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

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**Abstract**

*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.

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

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 (exx) toxin is one of twelve proteinaceous 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 (10⁶–10⁸ 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)
Detection and typing of \textit{Clostridium perfringens} in some retail chicken meat products

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)

Detection of \textit{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 \textit{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 \textit{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 \textit{C. perfringens} type D using multiplex PCR; In addition, it was determined that none of the isolates carried \textit{C. perfringens} enterotoxin (cpe) genes.

<table>
<thead>
<tr>
<th>Target toxin gene</th>
<th>Primer</th>
<th>Oligonucleotide sequence (5’ → 3’)</th>
<th>Amplicon length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>cpa (F)</td>
<td>3’ AAG ATT TGT AAG GCG CTT ’5</td>
<td>402</td>
<td>Kalender et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>cpa (R)</td>
<td>3’ ATT TCC TGA AAT CCA CTC ’5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>etx (F)</td>
<td>3’GCCTGATATCCATCTATTC ’5</td>
<td>541</td>
<td>Moller and Ahrens (1996)</td>
</tr>
<tr>
<td></td>
<td>etx (R)</td>
<td>3’CCACTTACTTGTCTCTACTAAC ’5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>enterotoxin</td>
<td>cpe (F)</td>
<td>3’GGAGATGGTTGGATATTAGG’5</td>
<td>233</td>
<td>Meer and Songer (1997)</td>
</tr>
<tr>
<td></td>
<td>cpe (R)</td>
<td>3’GGACCAGCAGTTGTAGATA’5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table (2): incidence and typing of *C. perfringens* in the examined chicken meat product samples (n=25).

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

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

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 (*etx*), 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 health threatening 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.

5. REFERENCES


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


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