Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

# Molecular typing of *Clostridium perfringens* isolated from turkey meat by multiplex PCR

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**Keywords**
*Clostridium perfringens*, toxin genes, turkey meat.

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2007/1607: received 3 October 2007, revised 14 March 2008 and accepted 25 March 2008

doi:10.1111/j.1472-765X.2008.02379.x

## Abstract

**Aims:** To determine the presence of toxin genes in 22 *Clostridium perfringens* isolated from turkey meat samples by molecular typing.
**Methods and Results:** For this purpose, alpha (*cpa*), beta (*cpb*), beta 2 (*cpb2*), epsilon (*etx*), iota (*iA*) and enterotoxin (*cpe*) toxin genes were analysed by multiplex PCR. All 22 turkey meat *Cl. perfringens* isolates were found to carry the *cpa*, gene but in none of the isolates *cpb*, *etx*, *iap* or *cpe* genes were detected. Results showed that all isolates represented type A and were *cpe* negative.
**Conclusions:** Our results indicate that *Cl. perfringens* type A is the most common type in turkey meat. Also multiplex PCR is effective and rapid method for typing of *Cl. perfringens*.
**Significance and Impact of the Study:** It is the first study about molecular typing of *Cl. perfringens* using multiplex PCR in turkey meat samples in Turkey.

## 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 diarrhoea 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 ($\alpha$), beta ($\beta$), epsilon ($\epsilon$) and iota ($\iota$) 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).

*Clostridium perfringens* is classified into five types (A–E) on the basis of their ability to produce major lethal toxins. Within these five types, all *Cl. perfringens* produce $\alpha$ toxin. In addition, type B strains produce $\beta$ and $\epsilon$ toxins, type C produces $\beta$ toxin, type D produces $\epsilon$ toxin and type E produces $\iota$ toxin. In addition to the major lethal toxins, a minority of *Cl. perfringens* strains produce a *Cl. perfringens* enterotoxin (CPE), which is responsible for the symptoms of common *Cl. perfringens* type A food poisoning. Different meats, including poultry meat, 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).

About 2–5% of all *Cl. perfringens* isolates, mostly belonging to type A, produce CPE, a 35-kDa single polypeptide (Songer and Meer 1996). CPE positive *Cl. perfringens* type A is one of the most commonly reported foodborne pathogen in the US, EU and Turkey (McClane 2001; Çakmak *et al.* 2006; Hughes *et al.* 2007). Thus, detection of *Cl. perfringens* toxin types and subtypes is critical for a better understanding of the epidemiology of *Cl. perfringens* infections and may be helpful in the development of effective preventive measures.

The classification of *Cl. 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 assays, have been established to genotype *Cl. perfringens* isolates with respect to the genes *cpa*, *cpb*, *etx*, *iap*, *cpe* and *cpb*2, encoding the $\alpha$, $\beta$, $\epsilon$, $\iota$, entero and $\beta$2 toxin, 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 *Cl. perfringens* in poultry meat in Turkey. In the present study, the multiplex PCR was used in order to determine the presence of alpha (*cpa*), beta (*cpb*), beta 2 (*cpb2*), epsilon (*etx*), iota (*iA*) and enterotoxin (*cpe*) toxin genes from a total of 22 *Cl. perfringens* strains isolated from turkey meat.

## Materials and methods

### Bacterial strain

*Clostridium perfringens* ATCC 13124 (for *cpa* gene), NCTC 8239 (*cpa*, *cpe* genes), ATCC 3626 (*etx*, *cpb* genes) and CCUG 44727 (*iA* gene) were used as positive controls in this study.

### Isolates

A total of 22 *Cl. perfringens*, isolated from 180 turkey meat samples collected from different supermarkets located in Ankara over a year period, were analysed by molecular typing for the detection of major toxin genes using multiplex PCR. The techniques described by Baumgart *et al.* (1990), Schalch *et al.* (1996) and Baumgart (1997) were used to isolate and identify *Cl. perfringens*.

For enrichment of *Cl. perfringens*, a 25-g portion of each sample was aseptically placed in a sterile plastic bag containing 225 ml of Perfringens Enrichment Medium [PEM; Fluid Thioglycollate Medium, supplemented with Perfringens (TSC) supplement, Oxoid SR 88, Oxoid, Hampshire, UK] and homogenized by a stomacher (AESAP 1068-Easy Mix; AES Laboratories, Combourg, France) for approx. 2 min and then incubated at 46°C for 20 h in an anaerobic condition (Gas generating kit, B 36, Oxoid). After the samples were enriched in PEM, one loopful of enrichment was streaked onto TSC agar (Tryptose Sulphite Cycloserine agar, Oxoid CM 857; Oxoid) and the plates were further incubated at 46°C for 20 h in a Gas Pak system (Gas generating kit, B 36, Oxoid) anaerobically. In order to confirm of *Cl. perfringens*, up to five suspect black colonies from each positive TSC agar plate were purified and identified biochemically by using catalase test, lactose fermentation, gelatinize production, nitrate reduction, motility test, acid phosphatase reaction, haemolysis test and the reverse CAMP testing as described by Schalch *et al.* (1996).

### Typing by multiplex PCR

Molecular typing of *Cl. perfringens* was performed by multiplex PCR (Meer and Songer 1997). In the PCR assay CPA, CPB, ETX, IA, CPE and CPB2 primer pairs (Promega, Madison, WI, USA) were used as shown in Table 1.

**Table 1** Primers for multiplex PCR detection of *Clostridium perfringens* toxin genes (Meer and Songer 1997)

| Primers | Primer sequence (5′–3′) | Size (bp) | Gene |
|---|---|---|---|
| CPA F | GCTAATGTTACTGCCGTTGA | 324 | *cpa* |
| CPA R | CCTCTGATACATCGTGTAAG | | |
| CPB F | GCGAATATGCTGAATCATCTA | 196 | *cpb* |
| CPB R | GCAGGAACATTAGTATATCTTC | | |
| ETX F | GCGGTGATATCCATCTATTC | 655 | *etx* |
| ETX R | CCACTTACTTGTCCTACTAAC | | |
| IA F | ACTACTCTCAGACAAGACAG | 446 | *iA* |
| IA R | CTTTCCTTCTATTACTATACG | | |
| CPE F | GGAGATGGTTGGATATTAGG | 233 | *cpe* |
| CPE R | GGACCAGCAGTTGTAGATA | | |
| CPB2 F | AGATTTTAAATATGATCCTAACC | 567 | *cpb2* |
| CPB2 R | CAATACCCTTCACCAAATACTC | | |

### DNA extraction

Extraction of DNA from all of the isolates was performed using a boiling technique. All isolates, stored at −80°C in cryovials, were incubated in cooked meat broth (Oxoid CM0081) at 37°C for 24 h in anaerobic conditions (top of tubes were covered by sterile liquid paraffin). One millilitre of each enrichment culture was separately transferred to a microcentrifuge tube. All tubes were centrifuged (Eppendorf Centrifuge 5417R) for 3 min at 12 000 ***g***. The pellets were resuspended in 200 $\mu$l sterile distilled water. The suspensions were mixed and heated to 95°C for 20 min in a water bath (Memmert WB/OB 7-45, WBU 45, Schwabagh, Germany) and centrifuged for 3 min at 12 000 ***g*** and cooled on ice. A volume of 10 $\mu$l was used as a template in the PCR.

### Multiplex PCR

The multiplex PCR was performed in a total volume of 50 $\mu$l. Reaction mixture contains 1× Reaction buffer (Promega), 1·5 mmol $l^{-1}$ $MgCl_2$, 0·12 mmol $l^{-1}$ dNTPs, 0·34 $\mu$mol $l^{-1}$ of each *cpe* primers, 0·36 $\mu$mol $l^{-1}$ of each *cpb* primers, 0·36 $\mu$mol $l^{-1}$ of each *cpb2* primers, 0·44 $\mu$mol $l^{-1}$ of each *etx* primers, 0·5 $\mu$mol $l^{-1}$ of each *cpa* primers, 0·52 $\mu$mol $l^{-1}$ of each *iA* primers, 5 units of Taq DNA polymerase (Promega, Madison, WI) and 10 $\mu$l template DNA. Thermal cycling (Biometra Personel Cycler, Goettingen, Germany) was carried out with 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A 10-$\mu$l aliquot of each PCR product was subjected to 1·5% agarose gel electrophoresis containing 0·1 $\mu$g $ml^{-1}$ ethidium bromide for 1 h at 100 V. Amplicon visualization and documentation were performed using gel documentation and analysis system (Syngene Ingenius, Cambridge, UK).

## Results

The 22 *Cl. perfringens* isolated from turkey meat were analysed by multiplex PCR in order to determine the toxin genes for the molecular typing. In all 22 *Cl. perfringens*, the *cpa* gene was detected (Fig. 1), so all the isolates were confirmed as *Cl. perfringens*. However, the *cpb*, *etx*, *iA*, *cpe* and *cpb2* genes were not detected in any of the isolates (data not shown).

According to the multiplex PCR analyses, results showed that all the isolates were *Cl. perfringens* type A and *cpe* negative.

## Discussion

In recent years, many authors have focused on the molecular typing of *Cl. perfringens* by the detection of toxin genes using multiplex PCR. Several studies reported that type A is the predominant type in poultry. The enterotoxins of type A have been reported to cause food-borne infections in humans (Lukinmaa *et al.* 2002; Engström *et al.* 2003; Wen and McClane 2004).

Our results suggested that toxin type A of *Cl. perfringens* was the most prevalent causative agent in turkey meat samples. Also *Cl. perfringens* types prevalent in chicken were similar to those found in other studies (Songer 1996; Yoo *et al.* 1997).

A study conducted in Turkey reported that eight *Cl. perfringens* from 160 chicken intestinal content were isolated by conventional methods and PCR. Isolates were characterized by the toxin neutralization test. According to their results, six of the eight isolates were found to be type A and two of them could not be typed (Kalender and Ertas 2005).

Similar to our study, molecular typing was managed with a total of 118 *Cl. perfringens* isolates from Finnish broiler chickens. All isolates were shown to carry the *cpa* gene but not the other toxin genes detected by the assay (Heikinheimo and Korkeala 2005). In various studies, type A has been reported to be the dominant type of *Cl. perfringens* worldwide (Meer and Songer 1997; Yoo *et al.* 1997; Engström *et al.* 2003; Nauerby *et al.* 2003). Lin and Labbe (2003) showed that all isolates from retail foods possessed *cpa* encoding α toxin. None of the isolates have the *cpe* gene.

Wen and McClane (2004) found that 80% of 278 *Cl. perfringens* isolates collected from contaminated foods were determined as type A. Nearly 20% of the food samples contaminated with type A isolates carried the *cpb2* gene. They also found that 4·3% of the isolates carried *cpe* gene by multiplex PCR, which was different from our study. In a different study, all 53 isolates analysed by PCR belonged to the toxin type A of *Cl. perfringens*, with the gene coding for α-toxin production. Two isolates carried the β2 gene as well, likewise none had the other toxin genes including *cpe* (Engström *et al.* 2003). Toxin gene profiles of poultry isolates indicated that all of the isolates were positive for *Cl. perfringens* α toxin gene and 46 of 48 isolates were β2 toxin gene. All strains were negative for β and ϵ toxin genes (Siragusa *et al.* 2006). Johansson *et al.* (2006) reported that the *cpb2* gene was found in 20% of the food poisoning outbreaks and 10% of the poultry. The *cpe* gene was found in all isolates originating from food poisoning outbreaks. From cases of necrotic enteritis, both *Cl. perfringens* type A and C have been isolated (Ficken and Wages 1997).

Our results indicate that *Cl. perfringens* type A is the most common type in turkey meat. Also multiplex PCR is effective and rapid method for typing of *Cl. perfringens*. In our study, all types of isolates were negative for *cpe*, this result might be due to the rare detection ratio (0–5%) of all *Cl. perfringens* as mentioned above.

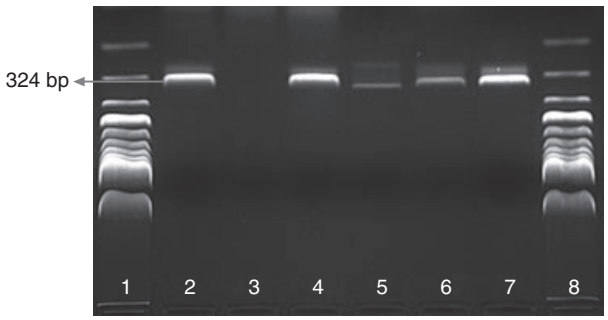


**Figure 1** *Cpa* gene detected *Clostridium perfringens* isolates (1 and 8, 100-bp DNA marker; 2, positive control; *Cl. perfringens*ATCC 13124; 3, negative control; 4–7, *Cpa*-positive *Cl. perfringens* isolates).

## Acknowledgements

Part of this study was supported by a grant of Ankara University Scientific Research Projects (BAP-0810072). The authors are grateful to Hannu Korkeala and Yağmur Derman from University of Helsinki Department of Food and Environmental Hygiene for providing the *Cl. perfringens* strains NCTC 8239, ATCC 3626 and CCUG 44727.

## References

Augustynowicz, E., Gzyl, A. and Slusarczyk, J. (2000) Molecular epidemiology survey of toxinogenic *Clostridium perfringens* strain types by multiplex PCR. *Scand J Infect Dis* **32**, 637–641.

Baumgart, J. (1997) *Mikrobiologische Untersuchung von Lebensmitteln*. Hamburg: Behr's Verlag.

Baumgart, J., Baum, O. and Lippert, S. (1990) Schneller und direkter Nachweis von *Clostridium perfringens*. *Fleischwirtschaft* **70**, 1010–1014.

Çakmak, Ö., Bilir Ormancı, F.S., Tayfur, M. and Erol, İ. (2006) Presence and contamination level of *Clostridium perfringens* in raw frozen ground poultry and poultry burgers. *Turk J Vet Anim Sci* **30**, 101–105.

Daube, G., China, B., Simon, P., Hvala, K. and Mainil, J. (1994) Typing of *Clostridium perfringens* by in vitro amplification of toxin genes. *J Appl Bacteriol* **77**, 650–655.

Eisgruber, H. and Hauner, G. (2001) Minced beef heart associated with a *Clostridium perfringens* food poisoning in a Munich old people's home. *Arch Lebensmittelhyg* **52**, 63–66.

Engström, B.E., Fermér, C., Lindberg, A., Saarinen, E., Båverud, V. and Gunnarsson, A. (2003) Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. *Vet Microbiol* **94**, 225–235.

Ficken, M.D. and Wages, D.P. (1997) Necrotic enteritis. In *Diseases of Poultry*, 10th edn ed. Calnek, B.W. pp. 261–264. Ames, IA: Mosby-Wolfe.

Garmory, H.S., Chanter, N., French, N.P., Bueschel, D., Songer, J.G. and Titball, R.W. (2000) Occurrence of *Clostridium perfringens* β2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol Infect* **124**, 61–67.

Hatakka, M. and Halonen, H. (2000) *Food-borne and Waterborne Outbreaks in Finland in 1999*. National Food Administration Research Notes, Vol. 7. Helsinki, Finland: National Food Administration.

Hatheway, C.L. (1990) Toxigenic clostridia. *Clin Microbiol Rev* **3**, 66–98.

Heikinheimo, A. and Korkeala, H. (2005) Multiplex PCR assay for toxinotyping *Clostridium perfringens* isolates obtained from Finnish broiler chickens. *Lett Appl Microbiol* **40**, 407–411.

Hughes, C., Gillespie, I.A. and O'Brien, S.J. (2007) Foodborne transmission of intestinal disease in England and Wales, 1992–2003. *Food Control* **18**, 766–772.

Johansson, A., Aspan, A., Bagge, E., Båverud, V., Engström, B.E. and Johansson, K.E. (2006) Genetic diversity of *Clostridium perfringens* type A isolates from animals, food poisoning outbreaks and sludge. *BMC Microbiol* **6**, 47.

Kadra, B., Gouillou, J.P., Popoff, M. and Bourlioux, P. (1999) Typing of sheep clinical isolates and identification of enterotoxigenic *Clostridium perfringens* strains by classical methods and by polymerase chain reaction (PCR). *FEMS Immunol Med Microbiol* **24**, 259–266.

Kalender, H. and Ertas, H.B. (2005) Isolation of *Clostridium perfringens* from chickens and detection of the alpha toxin gene by polymerase chain reaction (PCR). *Turk* J Vet Anim Sci **29**, 847–851.

Kanakaraj, R., Harris, D.L., Songer, J.G. and Bosworth, B. (1998) Multiplex PCR assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and in swine feed. *Vet Microbiol* **63**, 29–38.

Lin, Y.T. and Labbe, R. (2003) Enterotoxigenicity and genetic relatedness of *Clostridium perfringens* isolates from retail foods in the United States. *Appl Environ Microbiol* **69**, 1642–1646.

Lukinmaa, S., Takkunen, E. and Siitonen, A. (2002) Molecular epidemiology of *Clostridium perfringens* related to foodborne outbreaks of disease in Finland from 1984 to 1999. *Appl Environ Microbiol* **68**, 3744–3749.

McClane, B.A. (2001) *Clostridium perfringens*. In *Food Microbiology: Fundamentals and Frontiers* ed. Doyle, M.P., Beuchat, L.R. and Montville, T.J. pp. 351–372. Washington, DC: ASM Press.

McDonel, J.L. (1986) Toxins of *Clostridium perfringens* types A, B, C, D, and E. In *Pharmacology of Bacterial Toxins* ed. Dorner, F. and Drews, H. pp. 477–517. Oxford: Pergamon Press.

Meer, R.R. and Songer, J.G. (1997) Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. *Am J Vet Res* **58**, 702–705.

Nauerby, B., Pedersen, K. and Madsen, M. (2003) Analysis by pulsed field gel electrophoresis of the genetic diversity among *Clostridium perfringens* isolates from chickens. *Vet Microbiol* **94**, 257–266.

Oakley, C. and Wayrack, G. (1953) Routine typing of *Clostridium welchii*. *J Hyg* **51**, 102–107.

Ridell, J., Björkroth, J., Eisgrüber, H., Schalch, B., Stolle, A. and Korkeala, H. (1998) Prevalence of the enterotoxin gene and clonality of *Clostridium perfringens* strains associated with food-poisoning outbreaks. *J Food Prot* **61**, 240–243.

Schalch, B., Eisgruber, H., Geppert, P. and Stolle, A. (1996) Vergleich von vier Routineverfahren zur Bestaetigung von *Clostridium perfringens* aus Lebensmitteln. *Arch Lebensmittelhyg* **47**, 27–30.

Siragusa, G.R., Danyluk, M.D., Hiett, K.L., Wise, M.G. and Craven, S.E. (2006) Molecular subtyping of poultry-associated type A *Clostridium perfringens* isolates by repetitive-element PCR. *J Clin Microbiol* **44**, 1065–1073.

Songer, J.G. (1996) Clostridial enteric diseases of domestic animals. *Clin Microbiol Rev* **9**, 216–234.

Songer, J.G. and Meer, R.M. (1996) Genotyping of *Clostridium perfringens* by polymerase chain reaction is a useful adjunct to diagnosis of clostridial enteric disease in animals. *Anaerobe* **2**, 197–203.

Sterne, M. and Batty, I. (1975) Criteria for diagnosing clostridial infection. In *Pathogenic Clostridia* ed. Sterne, M. and Batty, L. pp. 79–122. London: Butterworths.

Wen, Q. and McClane, B.A. (2004) Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. *Appl Environ Microbiol* **70**, 2685–2691.

Yoo, H.S., Lee, S.U., Park, K.Y. and Park, Y.H. (1997) Molecular typing and epidemiological survey of prevalence of *Clostridium perfringens* types by multiplex PCR. *J Clin Microbiol* **35**, 228–232.