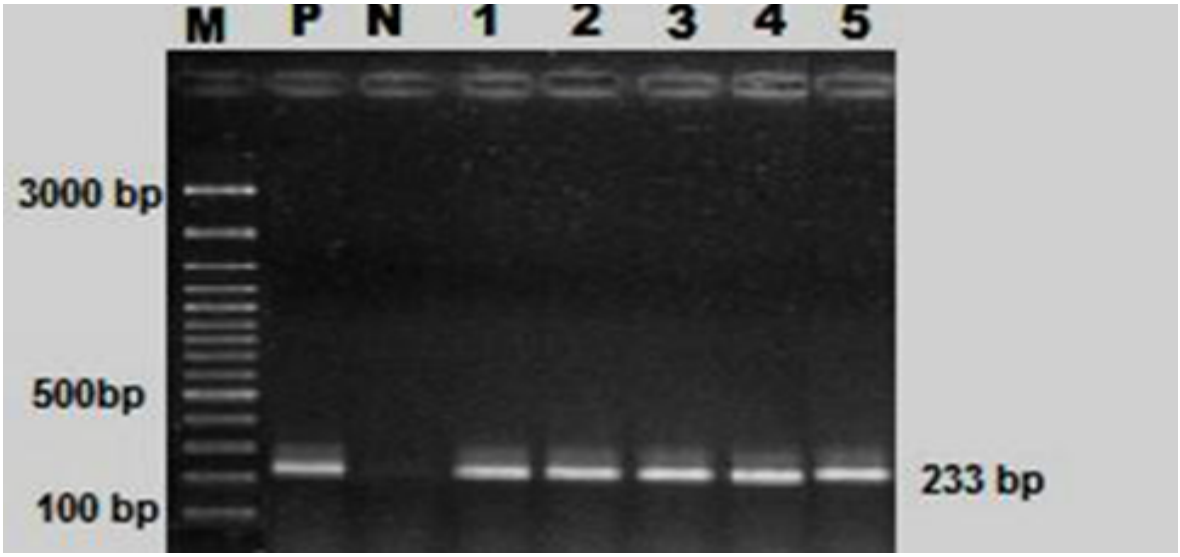

Fig. (1): Detection of the presence of cpe gene in positive C. *perfringens* isolates by PCR.

Lane (M): Standard molecular weight marker (100 bp), Lane **(P):** positive control, Lane **(N):** Negative control, Lanes (1, 2, 3, 4 and 5): Amplified C. *perfringens* enterotoxin genes (cpe) at 233 bp.

DISCUSSION

Clostridium *perfringens* is one of the most important causes of foodborne infections in human worldwide. C. *perfringens* is ubiquitous and widely distributed in soil, sewage and water and it is commonly present in foods particularly raw meats and poultry. Meat and meat products are the foods most frequently implicated as vehicles for C. *perfringens* food poisoning outbreaks **(McNamara *et al.*, 2011 and Wahl *et al.*, 2013)**. Minced beef are used in preparation of many popular food items such as beef burger, kofta, sausage and kobeba in Damietta governorate. Therefore, this study was aimed to determine the prevalence of enterotoxigenic C. *perfringens* in raw and minced beef marketed in Damietta governorate. The results of the present study demonstrated that out of 50 raw beef samples, 11 (22%) were found to be contaminated with C. *perfringens*. Whereas of the 50 raw minced beef samples, C. *perfringens* was isolated from 14 (28%) as illustrated in (Table 1). On the other hand, 2 (4%) and 3 (6%) of isolated C. *perfringens* strains from raw beef and minced beef samples, respectively were positive for the presence of cpe gene by using polymerase chain reaction as shown in Fig. (1). Concerning, the incidence of C. *perfringens* in raw beef samples, the obtained results were in agreement with previous study reported by **(Wen and McClane, 2004)** which found that C. *perfringens* could be detected in 17 of 83 (21%) examined raw beef samples. Moreover, nearly similar results were recorded by **(Miwa *et al.*, 1998)** who mentioned that C. *perfringens* could be recovered from (16%) of analyzed raw beef samples. On the other hand, in some reports, substantially higher prevalence of C. *perfringens* in raw beef has been recorded than were encountered in the present study. For instance, **(Miki *et al.*, 2008)** examined 35 raw beef samples for C. *perfringens* and the isolation rate was (45.7%). On the contrary, significantly lower prevalence of C. *perfringens* were reported by other authors **(Khan *et al.*, 2015)** who stated that (1%) of raw beef samples was positive for C. *perfringens*. Regarding the prevalence of C. *perfringens* in raw minced beef samples, similar findings were recorded by **(Stagnitta *et al.*, 2006)** which found C. *perfringens* could be isolated from 24 (24%) of tested raw minced beef samples. The recorded results were also in accordance with that obtained by **(Wen and McClane, 2004)** stated that out of 108 examined raw minced beef samples 25 (23%) were contaminated with C. *perfringens*. In addition, the results were comparable with that mentioned by **(Lin and Labbe, 2003 and Torky, 2004)** as they could isolate C. *perfringens* from raw minced beef at rates of (36%) and

(35%), respectively. However, significantly lower isolation rate of C. *perfringens* than those recorded in this study was detected by **(Abd Al-Tawab *et al.*, 2015)** with an incidence (16%) out of investigated raw minced beef. In addition**, Phillips *et al.*, (2008)** reported that C. *perfringens* was not recovered from any of the examined 94 chilled raw ground beef samples. On the contrary, much lower findings were compared with the study of **(Miki *et al.*, 2008)** who found that, the incidence of C. *perfringens* was (81.8%) in raw ground beef. Moreover,**Atwa *et al.*, (2011)** examined 125 samples of ready to cook beef products including minced beef; C. *perfringens* was isolated with an incidence of (48.8%). Contamination of raw beef with C. *perfringens* may be through different sources; mainly from animals during and after the process of slaughtering from intestinal contents, skin of animals, contaminated hands, soil, water and processing equipment **(Satio, 1990).** However, human may serve as an important reservoir of cpe-positive C. *perfringens*, introducing a contamination hazard into meat and meat products through improper handling **(Heikinheimo *et al.*, 2006 and Lindström *et al.*, 2011)**. The higher prevalence rate of C. *perfringens* in raw minced beef compared to raw beef samples could be attributed to bad hygienic practices in butcher's shops premises. Contaminated hands, cutting knives and grinders can contribute additional contamination to final ground meat product. The differences between the findings of various authors and those of this study may reflect true variations in the prevalence of C. *perfringens* in raw and minced beef in different geographical areas. However, samples with high prevalence of C. *perfringens* may suggest increased contamination of the collected samples associated with poor sanitary conditions during preparation, processing and storage of beef and beef products. On the other hand, low incidence of C. *perfringens* recovered in some other surveys may be related to differences in procedures of isolation used, which may cause increase or decrease in the prevalence of C. *perfringens* recovered. Many methods of isolation have been described for detection of C. *perfringens* from meat samples. It appears that some isolation procedures are yielded better recovery than others. For instance, most studies with low incidences used heat shocking for isolation of this organism by placing meat homogenates in a water bath for 10-15 min at 80°C or at 75°C for 20 minutes to kill non-spore forming aerobic bacteria and detect only spores. This is in consistent with the work by **(Wen and McClane, 2004)** who reported that about (2%) of meat sampled C. *perfringens* grew after heat shocking, clearly indicating they contained spores of this bacterium and about

29% of C. *perfringens* grew only in the absence of heat shocking, proving they had been contaminated with vegetative cells. It was suggested that vegetative cells were killed by heat shocking. Moreover, low isolation rate of C. *perfringens* in some studies may probably due to type of beef samples (fresh or chilled). C. *perfringens* cells lose their viability when foods are frozen or held under prolonged refrigeration make it difficult to detect **(FDA, 2001)**. Microbiological detection of C. *perfringens* in food without regard to whether isolates were enterotoxigenic has limited value in ensuring food safety. It is now known that not all strains of C. *perfringens* possess the enterotoxin gene and have the capacity to produce enterotoxin responsible for illness. Moreover, cultural methods detect all C. *perfringens* and do not differentiate between enterotoxigenic and non-enterotoxigenic C. *perfringens* strains **(Lin and Labbe, 2003; Wen and McClane, 2004 and EFSA, 2005)**. Therefore, a rapid, sensitive and reliable method for identification of enterotoxigenic C. *perfringens* strains in meat and meat products is essential in the food industry for preventing C. *perfringens* food poisoning outbreaks. Investigation of the presence cpe gene which encodes enterotoxin of C. *perfringens* strains isolated from beef samples was performed using PCR assay. The results in (Table 1), revealed that 2 (4%) and 3 (6%) of isolated C. *perfringens* strains from raw beef and raw minced beef samples were positive for the presence of cpe gene respectively. These results were consistent with the findings of **(Wen and McClane, 2004)** who recorded that (4.3%) cpe-positive of all C. *perfringens* isolates obtained from meat samples. Moreover, these results also in agreement with the incidence determined by another recent study **(Miki *et al.*, 2008)** who found that (4%) of isolated C. *perfringens* strains from raw beef samples were positive for the presence of cpe gene by the PCR assay. However, **Saito, (1990) and Miwa *et al.*, (1998)** reported that only (2%) of all C. *perfringens* strains isolated from beef were cpe-positive. These results are consistent with the fact that while C. *perfringens* has widespread distribution in the environment, only about 1 to 6% of the global C. *perfringens* population carries the enterotoxin (cpe) gene **(Van Damme-Jongsten *et al.*, 1989; Daube *et al.*, 1996; Smedley *et al.*, 2004 and Lindström *et al.*, 2011)**.

CONCLUSION AND RECOMMENDATIONS

The obtained results showed that PCR is a rapid, sensitive and reliable technique which can be used to discriminate the enterotoxigenic strains of isolated Clostridium *perfringens* from meat and meat products. In addition, the findings of the present study indicate that some meat

samples are contaminated, at the time of retail purchase, with C. *perfringens* organism having full potential to cause food poisoning which represents a health risk to consumers. To prevent foodborne diseases caused by C. *perfringens* and to improve quality of raw and minced beef, it is suggested that:

-Preventive measures in the slaughterhouses during slaughtering and dressing operations to avoid hazards of contamination of carcasses and meat through sanitary condition of the equipment and personal hygiene precautions.

-Good hygienic practices in butcher's shops premises to minimize hazards of contamination, hands, cutting knives and other tools of butcher should be clean and sanitized.

-Training courses on food hygiene for butchers about proper cleaning and sanitizing of equipment, utensils, work surfaces and proper food handling procedures.

Consumers can make a role to reducing the risk of an infection with C. *perfringens* by:

-Avoiding cross contamination of raw meat during food preparation.

-Appropriate cooking of raw meat and meat products at temperatures high enough to kill vegetative cells of enterotoxin-producing C. *perfringens*.

-Cooling rapidly through the temperature range 55°C to 12°C, holding foods at temperatures <10-12°C to prevent extremely heat-resistant C. *perfringens* spores that survive normal cooking temperatures from germinating and multiplying to food-poisoning levels during slow cooling and unrefrigerated storage of prepared foods.

-Re-heating cooked meat to an internal temperature of 72°C before consumption to destroy vegetative cells of C. *perfringens* which may be germinated during food storage.

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مدى تواجد الكلوستريديوم بيرفرنجنز المفرزة للإنتيروتوكسين في اللحم البقري الطازج والمفروم

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المُلخـــــص

الكلوستريديوم بيرفرنجنز هي من بين الميكروبات الأكثر شيوعاً المسببة للأمراض المنقولة عن طريق الأغذية للإنسان في جميع أنحاء العالم, حيث تظهر الأعراض المعدية المعوية لهذا المرض بسبب إفراز هذا الميكروب للإنتيروتوكسين CPE الذي يتم إنتاجه فقط خلال عملية التجرثم لخلايا الميكروب الحية داخل الأمعاء، وتعتبر اللحوم والدواجن المطبوخة من الأطعمة الأكثر شيوعاً في نقل الكلوستريديوم بيرفرنجنز المفرزة للإنتيروتوكسين, وقد أجريت هذه الدراسة لتحديد مدى انتشار ميكروب الكلوستريديوم بيرفرنجنز في اللحم البقري الطازج والمفروم المسوق في محافظة دمياط, باستخدام الطريقة التقليدية طبقاً لـ (FDA, 2001) القياسية لعزل وتأكيد الكلوستريديوم بيرفرنجنز في عينات اللحوم, والتعرف على الكلوستريديوم بيرفرنجنز المفرزة للإنتيروتوكسين باستخدام الطريقة الحديثة تقنية إنزيم البلمرة متسلسل التفاعل (PCR) للكشف عن الجين cpe, حيث تم تجميع وفحص ما مجموعه 100 عينة عشوائية (50 عينة من كلٍ من اللحم البقري الطازج والمفروم), ودلت النتائج التي تم الحصول عليها باستخدام الطريقة التقليدية على تواجد بكتيريا الكلوستريديوم بيرفرنجنز بنسب عزل 22٪ و28٪, في كلٍ من اللحم البقري الطازج والمفروم على التوالي, في حين تواجدت نسب 4٪ و6٪ من الكلوستريديوم بيرفرنجنز المفرزة للإنتيروتوكسين في كلٍ من اللحم البقري الطازج واللحم البقري المفروم على التوالي باستخدام تقنية إنزيم البلمرة متسلسل التفاعل (PCR) للكشف عن وجود الجين cpe، حيث تبين أن تقنية إنزيم البلمرة متسلسل التفاعل (PCR) طريقة سريعة وحساسة وموثوق بها والتي يمكن استخدامها للتعرف على سلالات الكلوستريديوم بيرفرنجنز المفرزة للإنتيروتوكسين المعزولة من اللحوم ومنتجاتها, وقد أظهرت نتائج هذه الدراسة أن بعض عينات اللحوم الطازجة المسوقة للاستهلاك الآدمي ملوثة بميكروب الكلوستريديوم بيرفرنجنز المفرزة للإنتيروتوكسين المسبب للتسمم الغذائي والتي تمثل خطراً على صحة المستهلكين, وعليه تم مناقشة النتائج والأهمية الصحية وتلك التي يجب أن يوصى بها.

الكلمات الدالة:

اللحم البقري الطازج والمفروم الطازج - الكلوستريديوم بيرفرنجنز - الجينات السمية (toxin genes) - طريقة الزرع التقليدية - تقنية إنزيم البلمرة متسلسل التفاعل (PCR).