Molecular typing of *Clostridium perfringens* isolated from minced meat

Asma Afshari¹, Abdollah Jamshidi²*, Jamshid Razmyar³, Mehrnaz Rad⁴

¹Ph.D Student of Food Hygiene, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
²Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
³Department of Clinical Science, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
⁴Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

Received: 4 September 2014         Accepted:  2 June 2015

Abstract

The aim of this study was to determine the presence of toxin genes in *Clostridium perfringens* isolated from 200 minced meat samples using molecular typing. For this purpose, after isolation of *Clostridium perfringens* using conventional culture method and confirmation by specific 16S rDNA gene PCR, alpha (*cpa*), beta (*cpb*), beta 2 (*cpb2*), epsilon (*etx*), iota (*iA*) and enterotoxin (*cpe*) genes were investigated by multiplex PCR. Out of 200 minced meat samples, 25 (12.5%) were determined as contaminated with *C. perfringens* and m-PCR results demonstrated that, out of these 25 isolates, 18 (81 %) possessed only the *cpa* gene (type A), while 4 (18 %) carried the *cpa* and *cpb2* toxin genes (type A-*cpb2*). Furthermore, the results showed 1 isolate (4 %) as type B, 1 isolate (4 %) as type D and 1 isolate (4 %) as type E. This study reports the first *cpb2* positive type B of *C. perfringens* in minced meat. Our results also indicate that *C. perfringens* type A is the most common type in minced meat.

Keywords: *Clostridium perfringens*, toxin genes, minced meat

*Corresponding author: Abdollah Jamshidi
Email: ajamshid@um.ac.ir
Tel: +98 51 38805607
Fax: +98 51 38763852
Introduction

Clostridium perfringens is a Gram-positive, spore-forming, anaerobic rod and ubiquitous pathogen, responsible for different diseases such as gas gangrene, food poisoning and diarrhea in humans as well as for enterotoxemia and haemorrhagic gastroenteritis in many domestic and wild animals (Daube et al., 1994, Songer, 1996). The pathogenicity of the organism is associated with several toxins which are used for toxin typing of the bacteria. The alpha (α), beta (β), epsilon (ε) and iota (i) toxins are the major lethal toxins produced by the organism that are closely related to its virulence, even though they produce several minor extracellular toxins (Hatheway, 1990).

C. perfringens is classified into five types (A–E) on the basis of their ability to produce major lethal toxins. Within these five types, all C. perfringens produce alpha toxin. In addition, type B strains produce b and ε toxins, type C produces b toxin, type D produces ε toxin and type E produces i toxin (Baums et al., 2004). In addition to the major lethal toxins, a minority of C. perfringens strains produce anenterotoxin (cpe), which is responsible for the symptoms of common C. perfringens type A food poisoning (Erol et al., 2008). Different meats and meat products have been frequently reported as the most common food vehicles (Hatheway, 1990, Ridell et al., 1998, Hatakka and Halonen, 2000, Eisgruber and Hauner, 2001, McClane, 2001, McCloud et al., 2001). About 2–5% of all C. perfringens isolates, mostly belonging to type A, produce cpe, a 35-kDa single polypeptide (Songer and Meer, 1996). Cpe positive C. perfringens type A is one of the most commonly reported food-borne pathogen in the US, EU and Turkey (McClane, 2001, Cakmak et al., 2006, Hughes et al., 2007).

The classification of C. perfringens isolates into toxigenic types has been traditionally performed by sero-neutralization with mice or guinea pigs (Oakley and Wayrack, 1953, Sterne and Batty, 1975; McDonel, 1986). Because these methods are time-consuming and expensive, they have largely been replaced by PCR-based detection methods. Various PCR protocols, including multiplex PCR assay, have been established for genotyping of C. perfringens isolates with respect to the cpa, cpb, etx, iap, cpe and cpb2 genes, encoding the α, β, ε, i, enterotoxins and b2 toxins, respectively (Daube et al., 1994, Songer and Meer, 1996, Meer and Songer, 1997, Yoo et al., 1997, Kanakaraj et al., 1998, Kadra et al., 1999, Augustynowicz et al., 2000, Garmory et al., 2000).

However, there are no published data on molecular typing of C. perfringens in minced meat presented in retail markets and butcheries in Iran. In present study, the multiplex PCR assay was used in order to determine the presence of alpha (cpa), beta (cpb), beta 2 (cpb2), epsilon (etx), iota (iA) and enterotoxin (cpe) genes in isolated C. perfringens from minced meat samples.

Materials and methods

Bacterial isolation: Over three months period (fall) total of 200 minced meat samples (10 g of mixed beef and sheep meat) were collected aseptically from different retail markets and butcheries in Mashhad. Samples were placed into a 50-ml sterile plastic tube for transfer to the laboratory. Upon arriving to the laboratory, processing of each meat sample started with homogenization step. Ten milliliters of sterile fluid thioglycolate (FTG) medium was then added to the 50-ml tube containing 10 g of the homogenized minced meat. An aliquot (1 ml) of each FTG meat suspension was added to each of two tubes containing 10 ml of sterile FTG medium. To enrich for any C. perfringens spores in the meat samples, one of those two tubes was heat shocked at 72°C for 20 minutes before
incubation at 37°C for 18 to 24 h. The other tube was directly incubated at 37°C for 18 to 24 h to enrich primarily for C. perfringens vegetative cells presented in meat samples (Wen and McClane, 2004).

Each FTG enrichment culture was streaked onto nutrient blood agar containing 7% defibrinated sheep blood and incubated anaerobically at 37°C for 48 hr. Colonies which showed characteristic dual hemolytic zones were picked up and sub-cultured in Tryptose Sulfite Cycloserine agar (TSC) and Tryptose Sulfite Neomycin agar (TSN) for purification. The identity of the isolates was confirmed by their colonial and microscopical morphology, hemolytic pattern and Gram staining (Razmyar et al., 2013).

Preparation of cell lysates: From each plate which had single typical colony, one colony was suspended in 100 μl distilled water, boiled for 10 min and then centrifuged at 10,000 rpm for 10 min. The supernatants were collected carefully and used as template for PCR assay (Razmyar et al., 2013).

Genus-specific PCR: The identity of the 25 recovered isolates was confirmed as C. perfringens based on the amplification of specific 16S rDNA gene, using specific primers (Table 1) (Wu et al., 2009). Toxin genotyping of isolates: PCR amplification yielded the expected products for the 16S rDNA gene (279bp), and 12.5% of minced meat samples were determined as contaminated with C. perfringens. Toxin typing of isolated C. perfringens showed that type A was the most predominant type (88%), while type B, D and E displayed the incidence of 4%, for each type. Amount of 22 isolates (88%) were determined as type A, and 3 isolates were determined as type B (No=1(4%)), type C (No=1(4%)) and type E (No=1(4%)). From 22 type A isolates, 18 isolates (81.8%) were determined as simple type A (carrying neither the cpe nor cpb2 gene) and 4 (18.1%) isolates were determined as heterogeneous types (carrying cpb2 gene) but none of the isolates were found to carry both the cpe and cpb2 genes. cpe2 gene was found in 4 isolates of type A and in one isolate of type B (20%).

Discussion

Pilgrimage and tourist attractions of the Mashhad city in Northeastern of Iran, which is known as the World's second pilgrimage city after Mecca, and the amount of food which
Table 1. Primers sequence and product size used for species-specific PCR and multiplex PCR

<table>
<thead>
<tr>
<th>Primer sequences (5<code>-3</code>)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAAGATGGCATCATCATTCAAC</td>
<td>279 (Wu et al., 2009)</td>
</tr>
<tr>
<td>TACCTCATTACTTCCCTCCCCAAA</td>
<td></td>
</tr>
<tr>
<td>GCTAATGTATGGCTGGTGA</td>
<td>324 (Meer and Songer, 1997)</td>
</tr>
<tr>
<td>GCCAAGATGATGCACTATCTA</td>
<td>196 (Meer and Songer, 1997)</td>
</tr>
<tr>
<td>GCAGGAACATTATACCATTC</td>
<td>655 (Meer and Songer, 1997)</td>
</tr>
<tr>
<td>CCATTACCTGTCTGACTAAC</td>
<td></td>
</tr>
<tr>
<td>ACTACTCTAGAAAGACAG</td>
<td>446 (Meer and Songer, 1997)</td>
</tr>
<tr>
<td>CTTCCTCTATTCTATCTACG</td>
<td></td>
</tr>
<tr>
<td>GGAGATGGTGTGGATATTAGG</td>
<td>233 (Meer and Songer, 1997)</td>
</tr>
<tr>
<td>GAGCCAGCAGTGTGATA</td>
<td></td>
</tr>
<tr>
<td>AGATTITTTATATGATCCCTAAA</td>
<td>567 (Bueschel et al., 2003)</td>
</tr>
<tr>
<td>CAATACCCTCACCAATTACTC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Results of Multiplex PCR genotyping of C. perfringens isolated from minced meat.

<table>
<thead>
<tr>
<th>Type</th>
<th>Toxin genes of C. perfringens</th>
<th>Cpa</th>
<th>cpb</th>
<th>etx</th>
<th>iA</th>
<th>cpe</th>
<th>Cpb2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>4(18%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4(18%)</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td>18(81%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>1(4%)</td>
<td>1(4%)</td>
<td>1(4%)</td>
<td>-</td>
<td>-</td>
<td>1(4%)</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>1(4%)</td>
<td>-</td>
<td>1(4%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>1(4%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1(4%)</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>25(12.5%)</td>
<td>1(4%)</td>
<td>2(8%)</td>
<td>1(4%)</td>
<td>-</td>
<td>5(20%)</td>
</tr>
</tbody>
</table>

serves for these tourism population indicates the importance of attention that must be taken to food-borne diseases. Following Campylobacter and Salmonella Spp., Clostridium perfringens is the third most important bacterial food-borne pathogen (Novak and Juneja, 2002). Meat and meat products are excellent medium for growth of this bacterium (Wen and McClane, 2004). Foods like kebab which contain minced-meat have a high popularity in Middle East and are prepared with the application of low heat treatment. The quality of these foods depends on meat and additives quality, sanitary condition of the equipments and the processing procedures (Atwa and Abou El-Roos, 2011).

Traditionally, typing of C. perfringens strains involved sero-neutralisation of culture filtrates and mice or guinea pigs injection with culture supernatants of C. perfringens, along with antitoxin, and death (mice) or dermonecrosis (guinea pigs) was assessed (Sterne, 1975). This assay was extremely time consuming, as growth of the organism was required. It was also expensive as two of the toxins, epsilon and iota, required trypsin for activation, but a third toxin, beta toxin, was inactivated by trypsin. Therefore each culture supernatant was assayed numerous times, with and without trypsin, and with and without the five different preparations of neutralizing antisera (Hatheway, 1990). Multiplex PCR assay is a useful alternative to traditional assays and as a replacement for standard in vivo typing methods (Meer and Songer, 1997).

In our study the contamination rate of minced meat samples with C. perfringens, was 12.5%. Higher incidence has been reported by other researchers who reported the incidence of C. perfringens in minced meat samples as 35% (Torky and A. Sh, 2004) and 28% (Atwa and Abou El-Roos, 2011). Guran et al (2014)
reported that 96% of ground beef and 88% of ground sheep meat samples as contaminated with \textit{C. perfringens}. Wen and McClane (2004) showed that 23% of American ground beef were contaminated with \textit{C. perfringens}. Kamber et al (2007) also reported \textit{C. Perfringens} isolation from 17% of minced meat samples, with 12%, 1%, 4% and 2% astypes A, B, C and D, respectively. Variation in contamination rates reported by different researchers might be due to differences in the technology of slaughtering, personnel and equipment hygiene, number of collected samples and the method of bacterial isolation. The other possibility is that, the present study was conducted during a season with low rainfall, which may have resulted in a lower number of animals suffering from diseases induced by \textit{C. perfringens} and thus, lower contamination of carcasses with this bacteria.

In our study \textit{C. perfringens} types A (\textit{cpa} positive), B (\textit{cpa, cpb} and \textit{etx} positive), D (\textit{cpa} and \textit{etx} positive) and E (\textit{cpa} and \textit{iap} positive) were identified in the samples, whereas type A was the dominant type (88%-22 out of 25). To the best of our knowledge, the present study is the first investigation on \textit{C. perfringens} genotyping, isolated from minced meat (beef and sheep) in Iran. Guran et al (2014) by using the same method for toxintyping of the isolates in their study, demonstrated that, 77.4% of isolates possessed only the \textit{cpa} gene (simple type A), while 7.6% carried the \textit{cpa} and \textit{cpb}2 toxin genes (type A-\textit{cpb}2\textsuperscript{+}), and 2.2% the \textit{cpa} and \textit{cpe} toxin genes (type A-\textit{cpe}\textsuperscript{+}), furthermore they reported 1.5%, 3.8% and 7.2% of samples as types B, C and D respectively which are approximately similar to our findings.

\textit{C. perfringens}enterotoxin (Cpe) is the most important virulence factor when type A isolates cause human GI diseases, although less than 5% of all \textit{C. perfringens} produce this toxin (Heikinheimo et al., 2004). In this study, all isolates were identified \textit{ascope}-negative which may indicates that contamination during pre-processing is unlikely, but processing, cutting, handling, and wrapping operations may be responsible for the addition of \textit{C. perfringens} spores and vegetative cells (Hall and Angelotti, 1965). In this study, 5 out of 25 (20%) of \textit{C. perfringens} isolates were identified as positive for \textit{cpb}2 gene. \textit{Cpb}2 toxin can be produced by all types of \textit{C. perfringens} (Fisher, 2006). Therefore the presence of such genes is not considered a risk by itself and there are some predisposing factors that have been associated with the pathogenicity of toxigenic \textit{C. perfringens} and consequently the development of disease (Crespo et al., 2007). In another study the researchers did not find any \textit{cpb}2 positive \textit{C. perfringens} in ground beef samples (Wen and McClane, 2004). In contrast to our results that indicate the presence of \textit{cpb}2 gene in 4 isolates of type A and in one isolate of type B, until now all reported \textit{cpb}2 positive \textit{C. perfringens} isolates from various foods were from type A (Guran et al., 2014). This study reports the first \textit{cpb}2 positive type B of \textit{C. perfringens} in minced meat.

In conclusion, multiplex PCR assay provides a simple and rapid method for genotyping of \textit{C. perfringens} isolates. This study showed that type A strain of \textit{C. perfringens} is the most prevalent types in minced meat in this region of Iran. Further investigations are required to reveal the pathogenicity of \textit{C. perfringens} type B with \textit{cpb}2 gene.

**Acknowledgements**

This research was funded by a grant (No.28440) from the Research Council of the Ferdowsi University of Mashhad. We would like to thank Mr. A. Kargar and Mrs S. khajenasir for their assistance in laboratory works.
References


clinical isolates and identification of enterotoxigenic *Clostridium perfringens* strains by classical methods and by polymerase chain reaction (PCR). *FEMS Immunology and Medical Microbiology* **24**, 259–266.


Torky, A.A.S (2004). Trials for inhibition of some food poisoning microorganisms in meat products, Cairo University, Egypt.


جداسازی و ناپیوستگی مولکولی باکتری کلستریدیوم پرفرنجنس جدا شده از گوشت چرخ کرده

اسما افشازاده، علی‌اصغر جمشیدی، جمشید رزمیار، مهرناز راد

چکیده

هدف از این مطالعه تعیین زنده تاکسین‌‌زای باکتری کلستریدیوم پرفرنجنس جدا شده از 200 نمونه گوشت چرخ کرده به روش ناپیوستگی مولکولی بود. پس از جداسازی کلستریدیوم پرفرنجنس به روش کلت‌سنب و تأیید تشخیص با استفاده از PCR DNA ژن‌های ال‌اف‌ا و با 2 اپسیلون، پنیا واناتروکسین با روش مولتی‌پاکس PCR ردقیبی شدن نمونه 200 نمونه مورد بررسی کلستریدیوم پرفرنجنس از 200 نمونه (12.14%) از جداسازی گردید. نتایج حاصل از مولتی‌پاکس PCR نشان داد که 88٪ نمونه‌های به عنوان کلستریدیوم پرفرنجنس در 200 نمونه به عنوان دارای ژن cpb2 شناخته شدند. همچنین نتایج به دست آمده نشان می‌داد که نمونه‌های تیره نزن کلستریدیوم پرفرنجنس در گوشت چرخ کرده در ایران می‌باشد.

واژگان کلیدی: کلستریدیوم پرفرنجنس، PCR، چندگانه و گوشت چرخ کرده