Detection and typing of *Clostridium perfringens* in some retail chicken meat products

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A B S T R A C T

Clostridium perfringens is considered as one of major food poisoning bacteria; which may refer to different lethal toxins production including *C. perfringens* enterotoxin. *C. perfringens* toxins have been contributed in many diseases in human being especially *C. perfringens* type A enterotoxin food poisoning. A total of 125 random raw and half cooked chicken meat samples represented by (breast, thigh, nuggets, panée and frankfurter "25 of each") were collected from various retail stores and supermarkets in Qualyubia governorate to investigate the presence of *C. perfringens* bacteriologically and detect the *cpa, etx,* and *cpe* toxin genes by multiplex PCR. Results demonstrated that 6 out of 25 raw breast samples (24%), 8 out of 25 raw thigh samples (32%), 5 out of 25 nuggets samples (20%), 4 out of 25 panée samples (16%), and 4 out of 25 frankfurter samples (16%) were found to be contaminated with *C. perfringens*. Twenty-seven positive isolates obtained from these samples were identified as *C. perfringens* based on the microscopic examination and biochemical tests. It was detected that 8 (29.6%) out of 27 *C. perfringens* isolates carried only alpha toxin gene (type A), and only one isolate (3.7%) of them expressed both alpha and epsilon toxin genes (type D); while *cpe* gene never had been detected in any examined isolate, according to the multiplex PCR results.

Keywords: chicken meat products, *C. perfringens*, multiplex PCR.

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1. INTRODUCTION

Clostridium perfringens is a Gram-positive, spore-forming, anaerobic, non-motile bacilli that is commensally inhabit the intestine of animals and humans. It is frequently isolated from soil, skin, sewage and surface water. Animal and human feces are considered the natural source of contamination of food products. Because of its ability to produce spores under bad environmental conditions, it is one of the most widespread bacteria in nature as well as in the gastrointestinal tract of most animal species (Mokhtari and Doosti, 2015, Obrien and Melville, 2000).

Because of its commensal inhabitant of poultry intestinal tract, which contributed in feather, skin contamination followed by processing plant, defeathering machines, scalding tanks or pass from intestine during evisceration and contaminate carcass meat, so different stages of poultry processing line can add *C. perfringens* contamination source even starting from the hatchery (Voidarou *et al.*, 2011).

Transmission of *C. perfringens* may occur via foodborne, water borne, animal contact, person-to-person, and others, at the point of consumption. *C. perfringens* transmission was referred primarily to food through unhygienic food handling and cross-

contamination in the processing pathway (Butler *et al.*, 2015). Because of its ability to form a spore, this microorganism is able to survive adverse conditions such as aerobic and food processing procedures. Its spores may contaminate meat and meat products either before processing and survive cooking or after processing due to unhygienic handling of prepared food (Santos *et al.*, 2002 and Potter, 2001).

The virulence factor of *C. perfringens* strains is associated with several toxins production, within them, all strains produce alpha (α) toxin encoded by *cpa* gene; other major lethal toxins are beta (β), epsilon (ε) and iota (ι). In addition to these major lethal toxins, some strains, with a ratio of 0 to 5 %, have a capability of producing *C. perfringens* enterotoxin encoded by *cpe* gene that is the main cause of common *C. perfringens* type A food poisoning (McClane, 2007 and Juneja *et al.*, 2010).

All type A strains produce α toxin, type B produce α, β and ε toxins, type C produce α and β toxins, type D produce α and ε toxins, while type E produce α and ι toxins. *C. perfringens* types (B-E) are recognized as "frank pathogens" for animals and human, while type A strains are commensally inhabit the GIT of them. *C. perfringens* type A strains are implicated in numerous human diseases such as food-poisoning and gastrointestinal illness (Fisher *et al.*, 2005).

Alpha toxin is a necrotizing toxin produced by all *C. perfringens* strains; the purified toxin can cause serious acute pulmonary disease, as well as vascular leak, hemolysis, thrombocytopenia and liver damage. Epsilon (*etx*) toxin is one of twelve proteinaceus toxins produced by *C. perfringens* (types B and D); this toxin is a pore-forming protein; in addition to production of alpha toxin. Beta toxin is a lethal necrotizing toxin found in types B and C. Theta toxin is an oxygen–labile cytolysin; this toxin can damage blood vessels, resulting in leukostasis, thrombosis, decreased perfusion and tissue hypoxia; Theta toxin also stimulates cytokine release and can cause shock (The center for food security and public health, 2004).

C. perfringens type A foodborne illness occur after the ingestion of food contaminated with a large number (10^6–10^8 cells/g) of type A viable vegetative *C. perfringens* cells specially meat and meat products. After ingestion, *C. perfringens* enterotoxin (*cpe*) has been produced during inter-intestinal sporulation (McClane and Rood, 2001).

C. perfringens type A is contributed in much human foodborne gastroenteritis, such as food poisoning, sporadic diarrhea, antibiotic-associated diarrhea, and nosocomial diarrhea outbreaks. *C. perfringens* was estimated to cause 10% (1 million cases) of recorded USA food poisoning cases/year. Deaths from *C. perfringens* type A food poisoning are not common but may occur in the elderly and debilitated patients. *C. perfringens* type A food poisoning is contributed to kill seven people in the USA and between 100 people in the UK (Byrne *et al.*, 2008 and Scallan *et al.*, 2011).

C. perfringens enterotoxin is thought to be the main causative agent of *C. perfringens* food-borne gastroenteritis. Recently, some outbreaks of food poisoning, non-CPE producers of *C. perfringens* were isolated. These results suggest that enterotoxin of *C. perfringens* can be causative agents of acute gastroenteritis in humans (Yongi *et al.*, 2014).

C. perfringens type A food poisoning is characterized by symptoms of diarrhea and abdominal cramps with rare signs of fever or vomition; the course of disease can

be peracute, acute, or chronic, with signs of the acute and peracute condition including intense abdominal pain, depression, and bloody diarrhea (Shimizu *et al.,* 2002 and Rahimi *et al.,* 2011).

Classical typing of *C. perfringens* has been performed by toxin neutralization with mice or guinea pigs; because these methods are time consuming and expensive, they have largely been replaced by PCR-based detection methods. In recent years, various PCR protocols, including multiplex PCR assays, have been established to toxin-typing of *C. perfringens* isolates with respect to the genes *cpa, cpb, etx, iA, cpe* and *cpb2*, encoding the alpha, beta, epsilon, iota, entero- and b2-toxin, respectively (Al-Khaldi *et al.* 2004; Baums *et al.*, 2004).

C. perfringens strains that associated with food poisoning outbreaks are carrying their enterotoxin gene, *cpe,* on their chromosome, while *C. perfringens* strains isolated from non-foodborne cases, such as antibiotic-associated diarrhea (AAD) and sporadic diarrhea, carry *cpe* on the plasmid. Investigation of 31 clinical and non-clinical *C. perfringens* isolates to locate the *cpe* gene by PCR is performed; where *cpe* of nine heat-sensitive strains isolated from three outbreaks of food poisoning were located on the plasmid, while those of heat-resistant strains from other food poisoning outbreaks were located on the chromosome. Moreover, the *cpe* of 5 heat-sensitive strains isolated from healthy human feces were located on the plasmid. They concluded that heat-sensitive, *cpe*-plasmid-borne *C. perfringens* strains should not be disregarded as causative agents of food poisoning (Nakamura *et al.,* 2004).

Therefore, this study aimed to detect and typing of *C. perfringens* in some chicken meat products by biochemical and multiplex PCR methods.

2. MATERIAL AND METHODS

2.1. Collection of samples:

A total of 125 random samples of fresh raw and partially cooked chicken meat products represented by (breast, thigh, nuggets, panée and frankfurter (25 of each), respectively; were collected from different retail groceries and supermarkets in Qalyubiya governorate for bacteriological and molecular examination. All the collected samples were subjected to the following examination.

2.2. Preparation of the samples:

It was done according to (APHA, 1992).

2.3. Detection of C. perfringens:

It was done according to (ISO, 2004) using TSC media.

2.4. Isolation of C. perfringens:

It was done according to (Carter and Cole, 1990) using cooked meat media and 10% sheep blood agar.

2.5. Identification of C. perfringens:

It was done according to (MacFaddine, 1980 and Cato et al., 1986).

2.5.1. Staining:

It was done according to (Cruickshank et al., 1975).

2.5.2. Cultural characteristics:

It was done according to (Cruickshank et al., 1975):

2.5.2.1. Cooked meat media (BioMed).

2.5.2.2. Sheep blood agar media.

2.5.2.3. Egg yolk agar media (Nagler's reaction).

2.5.2.4. Nutrient gelatin media.

2.5.2.5. Biochemical reactions

2.5.2.6. Nitrate reduction test was done according to (Willis, 1977)

2.5.2.7. Zinc Test was done according to (Willis, 1977)

2.5.2.8. Indole production test it was done according to (MacFaddine, 1980)

2.5.2.9. Hydrogen sulphid test it was done according to (MacFaddine, 1980)

2.5.2.10. Sugar fermentation test it was done according to (Willis, 1977)

2.5.3. Detection of Clostridium perfringens toxins by using Multiplex PCR was done according to Kalender et al. (2005), Moller and Ahrens (1996), and Meer and Songer (1997).

3. RESULTS

As illustrated in table (2) and Pic. (1): Out of 125 raw and half processed chicken meat products, 27(21.6%) samples were found to be contaminated with C. perfringens in incidence of 24, 32, 20, 16 and 16% from examined raw breast, raw thigh, nuggets, panée and frankfurter samples, respectively. eight (29.6%) isolates were confirmed as C. perfringens type A after detection of alpha toxin gene that gave a characteristic ampilicon band at 402bp; and only one isolate (3.7%) gave the characteristic fragment of epsilon toxin encoded by etx at base pairs 541 indicated C. perfringens type D using multiplex PCR; In addition, it was determined that none of the isolates carried C. perfringens enterotoxin (cpe) genes.

Table (1): Primer sequences of *C. perfringens* enterotoxin genes used for Multiplex PCR identification system:

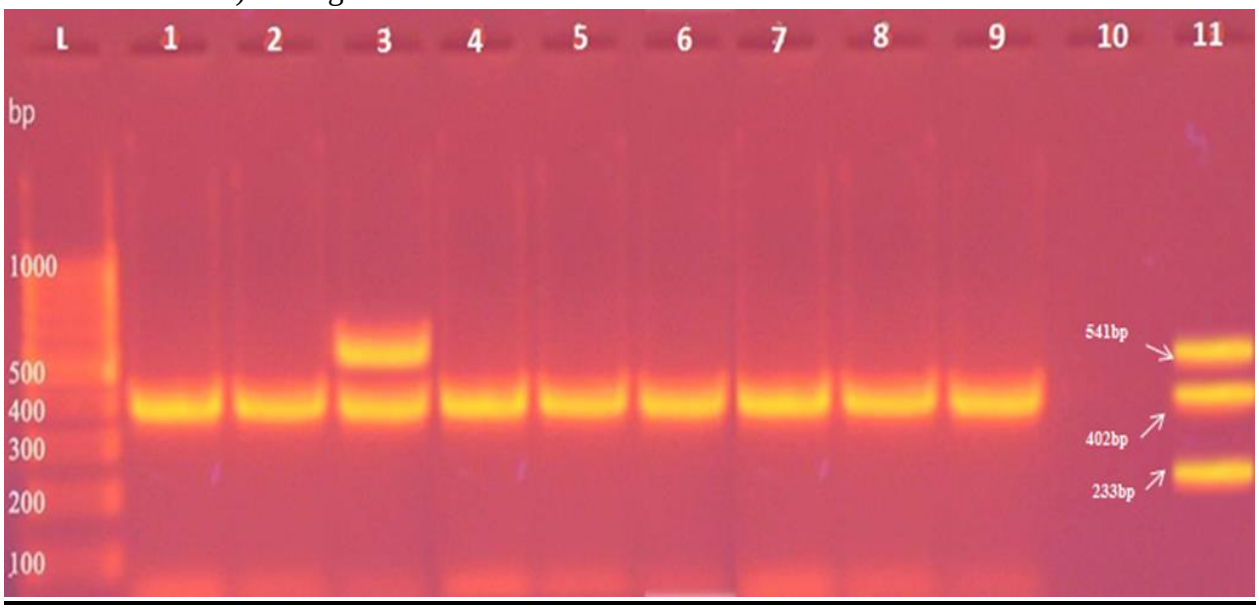
Target toxin gene	Primer	Oligonucleotide sequence (5′ → 3′)	Amplicon length (bp)	Reference
A	*cpa* (F)	3′ AAG ATT TGT AAG GCG CTT ′5	402	Kalender *et al.* (2005)
	cpa (R)	3′ ATT TCC TGA AAT CCA CTC ′5		
D	*etx* (F)	3′GCGGTGATATCCATCTATTC ′5	541	Moller and Ahrens (1996)
	etx (R)	3′CCACTTACTTGTCCTACTAAC ′5		
enterotoxin	*cpe* (F)	3′GGAGATGGTTGGATATTAGG′5	233	Meer and Songer (1997)
	cpe (R)	3′GGACCAGCAGTTGTAGATA′5		

Table (2): incidence and typing of *C. perfringens* in the examined chicken meat product samples (n=25).

Samples	Positive samples		Types of isolates					
	No.	%	A (*cpa*)		D (*etx*)		*cpe*	
			No.	%	No.	%	No.	%
Raw chicken meat								
Chicken breast	6	24	2	33.3	0	0	0	0
Chicken thigh	8	32	2	25	1	12.5	0	0
Half cooked chicken meat products								
Chicken nuggets	5	20	1	20	0	0	0	0
Chicken panée	4	16	2	50	0	0	0	0
Chicken frankfurter	4	16	1	25	0	0	0	0
Total	27	21.6	8	29.6*	1	3.7*	0	0

*- in relation to total number of isolates (27).

Pic. (1): Agarose gel electrophoresis of multiplex PCR for detection of *C. perfringens* (type A, D and enterotoxin) toxin genes.



Lane L: 100 bp ladder as molecular DNA size marker.
Lane 1: Control positive for cpa (402bp), etx (541bp) genes, and cpe (233bp).
Lane 11: Control negative.
Lanes 2, 3, 5, 6, 7, 8, 9 and 10: Positive samples for alpha toxin (*cpa*- type A).
Lanes 4: Positive samples for epsilon toxin (*etx*- type D).
Lane 11: negative control sample.

4. DISCUSSION

Foods of animal origin such as poultry meat, which are high in protein, have great importance in the occurrence of food poisoning depending on *C. perfringens*; factors are widely available in the products of raw meat and half cooked meats prepared with these contaminated meats.

Results tabulated in table (2) are in agree with (Zakaria, 2005) who recorded isolation of *C. perfringens* in examined chicken breast, thigh and frankfurter samples in prevalence of 25, 35, 10%; (Emara, 2014) who detected *C. perfringens* in 30% of examined fillet samples; (Nabil *et al.*, 2014) who detected *C. perfringens* in 13.3% of examined frankfurter samples; (Sobhy, 2016) who detected *C. perfringens* in 36.6% of examined raw samples.

Adversely, results were lower than that reported by (Prabhu *et al.*, 2013) who detected *C. perfringens* in 81.69% of examined samples; (Torky and Hassan, 2014) who detected *C. perfringens* in 70% of examined chicken meat samples; while, higher than those reported by (Thangamani and Subramanian, 2012) who detected *C. perfringens* in 3.81% of examined samples; (Afshari *et al.*, 2015) who detected *C. perfringens* in 15.5% of examined chicken meat samples. Moreover, reported results were disagreed with (Hashem, 2015) and (Ibrahim-Hemmat *et al.*, 2015) who failed to detect *C. perfringens* in any examined chicken meat sample; and (Nasr *et al.*, 2007) who did not detect *C. perfringens* in any examined nuggets samples.

Differences may be attributed to difference in circumstances of hygienic measures effectiveness during slaughtering, processing practices, handling from production to consumption. In case of chicken products, differences can be attributed to the difference in bacterial load of used raw materials; addition of additives, spices and preservatives as well as the conditions occurred before and after slaughtering of the birds affects the bacterial load in these products.

It is not wearing that, all examined *C. perfringens* isolates were found to be positive according to multiplex PCR results, where 29.6% of them were determined as type A. It can be explained by: (1) the fact that *cpa* toxin gene are commonly present gene in all *C. perfringens* types, (2) *C. perfringens* type A is dominant in almost all of the research concerning poultry meat as reported by (Lin and Labbe 2003; Nowell *et al.* 2010).

According to multiplex PCR results as showing in table (2) and pic.1, out of examined *C. perfringens* isolates, 29.6% of isolates were confirmed as *C. perfringens* type A (*cpa*), and 3.7% isolate was *C. perfringens* type D (*etx*); while failed to detect enterotoxin production gene (*cpe*); which were in agree with (Erol *et al.*, 2008) who reported that recent studies claimed that *C. perfringens* type A foodborne poisonings are rarely associated with enterotoxin encoding by the *cpe* gene it is only detected in (0-5%) of outbreaks isolates.

So, our results are logically in agree with (Abd Al-Tawab *et al.*, 2015) (Prabhu *et al.*, 2013), (Salah El-din *et al.*, 2015) who proved that all their PCR examined *C. perfringens* isolates gave a characteristic band at 402bp (*cpa* only); Torky and Hassan (2014) revealed 66% of multiplex PCR examined isolates gave bands at 402bp and 33.3% gave both bands at 402 and 541bp, which referring to *C. perfringens* type A (*cpa*) and *C. perfringens* type D (e*tx*), respectively; while failed to detect either *C. perfringens* type B or C. While it disagreed with (Afshari *et al.*, 2015) who detected type A (29.03%), type C (70.96%), and *cpe*

(25.00%) toxin genes in examined *C. perfringens* isolates.

These findings proved that presence of *C. perfringens* bacteria in either raw or half cooked chicken meat products in a great number may be responsible for inferior quality of meat products resulting in economic losses and the possibility of causing public health hazard. Meat and meat products can be contaminated with *C. perfringens* especially of type A through many sources which contributing a significant public heath threating factor. Recommendations include following a strict hygienic measure throughout the whole meat and meat products manufacturing starting from slaughtering to consumption to minimize bacterial contamination and safeguard human health.

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