

## Phenotypic and Genotypic Characterization of *Clostridium perfringens* Isolated from Cattle Meat

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### Abstract

The existence of *Clostridium perfringens* in cattle meat has a public health importance. This study was aimed to investigate the *Clostridia perfringens* prevalence in cattle meat. Forty meat samples from fore and hind quarter (20 for each) were randomly collected from abattoirs and butcher's markets at south Sinai province. The collected samples were subjected to the bacteriological examination; moreover, the total *C. perfringens* count from examined meat samples was  $2 \times 10^3$  C.F.U./g; in addition, the prevalence of *C. perfringens* was 30% in cattle meat samples. Molecular typing using multiplex PCR for detection of the major toxin genes of *C. perfringens* revealed that positive gene detection at 324 bp for *C. perfringens* type A. Also enterotoxin virulent gene was detected at 485 bp *C. perfringens* is considered one of the main spoilage organisms of meat leading to contaminations of great amount of decomposed meat, which ending with economic losses; bad hygienic conditions in slaughterhouses are the main cause for meat contamination with *Clostridium species*, since the gastrointestinal tract of slaughtered animals is their natural habitat, the abattoir processing represent a great source for meat surface contamination by these pathogens.

**Keywords:** *C. perfringens*, cattle meat, PCR, pathogens.

### Introduction

Meat is an excellent source of high quality protein and rich in minerals and vitamins which are essential for human health, power and vitality. Meat may affect the public health due to their contamination with spoilage microorganisms which

are responsible for objectionable changes as well as the presence of pathogenic organisms which may lead to either food infection or food intoxication (Libby, 1975; Hanninen, 1980 and FAO/WHO, 1983). Microbiological quality of fresh meat has been receiving

attention all over the world due to the occurrence of significant outbreaks of foodborne illness affecting consumers. Quality maintenance is important not only for consumer health protection but also to assure uniformity in fresh meat shelf-life, (Widders, 1995). Meat represented the main source of proteins and essential amino acids. So, it considered a highly favorable environment for the growth of pathogenic bacteria. The microbiological contamination of carcasses occurs mainly during processing and handling, such as skinning, evisceration, storage and distribution at slaughter houses and retail establishments (Gill, 1998 and Abdalla et al., 2009). Many public health hazards were detected due to presence of anaerobic *Clostridia*. Their spores enable it to survive the relatively high temperature (Barnes, 1985). *C. perfringens* as a food poisoning organism has a human health hazards. Due to the consumption of meat and meat products, most of foodborne diseases caused by *C. perfringens* occurred (Lynch et al. 2006; Wahl et al. 2013).

The abattoir process is considered the great source of meat surface contamination by *C. perfringens* organisms either internally from animal after slaughtering as postmortem invasion from gastrointestinal

tract or externally from contaminated hands, skin of animals, water, soil and processing equipment's. Illness and death caused by foodborne pathogens in developing countries need billions of dollars for medical care and social awareness (Fratmico et al., 2005).

The control and/or prophylaxis for food poisoning outbreaks associated with meat consumption depends mainly on detecting the causative agents in carcasses, eliminating them to ensure food safety and to protect public health from microbial contamination of food (Al Khaldi et al. 2004 and Albini et al. 2008).

The current preliminary study was aimed to investigate the prevalence, total count and molecular characterization of *C. perfringens* in cattle meat.

### **Material and Methods**

#### **1- Collection of samples:**

40 meat samples were collected randomly from abattoirs and butcher's markets at South Sinai province. Samples were collected separately in sterile plastic bags, and then were labeled and given serial numbers. They were preserved in an Ice box and transferred quickly to the laboratory with minimum of delay for anaerobic bacteriological examination according to Cruickshank et al., (1975).

## 2-Processing of collected samples:

### a) Preparation of homogenate (ICMSF, 1978).

Twenty-five grams of each sample were removed aseptically using a sterile forceps and scissors from different points of the sample then placed in sterile polyethylene bag, then adding 225 ml of sterile peptone water 0.1% aseptically; the content was homogenized in a stomacher (MPW.302 Poland) for one minute to provide a dilution of  $10^{-1}$ . Such homogenate was used for all bacterial investigations.

### b) Preparation of serial dilutions:

From the prepared homogenate; 1/10 dilution; 1 ml was taken by a sterile pipette and mixed with 9 ml sterile peptone water 0.1% in a sterile test tube and was shaken well using a test tube shaker to make a dilution of 1/100 using a new sterile pipette 1 ml was transferred from 1/100 dilution to next tube for making a dilution of 1/1000; the process repeated till dilution of  $10^{-5}$ .

### 3-Total *C. perfringens* count:

The method recommended by ICMSF (1978) for enumeration of *C. perfringens* in meat was done.

From each prepared dilution 0.1 ml was pipetted into the center of each plate of *C. perfringens* selected media (oxid) which was used for enumeration of the

total viable cell count of *C. perfringens* in meat and meat products. Aseptically, the counts of one vial of each Perfringens agar (OPSP) supplements A and B oxid (SR 76 and SR77) which had been dehydrated by the addition of 2ml of sterile distilled water were added, mixed well and poured into sterile dishes.

Perfringens (OPSP) selective supplement A oxid (Code SRO 76E): A box of freeze-dried selective supplement for the isolation of *C. perfringens* contained 10 vials. Each vial was sufficient to supplement 500 ml of perfringens Agar base, and contained 50 mg of sodium sulphadiazine.

Perfringens (OPSP) selective supplement B oxid (Code SRO 77E). A box of freeze-dried selective supplement for the isolation of *C. Perfringens* contained 10 vials. Each vial was sufficient to supplement 500 ml of *C. Perfringens* Agar Base. The vial contained 0.25 mg of Oleandomycin phosphate, and 5000 IU polymyxin B sulphate to each vial 2 ml of sterile distilled water were aseptically added, and mixed gently to dissolve. Aseptically the vial contents from the supplements A and B were added to 500 ml of perfringens Agar Base and cooled to 50°C, mixed well and poured into sterile Petri dishes. After making count on

perfringens agar media then incubated at 37°C for 24 hs in Gas-Pack Anaerobic Jar according to *Brewer and Allgeier (1966)*. OPSP agar plates having black colonies were selected and counted.

#### **4- Isolation of Clostridial organism (ICMSF. 1978):**

The surface of each sample was sterilized by using hot spatula and two bean size pieces were obtained from the deeper parts, then inoculated separately into two tubes of freshly prepared, previously boiled and cooled cooked meat medium. One of the two inoculated tubes was heated at 80°C for 15 minutes in water bath with a depth of water more than the level of the material in tubes, while the second tube was left unheated. Both tubes were then incubated anaerobically at 37°C for 48 hrs. A loopful from each unheated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200 mg /ml) for isolation of *C. perfringens* (*Smith and Holdeman. 1968*)

While, the heated tubes were then streaked on 10% sheep blood agar plates for isolation of other clostridia. Inoculated plates were immediately incubated anaerobically at 37°C for 24 - 48 hrs. After incubation, the anaerobic growth on the blood agar plates was examined macroscopically and microscopically. Selected

suspected colonies were transferred to tubes of freshly boiled and cooled cooked meat medium and incubated anaerobically at 37°C for 24 hours to have a pure culture of isolates for further identification.

#### **5- Identification of Clostridial organism:**

Isolates were identified according to *Smith and Holdeman (1968)*, *Willis (1977)*, *Macfaddin (1980)*, *Smith and Williams (1984)* as the following; Staining, cultural characteristics, Nagler's reaction, and different biochemical reactions.

#### **6- Methods of polymerase chain reaction PCR:**

##### **DNA Extraction and purification:**

Strains of *C. perfringens* types A, B, C, D and E were considered positive while *C. septicum*, *C. sordellii*, *C. sporogenes*, *C. leptum* and *C. ramosum* were the negative controls. Isolated strains were cultured on blood agar and total DNA was extracted as described by (*Sambrook and Russell, 2001*). Two or three colonies that grown on blood agar were suspended 350 µL of STET buffer (100 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 5% Triton® X-100) in 1.5-mL microtubes. Then, 25µL of lysozyme (10 mg/ mL) was added to each solution and the content was mixed,

subsequently, the microtubes were placed in a boiling water bath for 40 seconds.

The bacterial lysate was centrifuged at 13000 rpm for 15 minutes at room temperature in a Microfuge® refrigerated microcentrifuge (Beckman Coulter, USA). After that, the supernatant was poured into a fresh microcentrifuge tube and the nucleic acids were precipitated from it by adding 40 µL of 2.5 M sodium acetate (PH 5.2) and 420 µL of isopropanol. Precipitated nucleic acids were recovered by centrifugation at 13000 rpm for ten minutes at 4 °C. Next, the supernatant was removed and the pellet of nucleic acid was rinsed with 1mL of 70% ethanol at 4 °C. finally, the pellet was dried and resuspended in 50µL of TE buffer containing RNase.

**PCR amplification (Van Asten et al., 2009):**

DNA samples were tested in 50 µl, reaction volume in a 0.2 ml.

eppendorf tube, containing PCR buffer which was composed of: 25 µl M. Mix [Cosomo PCR red Master Mix (2x)] willow fort W1020300x), England, 2 µl target DNA, 1 ml of each primer (containing 10 P mole/µl) and the mixture was completed by water nuclease free to 50 µl.

**Identification of the PCR products by agarose gel electrophoresis:**

Agarose gel preparation (Applichem, Germany, GmbH), placement of combs and gel pouring. The gel was run until the bromothymol blue and xylene cyanol had migrated the appropriate distance through the gel (2/3 of the gel length), the lid of the gel tank was closed and attached to the power supply. After the electric current was turned off, the lid of the gel was removed and the gel was transferred to trans-illuminator to observe the amplified DNA on the gel in comparison to molecular weight markers. The gel was photographed.

*PCR protocol for amplification conditions of PCR products.*

Gene	Stage (°C)	Temp.	Time	No. of cycle
toxin of <i>C. perfringens</i>	Initial denaturation	94	2	1
	Denaturation	94	15 sec.	35
	Annealing	55	30 sec.	
	extension	68	1 min.	
Cpe	Initial denaturation	95	15 min.	1
	Denaturation	94	30 sec.	40
	Annealing	53	90 sec.	
	extension	72	90 sec.	
	Final extension	72	10 min	

PCR products were analyzed for the presence of specific fragments of the expected length in a 1.5 % agarose gel electrophoresis stained with Ethidium bromide.

## RESULTS

### 1-The total count of *C. perfringens* from cattle meat samples:

**Table (1):** Statistically analytical results of *C. perfringens* count of examined meat samples.

Cattle meat samples No=40 (20 for each quarter)	Count C.F.U./g		
	Min.	Max.	Mean $\pm$ S.E.
Fore quarter	1x10	6.4x10 <sup>2</sup>	3.4x10 <sup>2</sup> $\pm$ 1.4x10 <sup>2</sup>
Hind quarter	1.7x10 <sup>2</sup>	4.2x10 <sup>3</sup>	1.6x10 <sup>3</sup> $\pm$ 8.3x10 <sup>2</sup>

### 2-Phenotypic characteristics of *C. perfringens*

**Table (2):** Phenotypic Identification of *C. perfringens*:

Biochemical test	Xylose	Mannose	Maltose	H <sub>2</sub> S	Nitrate reduction	Indol	Mannitol	Sucrose	Lactose	Glucose	Gelatine	Spores
<i>C. perfringens</i>	-	+	+	+	+	-	-	+	+	+	+	Co

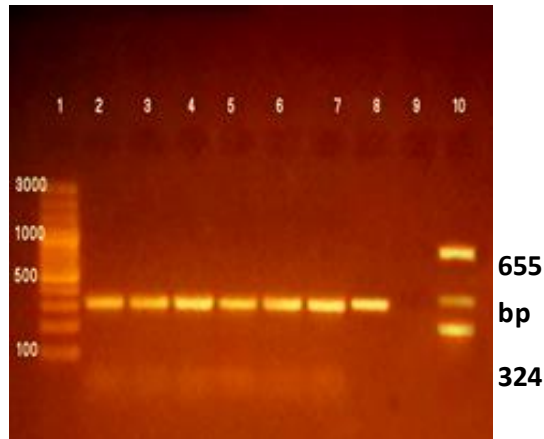
+ = Positive reaction.      - = Negative reaction.      Co  
= Central oval.

### 3-Prevalence of *C. perfringens* in the examined Cattle meat samples:

**Table (3):** Prevalence of *C. perfringens* in the examined cattle meat samples.

Cattle meat samples		<i>C. perfringens</i>	
		No.	%
No =40 (20 for each quarter)	Fore quarter	5	25
	Hind quarter	7	35

#### 4- Molecular characterization of *C. perfringens* strains :



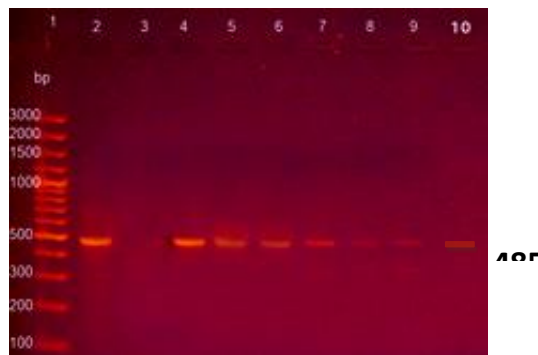
**Figure (1):** Positive gene amplification for *C. perfringens* type A (cpa) at 324 bp.

Lane 1: DNA marker (GeneRuler 100 bp DNA Ladder, Fermentas)

Lane 2-8: Positive *C. perfringens* type A (cpa) at 324 bp.

Lane 9: Control Negative (mix of various toxin types).

Lane 10: Control positive (toxin of *C. perfringens* of type A)



**Figure (2):** Positive gene amplification of enterotoxin virulent gene (cpe) at 485 bp.

Lane 1: DNA marker (GeneRuler 100 bp DNA Ladder, Fermentas)

Lane 2: Control Positive.

Lane 3: Control Negative

Lane 4-10: Positive enterotoxin virulent gene (cpe) at 485 bp.

#### Discussion

Meat may be contaminated with clostridial spores during the slaughtering process and the

following handling. Since *C. perfringens* present in the normal flora of intestinal tract of animals. Contamination of the

carcass from the intestinal contents, as well as, dust, soil or from workers is virtually unavoidable. In the present work, as illustrated in Table (1), the *C. perfringens* count (C.F.U./g) of the examined samples of cattle meat were  $1 \times 10^1$  to  $6.4 \times 10^2$  with an average of  $3.4 \times 10^2 + 1.4 \times 10^2$  obtained from four (20%) positive meat samples for fore quarter,  $1.7 \times 10^2$  to  $4.2 \times 10^3$  with an average  $1.6 \times 10^3 + 8.3 \times 10^2$  obtained from five (25%) positive meat samples for hind quarter. **Hassan (1994)** disagreed with this count who detected the *C. perfringens* count of cattle meat samples with a mean value of  $1.17 \times 10^3 + 0.35$  and  $1.90 \times 10^3 + 0.74$  in basateen and moneeb abattoir, respectively. **Hedia (2009)** mentioned lower prevalence of *C. perfringens* in cattle meat samples for fore quarter with an average of  $1.7 \times 10^2$  and higher prevalence for hind quarter with an average  $2.1 \times 10^3$ .

The recorded data in table (3) demonstrated the prevalence of *C. perfringens* in the examined cattle meat samples. The percentage of isolation of *C. perfringens* from cattle meat samples were 25% and 35% (fore & hind quarter), respectively. Much higher results were recorded by **El-Naenaeey (1989)** who isolated *C. perfringens* from cattle meat

with an incidence of 56.5%. Lower results were obtained by **Cohen et al., (2006)** who isolated the organism from beef meat with an incidence of 4.5%. While **Phillips et al. (2008)** failed to detect *C. perfringens* from retail ground beef samples. These results were in harmony with the results detected by **Hedia (2009)** who isolated *C. perfringens* by percentage 20% & 33.4% from examined cattle raw meat (fore and hind quarter). Much higher result were obtained by **Khalid (2013)** who isolated *C. perfringens* from cattle meat samples by percentage 20%. Very high incidence of *C. perfringens* in ground beef samples (96%) were detected by **Guran et al., (2014)**. **Fahim et al. (2017)** detected lower incidence of *C. perfringens* in raw beef samples (16%).

Results of molecular detection of major toxin gene (cpa) using multiplex PCR revealed positive gene amplification at 324 bp for all tested *C. perfringens* type A figure (1). This result agrees with **Nauerby (2003) and Crespo (2007)** who applied toxin typing for isolates by PCR and all were found to be type (A). **Fisher et al. (2005)** disagreed with this result and revealed that, most type C isolates produce at least three lethal toxins, alpha, beta and perfringolysin O, and several isolates also produced



beta 2 toxin. *Ahsani (2010)* used multiplex PCR and isolated *C. perfringens* type (A) by lower ratio (17.39%). *Mohamed, et al. (2010)* used multiplex PCR and revealed that *C. perfringens* type A at the rate of (65%). This result in harmony with the result obtained by *Guran and Oksuztepe (2013)* who detected that 97.6% of *C. perfringens* isolates carried only CPa toxin gene (type A) by using multiplex PCR. Nearly similar result obtained by *Guran et al. (2014)* who used Multiplex PCR and demonstrated that, out of the 262 *C. perfringens* isolates, 203 (77.4 %) possessed only the cpa gene (type A). *Afshari, Asma; et al. (2015)* used multiplex PCR and indicated that *C. perfringens* type A is the most common type in minced meat. *Fayez, et al. (2020)* detected *C. perfringens* type A by lower percentage (75.2%).

The result in figure (2) revealed the detection of enterotoxin virulent gene (cpe) in all examined *C. perfringens* isolates by using multiplex PCR. *Nasr (2007)* reported that enterotoxin gene was detected in all enterotoxigenic type A isolates. In contrast, this result disagrees with *Prabhu (2013)* who found that all isolates of *C. perfringens* have alpha ( $\alpha$ ) toxin gene. *Gamal A. et al. (2018)* stated that all toxigenic strains of *C. perfringens* type A (33, 33.7%)

were positive for alpha toxin (cpa) and enterotoxin (cpe) genes. While finding of *Yibar et al. (2018)* disagree with this result and proved that of the 81 suspicious isolates tested by RT-PCR, 22 (27.2%) carried the cpe gene either on the plasmid or chromosome. On the contrary *Fayez, et al. (2020)* concluded that *C. perfringens* type A with only cpa+ gene was the most prevalent isolated toxin type found in 191 (72.9%) isolates.

In conclusion, *C. perfringens* is considered one of the main spoilage organisms of meat leading to contaminations of great amount of decomposed meat, which ending with economic losses. Poor hygienic procedures in slaughterhouses are the main cause for meat contamination with *Clostridium species*. Since the natural habitat of *C. perfringens* in the gastrointestinal tract of slaughtered animals, the abattoir processing represents a great source for meat surface contamination by *C. perfringens* organisms.

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