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Typing of Toxigenic Isolates of *Clostridium perfringens* by Multiplex PCR in Ostrich

Research Article

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ABSTRACT

Clostridium perfringens is an important pathogen that provokes numerous different diseases. This bacterium is classified into five various types, each of which capable of causing a distinct disease. There are various methods for the bacterial identification, many are labor-intensive, time-consuming, expensive and also show low sensitivity and specificity. The aim of this research was to identify the unlike types of *Clostridium perfringens* using PCR method. In this study, 120 ostrich-dung samples were randomly collected from areas around the city of Kerman, southeastern Iran. After processing and culturing of samples, the produced colonies were morphologically studied, gram stain test was also carried out and the genera of these bacteria were identified through biochemical tests. DNA extracted from isolated bacteria for genotyping was tested by multiplex PCR with specific primers. Based on length of synthesized fragments by PCR, toxin types and bacterial strains were detected. *Clostridium perfringens* isolated types were divided as follows: 100% type A, 0% type B, 0% type C and 0% type D. It should be emphasized that, up to now, *Clostridium perfringens* type A has not been reported in Iran.

KEY WORDS biochemical tests, *Clostridium perfringens*, genetic typing, multiplex PCR.

INTRODUCTION

Clostridium perfringens is a gram-positive anaerobic bacterium that is able to form spores. It is widespread in the environment (e.g. in soil and sewage) and is commonly found in the intestines of animals, including humans, where it is pathogenic in certain circumstances. In humans, it can cause gangrene and gastrointestinal disease (e.g. food poisoning and necrotic enteritis), whereas in other animals, gastrointestinal and enterotoxemic diseases occur more frequently. *C. perfringens* does not invade healthy cells but produces various toxins and enzymes that are responsible for the associated lesions and symptoms (Sawires and Songer, 2006). The toxins produced depend on the *C. perfringens* strain involved, and each type of toxin induces a specific syndrome. Therefore, the correct identification of *C. perfringens* pathovars is critical for epidemiological studies and for the development of effective preventative measures, including vaccination.

Outbreaks of necrotic enteritis, caused by *C. perfringens*, have been frequently reported in chickens throughout the world including Iran (Gokce *et al*. 2007; Greco *et al*. 2005; Settanni and Corsetti, 2007; Ahsani *et al*. 2010a; Ahsani *et al*. 2011). However, there were no reports of necrotic enteritis in the ostrich since the ostrich has been introduced in 1996. The population of ostrich was rapidly increased and now regarded as one of the major domestic industries in Iran. In some laboratories, a serum neutralization test on mice or guinea pigs is employed to determine and diagnose bacterial toxins (Greco *et al*. 2005).

This method is tedious, time-consuming, expensive and monovalent. Furthermore, it is improper and unethical to apply it at the expense of laboratory animals (Piatti *et al*. 2004; Greco *et al*. 2005; Miyashiro *et al*. 2007).

According to Babe *et al*. (2012) from feces samples collected from ostrich farm only 40% strains of *C. perfringens* were isolated regard to biochemical tests. In their strains only 4% strains were toxigenic and ELISA test of their sample for alpha toxin is positive and ELISA test for epsilon and beta toxin were negative. The kits for detection of iota toxin were not available and therefore detection of type E was not contemplated. ELISA utilizes polyclonal antibodies to identify *C. perfringens* toxins (Ahsani *et al*. 2010a). However, its disadvantage is that the interaction reaction among the produced antibodies works against the toxins, which may difficult the identification of toxin types. Moreover, ELISA falls short of identifying ß2 toxins and in order to identify toxins from spore-forming bacteria, it must be activated by means of special culture methods (Baums *et al*. 2004).

Biochemical tests are also incapable of distinguishing different types of *C. perfringens* (Mac Fadin, 2000). PCR is the most modern practical technology in diagnosing infectious diseases and compared with classical techniques, it has been shown to be more rapid, with results obtained in a few hours, and also more reliable (Miyashiro *et al*. 2007; Ahsani *et al*. 2010a).

PCR allows a faster bacterial identification directly from clinical samples (Ahsani *et al*. 2010b). The relatedness of bacterial isolates has in the past been determined by testing for one or several phenotypic properties. They reflect the bacteria's production of certain proteins, which may not be expressed under certain environmental or culture conditions. In contrast, some of the newer molecular typing methods involving the analysis of DNA offer many advantages over traditional techniques.

Genotyping, which is based on a more stable marker, DNA, is not dependent on gene expression. Another advantage of genotyping methods is that the discriminatory power of DNA-based methods is generally superior to that of phenotypic methods.

The ability to distinguish between genomes is important to several disciplines of microbiological research, for example in studies on population genetics and microbial epidemiology (Baums *et al*. 2004).

Of great importance when choosing a method for genotyping are the typability, reproducibility, discriminatory power and also the ease and cost of performing the analysis. With polymerase chain reaction (PCR), selected segments of any DNA molecule can be amplified exponentially. Several polymerase chain reaction (PCR) methods have since been established (Table 1).

Table 1 PCR methods to detect *clostridium perfringens* toxin genes (Ahsani, 2009)

Gene (s)	Specimen (s)	Application	Reference
cpe	Bacterial cell DNA lysate	Single PCR	(Kokai-Kun *et al*. 1994; Ridell *et al*. 1998)
	Feces	Single PCR	(Saito *et al*. 1992)
	Meat, feces	Nested PCR	(Miwa *et al*. 1996)
	Intestinal contents	MPN-PCR	(Miwa *et al*. 1997)
	Various foods	Single PCR	(Kim *et al*. 2000)
	Meat	PCR-ELISA	(Baez *et al*. 1996)
cpe, cpa	Bacterial cell lysate, DNA	Duplex PCR	(Tansuphasiri 2001; Augustynowicz 2002)
	Food, feces	Duplex PCR	(Fach and Popoff, 1997)
	Feces, intestinal contents	Duplex PCR	(Kanakaraj *et al*. 1998)
	Feces	Duplex PCR	(Tansuphasiri *et al*. 2002)
	Water, food, samples stool	Real-time FRET-PCR	(Cruz *et al*. 2006)
cpe, pa, cpb, tx, ia	Bacterial cell lysate, DNA	Multiplex PCR	(Daube *et al*. 1994; Songer and Meer 1996; Meer and Songer 1997; Kadra *et al*. 1999; Kalender *et al*. 2005)
cpe, pa, cpb, tx, ia, cpb2	Bacterial cell lysate, DNA	Multiplex PCR	(Herholz *et al*. 1999; Garmory *et al*. 2000; Gkiourtzidis *et al*. 2001; Baums *et al*. 2004)

MATERIALS AND METHODS

Sampling and biochemical tests

In this study, ostrich dung samples were obtained from 120 ostrich, from eight locations in southeastern Iran. The ostriches were from both genders and their ages ranged from 1 month to 6 years old.

The samples were collected aseptically in sterile plastic bags and transferred to the laboratory within 1 to 2 hours, after which they were accordingly processed. Samples were diluted in PBS (1:10), the bath temperature was maintained at 80 ˚C for ten minutes in order to eliminate the non-spore-forming bacteria, subsequently they were cultivated on 5% sheep blood agar and anaerobically incubated using Anoxomat® (Mart Microbiology, Netherlands) at 37 ˚C for 48 hours.

The suspected colonies were identified by characteristic colony morphology, gram staining and biochemical tests as described by Mac Fadin (2000). Biochemical identification consisted of gelatin and lecithin hydrolyses; and catalase, lipase and motility tests. Moreover, indole production, litmus milk reaction and carbohydrate fermentation (sucrose, glucose, lactose and maltose) were carried out in order to identify the bacterial genus according to Mac Faddin (2000) (Table 2).

DNA extraction and PCR

Isolated strains (only *C. perfringens*) were cultured on blood agar and total DNA was extracted as described by Sambrook and Russell (2002).

Two or three colonies that grown on blood agar were suspended in 350 µL of STET buffer (100 m*M* Tris-HCl, 10 m*M* EDTA, 100 m*M* NaCl, 5% Triton® X-100) in 1.5 mL microtubes. Then, 25 µL 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 1 mL of 70% ethanol at 4 ˚C. Finally, the pellet was dried and resuspended in 50 µL of TE buffer containing RNase. Four specific primer sets corresponding to each toxin of *C. perfringens* were evaluated for their ability to detect *C. perfringens* and to discriminate between *C. per fringens* and other clostridia species (Table 3), according to Komoriya *et al.* (2007) and Van Asten *et al.* (2009).

The PCR was performed in a Thermal Cycler® (Bio-Rad, USA) in a total reaction volume of 50 µL containing: 5 µL of 10X PCR buffer (10 m*M* Tris-HCL, pH 9.0, 50 m*M* KCl), 2 µL 50 m*M* MgCl2, 250 µ*M* of each deoxynucleotide triphosphate, 5 U of *Taq* DNA polymerase, 100 pmol of primers and 5 µL of template DNA. Amplification was obtained with 35 cycles following an initial denaturizing step at 95 ˚C for ten minutes.

Each cycle comprised denaturation at 94 ˚C for 45 seconds, annealing at 55 ˚C for 30 seconds, and synthesis at 72 ˚C for 90 seconds. The final extension step occurred at 72 ˚C for ten minutes.

Then, 10 µL of the amplified product was electrophoresed in a 1.5% agarose gel and stained with DNA safe satin. Amplified bands were visualized and photographed under UV illumination.

RESULTS AND DISCUSSION

In this study, 120 intestinal contents were analyzed and well-grounded results 25 percent of samples were positive for *Clostridium* types that show sensitivity of Ostrich towards clostridial diseases.

Also *C. perfringens* was more prevalent type of clostridia genus that was isolated and included 36 % of total clostridia isolates, including *C. perfringens, C. baratii*, *C. absonum*, *C. bifermentans*, *C. sporogenes*, *C. leptum*, *C. aurantibutyricum*, *C. sporosphaeroides C. symbiosum*, *C. scatologenes*, *C. ramosum* and *C. sordellii*.

Table 2 Biochemical tests for identification some clostridia species

Biochemical tests / Clostridial species	Catalase test	Egg yolk agar: Lecithinase produced	Egg yolk agar: Lipase produced	Gelatin hydrolyzed	Indole produced	Carbohydrate fermentation: Glucose	Carbohydrate fermentation: Lactose	Carbohydrate fermentation: Sucrose	Carbohydrate fermentation: Maltose	Motility	Milk reaction
C. perfringens	–	+	–	+	–	+	+	+	+	–	dc
C. baratii	–	+	–	–	–	+	+w	+	+w	–	c
C. absonum	–	+	–	+	–	+	+	+	+	±	c
C. bifermentans	–	+	–	+	+	+	–	–	–w	+	d
C. sporogenes	–	–	+	+	–	+	–	–	–w	±	d
C. leptum	–	–	–	–	–	–	±	±	+	–	–
C. aurantibutyricum	–	–	+	+	–	+	+	+	+	+	c
C. sporosphaeroides	–	–	–	–	–	–	–	–	–	–	–
C. symbiosum	–	–	–	–	–	+	±	–	–	±	–c
C. scatologenes	–	–	–	–	±	+	–	–	–	+	–
C. ramosum	–	–	–	–	–	+	+	+	+	–	c
C. sordellii	–	+	–	+	+	+	–	–	+w	±	d

c: curd; d: digestion; dc: first digestion then curd and w: weak.

Table 3 Primers employed in multiplex PCR

Toxin	Gene	Sequence 5'-3'	Primer position (bp)	Amplicon (bp)
à	plc (cpa)	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	663-968	324
ß	cpb	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	871-1045	196
ε	etx	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	267-862	655
ι	iap	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	1739-2161	446

The DNA extracted from all bacterial strains was identified by biochemical tests and standard strains. The quality of the extracted DNA was examined through 1% agarose gel and DNA fragments had good quality. PCR was carried out with four sets of specific primers that were complementary to a fragment of α, β, ε and ι encoding genes of *C. perfringens*. Primer annealing of complementary genes and synthesis of the fragment of interest revealed a specified toxin gene. Thus, various types were identified in accordance with their toxins. The toxin encoding gene was amplified by means of single PCR, the fragment of 324 bp belonged to α toxin gene, shared by all the types. The 196 bp fragment, on the other hand, was from the ß toxin encoding gene, existing in types B and C. The 655 bp fragment was from the ε toxin encoding gene and is observable in type D. The ι toxin encoding gene found in type E generates the fragment of 446 bp (Figure 1). PCR results corresponding to positive and negative controls are displayed in Figure 2. Out of 30 *C. perfringens* types, isolated by biochemical tests, all (100%) were type A, 0 (0%) were type B, 0 (0%) were type C and 0 (0%) were type D (Figure 3).

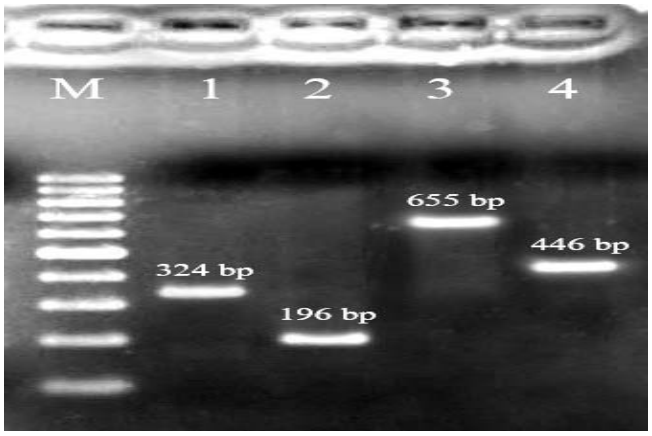

Figure 1 Detection of *C. perfringens* toxin genes amplified by single PCR M: marker (DNA ladder, 100 bp); Lane 1: a toxin encoding gene; Lane 2: β-toxin encoding gene; Lane 3: ε-toxin encoding gene and Lane 4: ι toxin encoding gene

The PCR technique is a powerful tool to detect and identify minimal numbers of microorganisms. A multiplex PCR, which can detect all *C. perfringens* major toxin genes, has been developed (Gurjar *et al*. 2008). Toxin typing of *C. perfringens* is important since particular toxin types are associated with specific enteric diseases in animals (van Asten *et al*. 2009). Traditionally, for classification of *C. perfringens*, serum neutralization tests on mice or guinea pigs are performed. However, as it was pointed (Gurjar *et al*. 2008) these tests are laborious, expensive, and no longer considered ethically acceptable. In some diagnostic laboratories, this differentiation has been replaced by rapid and easy to use enzyme-linked immunosorbent assays (ELISAs).

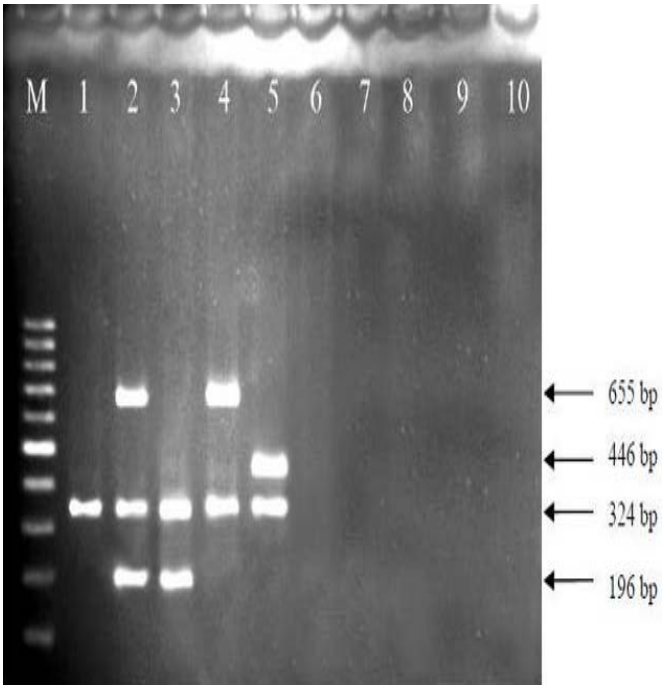

Figure 2 Standard strains, positive and negative controls. M: marker (DNA ladder, 100 bp); Lanes 1 to 4: positive controls, standard strain of *C. perfringens* types A, B, C and D; Lanes 5 to 9: negative controls, *C. septicum*, *C. sordellii*, *C. sporogenes*, *C. leptum* and *C. ramosum*

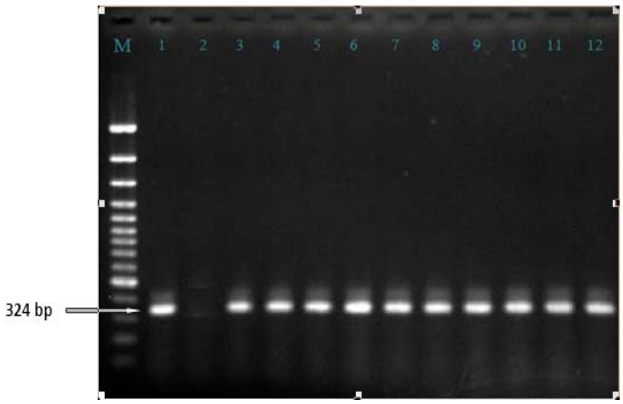

Figure 3 Detection of 324 bp fragement of alpha toxin genes of *perfringens* by PCR. Lane M: 100 bp marker DNA. Lane 1: positive control; Lane 2: negative control and Lanes 3 to 12: field isolates positive for alpha toxin genes

Although the ELISAs allow reliable typing of *C. perfringens* isolates, the options for subtyping are limited. For example, using ELISA is not possible to detect the beta toxin. In addition, high levels of enterotoxin are available only during sporulation .Furthermore, traditional methods are limited for subtyping. Thus, various PCR protocols including multiplex PCR assays have been established to genotype *C. perfringens* isolates (Babe *et al*. 2012). PCR typing is achieved by demonstrating the presence of the encoding gene(s) in the bacterialgenome (Van Asten *et al*. 2009) or in the plasmid since the epsilon toxin gene is thought to reside on a large plasmid (Songer, 2006). A number of the molecular tools allowing an easier *in vitro*

test and PCR method of typing *C. perfringens* have been developed (Yoo *et al*. 1997). Biological gauge comprises one of the important methods of identifying *C. perfringens*; however, it shows several disadvantages including the long time the test takes (24 to 72 hours) (Timoney *et al*. 1988). Another limitation consists of non-specific deaths that may lead to false diagnosis. Moreover, some *C. perfringens* strains are incapable of producing toxins in measurable amounts under laboratory conditions, which creates an obstacle for typing by classical methods (Kalender *et al*. 2005). Sensitivity and specificity are the two main characteristics of an efficient and practical technique, two qualities that PCR shows. Rapidity constitutes one of the major advantages of this method, so that bacterium identification and type determination lasts no more than four hours. Hence, the toxicogenic strain in the sample can be identified by means of a rapid evaluation by the PCR technique before it produces toxin.

In the current study, only *C. perfringens* type A was isolated from ostrich-dong samples, with type A being the most frequent in Kerman. Therefore, it is recommended that vaccination against enterotoxemia in this province should provide adequate immunity, especially against *C. perfringens* types A. Furthermore, enterotoxemia in ostrich is asymptomatic; thus, molecular Primers used in this study were perfectly specific, only complementary to *C. perfringens* encoding toxin gene. Up to our knowledge, no similar research has been conducted in Iran and the findings of this study disagree with those from different countries (Albini *et al*. 2008; Kalender *et al*. 2005; Wojdat *et al*. 2006; Yoo *et al*. 1997).

The difference comprises the fact that the existence and emergence of some bacterium types is closely related to geographic features, consequently several regions may totally lack one or more types of a certain bacterium. For instance, *C. perfringens* type A has been reported as most prevalent in North America, while types C, D and E are uncommon. Moreover, type A constitutes 97% of isolates in Belgium (Juneja *et al*. 2008). Although previous studies suggested that types A and E did not exist in Iran, our study detected and identified type A for the first time in Kerman, southeastern Iran (Ahsani *et al*. 2010a; Ahsani *et al*. 2010b; Ahsani *et al*. 2011). Primers used in this study were perfectly specific, only complementary to *C. perfringens* encoding toxin gene. In addition, PCR showed to be highly sensitive, so that the reaction was accomplished successfully with a small amount of DNA (less than 1 µL). In general, molecular methods provide a new insight into bacterial classification. They offer essential genetic information about the organism of interest, which comprises one of the most practical and helpful aspects of PCR. As PCR employs specific primers, an individual species or strain may be traced among several species or types of organisms (Ahsani *et al*. 2010b). Compared to other identification techniques that are based on large amounts of samples, PCR is safer for researchers; since sample preparation for PCR starts with cell lysis and DNA extraction. It is unnecessary to say that the cell cannot survive this stage and loses its pathogenic property (Ahsani *et al*. 2010b).

CONCLUSION

PCR has become an essential research and diagnostic tool, being a powerful technique with a vast and increasing range of applications. Prevention of clostridial diseases in ostriches is important in public health and food poisoning in humans. This study has been the first step for more and better identification of *clostridium* types in ostrich and we can produce specific *clostridium* vaccine and preventing from clostridial disease in this animal by identifying the important pathogen varieties in ostrich.

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REFERENCES

Ahsani M.R., Mohammadabadi M.R. and Shamsaldini M. (2010a). *Clostridium perfringens* isolate typing by multiplex PCR. *J. Venom. Anim. Toxin. Incl. Trop. Dis*. **16,** 573-578.

Ahsani M.R., Mohammadabadi M.R., Shamsaldini M., Ezatkhah M., Hasani M. and Esmailizadeh K.A. (2010b). Using triplex PCR for detecting of A, B and C types of *Clostridium perfringens*. *Iranian J. Anim. Sci. Res.* **2,** 184-189.

Ahsani M.R., Shamsadini M.B., Esmailizadeh A.K. and Mohammadabadi M.R. (2011). Genotyping of isolates of *Clostridium perfringens* from vaccinatedand unvaccinated sheep. *Small Rumin. Res.* **95,** 65-69.

Ahsani M.R. (2009). Molecular study of different native type of *Clostridium perfringens* in Kerman province: MS Thesis. Shahid Bahonar University of Kerman. Kerman, Iran.

Albini S., Brodard I., Jaussi A., Wollschlaeger N., Frey J. and Miserez R. (2008). Real-time multiplex PCR assays for reliable detection of *Clostridium perfringens* toxin genes in animal isolates. *Vet. Microbiol.* **127,** 179-185.

Augustynowicz E., Gzyl A. and Slusarczyk J. (2002). Detection of enterotoxigenic *Clostridium perfringens* with a duplex PCR. *J. Med. Microbiol.* **51,** 169-172.

Babe T., Jafari B. and Bahmanpour S. (2012). *Clostridium perfringens* isolated typing by ELISA in ostrich. *African J. Microbiol. Res.* **6,** 1766-1769.

Baez L.A. and Juneja V.K. (1995). Nonradioactive colony hybridization assay for detection and enumeration of enterotoxigenic *Clostridium perfringens* in raw beef. *Appl. Environ. Microbiol.* **61,** 807-810.

Baums C.G., Schotte U., Amtsberg G. and Goethe R. (2004). Diagnostic multiplex PCR for toxin genotyping of *Clostridium perfringens* isolates. *Vet. Microbiol.* **100,** 11-16.

Cruz W.P.D., Gozum M.M.A., Lineberry S.F., Stassen S.D., Daughtry M., Stassen N.A., Jones M.S. and Johnson O.L. (2006). Rapid detection of enterotoxigenic *Clostridium perfringens* by real-time fluorescence resonance energy transfer PCR. *J. Food Prot.* **69,** 1347-1353.

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.

Fach P. and Popoff M. (1997). Detection of enterotoxigenic *Clostridium perfringens* in food and fecal samples with a duplex PCR and the slide latex agglutination test. *Appl. Environ. Microbiol.* **63,** 4232-4236.

Garmory H.S., Chanter N., French N.P., Bueschel D., Songer J.G. and Titball R.W. (2000). Occurrence of *Clostridium perfringens* β-2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol. Infect.* **124,** 61-67.

Gkiourtzidis K., Frey J., Bourtzi-Hatzopoulou E., Iliadis N. and Sarris K. (2001). PCR detection and prevalence of alpha-, beta-, beta 2-, epsilon-, iota- and enterotoxin genes in *Clostridium perfringens* isolated from lambs with clostridial dysentery. *Vet. Microbiol.* **82,** 39-43.

Gokce H.I., Genc O., Sozmen M. and Gokce G. (2007). Determination of *Clostridium perfringens* toxin-types in sheep with suspected enterotoxemia in Kars Province, Turkey. *Turk J. Vet. Anim. Sci.* **31,** 355-360.

Greco G., Madio A., Buonavoglia D., Totaro M., Corrente M. and Martella V. (2005). *Clostridium perfringens* toxin-types in lambs and kids affected with gastroenteric pathologies in Italy. *Vet. J.* **170,** 346-350.

Gurjar A.A., Hegde N.V., Love B.C. and Jayarao B.M. (2008). Real-time multiplex PCR assay for rapid detection and toxin typing of *Clostridium perfringens* toxin producing strains in feces of dairy cattle. *Mol. Cell Prob.* **22,** 90-95.

Herholz C., Miserez R., Nicolet J., Frey J., Popoff M., Gibert M., Gerber H. and Straub R. (1999). Prevalence of beta 2-toxigenic *Clostridium perfringens* in horses with intestinal disorders. *J. Clin. Microbiol.* **37,** 358-361.

Juneja V.K., Marks H. and Thippareddi H. (2008). Predictive model for growth of *Clostridium perfringens* during cooling of cooked uncured beef. *Food Microbiol.* **25,** 42-55.

Kadra B., Guillou 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., Ertas H.B., Cetinkaya B., Muz A., Arslan N. and Kilic A. (2005). Typing of isolates of *Clostridium perfringens* from healthy and diseased sheep by multiplex PCR. *Vet. Med.* **50,** 439-442.

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.

Kim S., Labbe R.G. and Ryu S. (2000). Inhibitory Effects of Collagen on the PCR for Detection of *Clostridium perfringens*. *Appl. Environ. Microbiol.* **66,** 1213-1215.

Kokai-Kun J., Songer J., Czeczulin J., Chen F. and McClane B. (1994). Comparison of western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. *J. Clin. Microbiol.* **32,** 2533-2539.

Komoriya T., Hashimoto A., Shinozaki A., Inoue M. and Kohno H. (2007). Study on partial purification of α-toxin produced from obligate anaerobe *Clostridium perfringens*. Research Institute of Industrial Technology, Nihon University. **88,** 1-11.

MacFaddin J.F. (2000). Biochemical Tests for Identification of Medical Bacteria. Baltimore: Lippincott Williams and Wilkins.

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

Miwa N., Nishina T., Kubo S. and Atsumi M. (1997). Most probable number method combined with nested polymerase chain reaction for detection and enumeration of enterotoxigenic *Clostridium perfringens* in intestinal contents of cattle, pig and chicken. *J. Vet. Med. Sci.* **59,** 89-92.

Miwa N., Nis hina T., Kubo S. and Fujikura K. (1996). Nested polymerase chain reaction for detection of low levels of enterotoxigenic *Clostridium perfringens* in animal feces and meat. *J. Vet. Med. Sci.* **58,** 197-203.

Miyashiro S., Nassar A.F.C., Del Fava C., Cabral A.D. and Silva M. (2007). *Clostridium perfringens* types A and D associated with enterotoxemia in an 18-month-old goat. *J. Venom. Anim. Toxin. Incl. Trop. Dis.* **13,** 885-893.

Piatti M., Ikuno A.A. and Baldassi L. (2004). Detection of bovine *Clostridium perfringens* by polymerase chain reaction. *J. Venom. Anim. Toxin. Incl. Trop. Dis.* **10,** 154-160.

Ridell J., Björkroth J., Eisgruber 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.

Saito M., Matsumoto M. and Funabashi M. (1992). Detection of *Clostridium perfringens* enterotoxin gene by the polymerase chain reaction amplification procedure. *Int. J. Food Microbiol.* **17,** 47-55.

Sambrook J. and Russell D.W. (2002). Molecular Cloning a Laboratory Manual. Woodbury: Cold Spring Harbor Laboratory Press.

Sawires Y.S. and Songer J.G. (2006). *Clostridium perfringens*: insight into virulence evolution and population structure. *Anaerobe*. **12,** 23-43.

Settanni L. and Corsetti A. (2007). The use of multiplex PCR to detect and differentiate food and beverage-associated microorganisms: a review. *J. Microbiol. Method.* **69,** 1-22.

Songer J.G. (2006). Bovine enteritis and enterotoxemia. Pp 43-80 in Genus Clostridium Clostridia in Medical, Veterinary and Food Microbiology: Diagnosis and Typing. J. Mainil, Ed. Luxembourg: European Commission / EU Concerted Action.

Songer J.G. (1996). Clostridial enteric diseases of domestic anim-

als. *Clin. Microbial. Rev.* **9,** 216-234.

Songer J. and Meer R. (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.

Tansuphasiri U. (2001). Development of duplex PCR assay for rapid detection of enterotoxigenic isolates of *Clostridium perfringens*. *Southeast Asian J. Trop. Med. Public Health*. **32,** 105-113.

Tansuphasiri U., Wongsuvan G. and Eampokalap B. (2002). PCR detection and prevalence of enterotoxin (*cpe*) gene in *Clostridium perfringens* isolated from diarrhea patients. *J. Med. Assoc. Thailand.* **85,** 624-633.

Timoney J.F., Gillespie J.H., Scott F.W. and Barlough J.E. (1988). Hagan and Bruner's Microbiology and Infectious Diseases of Domestic Animals. Ithaca: Comstock Publishing Associates.

Van Asten A.J.A.M., van der Wiel C.W., Nikolaou G., Houwers D.J. and Grone A.A. (2009). Multiplex PCR for toxin typing of *Clostridium perfringens*. *Vet. Microbiol.* **136,** 411-412.

Wojdat E., Kwiatek K. and Kozak M. (2006). Occurrence and characterization of some Clostridium species isolated from animal feeding stuffs. *Bull Vet. Inst. Pulawy.* **50,** 63-67.

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.