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PCR-Based Detection and Molecular Genotyping of Enterotoxigenic *Clostridium perfringens* Isolates from Swine Diarrhea in Korea

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Abstract *Clostridium perfringens* strains were isolated from swine diarrhea in Korea. Three out of nineteen (15.8%) isolates of *C. perfringens* were found to be enterotoxigenic by PCR analysis. PCR-based genotyping of the three enterotoxigenic isolates of *C. perfringens* revealed that they were types A, C and D, respectively. These results suggest that various types of enterotoxigenic *C. perfringens* can cause swine diarrhea, and that the presence of enterotoxigenic type A strain, known to be strongly associated with food poisoning, may cause public health problem in Korea.

Key words: *Clostridium perfringens*, swine diarrhea, enterotoxin, PCR, genotyping, food poisoning

Clostridium perfringens is a spore-forming, rod-shaped, Gram-positive anaerobe commonly found in the intestine of humans and mammals, and is responsible for a wide range of diseases such as gas gangrene and necrotic enteritis in animals and humans [17, 24]. The virulence of this organism is associated with the production of several toxins (exotoxins and enterotoxins). Among them, four of the lethal toxins, including alpha (α), beta (β), epsilon (ε), and iota (ι), are considered to be the major toxins. *C. perfringens* is classified into five toxigenic types (types A to E) according to the major toxins produced by each subtype: Bacteria from type A produce only α-toxin, those from type B produce α-, β-, and ε-toxins, those from type C secrete α- and β-toxins, those from type D produce α- and ε-toxins, and bacteria from type E secrete α- and ι-toxins [23].

C. perfringens type A food poisoning has been known as one of the most common causes of foodborne disease in the United States and Europe. Usual symptoms of food poisoning by *C. perfringens* are diarrhea and severe abdominal pains. Although death rates from *C. perfringens* type A food poisoning are low, death is more prevalent in debilitated or institutionalized individuals, particularly the elderly [17]. Enterotoxin is the causative agent of *C. perfringens* type A food poisoning. Most of *C. perfringens* strains are non-enterotoxigenic strains, and surveys suggest that only about 6% of all *C. perfringens* isolates carry the gene encoding *C. perfringens* enterotoxin (CPE) [3, 11, 15, 33, 34]. It is necessary to distinguish the enterotoxigenic organisms from the non-enterotoxigenic ones to confirm food poisoning by *C. perfringens* [17]. Food poisoning by *C. perfringens* is strongly associated with enterotoxigenic *C. perfringens* type A, even though some type C and type D isolates can also express an enterotoxin [21]. CPE is a 36-kDa single polypeptide composed of 309 amino acids [28] and is produced in a large amount (20 to 30% of total protein), but only during sporulation of enterotoxigenic *C. perfringens* [5, 7, 16, 21, 24, 29]. *C. perfringens* can form large numbers of spores in some meat products, and enterotoxin formation in meat and poultry has also been shown to occur [2]. Although it is not considered as a major toxin in the classical sense, CPE is the principal toxin involved in foodborne illness caused by *C. perfringens*, and is considered by many to be a virulence attribute in animal strains of the organism [17, 21].

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Classically, classification (types A to E) of *C. perfringens* has been performed by two different methods: the seroneutralization of lethality by intravenous injection in mice and the seroneutralization of the dermonecrotic effect in guinea pigs [30, 31]. However, owing to their often-questionable sensitivity, specificity, and expense (a large amount of active toxin, specific neutralizing antiserum for each toxin, and a lot of laboratory animals), dependence on these *in vivo* methods of toxin detection has been a stumbling block to routine diagnosis of *C. perfringens*. Alternative *in vitro* methods, such as molecular approaches to identification of toxin genes and immunoassays for detection of toxins, provide simple, rapid, and accurate tests for diagnosis and typing of isolates [8, 13, 18]. Nucleic acid probes and polymerase chain reaction (PCR) assays have been reported as very useful methods for detection of *C. perfringens*. Specifically, PCR has been utilized for toxin typing as well as the detection of specific genes of *C. perfringens*. It is extremely sensitive and allows the detection of minimal quantities of target DNA, and an *in vitro* toxin typing method based on PCR has been developed to determine *C. perfringens* toxin types [1, 4, 6, 25].

Detection of Enterotoxigenic *C. perfringens* Strains from Swine Diarrhea

Fresh fecal samples were collected from piglets from the same farm (aged from 25 to 35 days) having clinical signs of postweaning diarrhea, and placed in a prereduced sterile transport medium [19]. The fecal samples were diluted serially from 10^{-1} to 10^{-7} with diluent A (KH_2PO_4 0.45 g, Na_2HPO_4 0.6 g, L-cysteine 0.05 g, Tween 80 0.05 g, agar 0.1 g per 100 ml) and 0.05 ml of aliquots was spread onto Neomycin-Nagler (NN) medium (peptone 4 g, Na_2HPO_4 0.5 g, KH_2PO_4 0.1 g, NaCl 0.2 g, $MgSO_4$ 0.01 g, glucose 0.2 g, 5% egg yolk, neomycin 20 mg, agar 2.5 g per 100 ml). The agar plate was incubated at 37°C for 48 h in an anaerobic "steel-wool" jar filled with oxygen-free CO_2 gas [26]. After incubation, colonies with a white precipitation ring, lecithinase-positive reaction [12, 19], were regarded as those of *C. perfringens*.

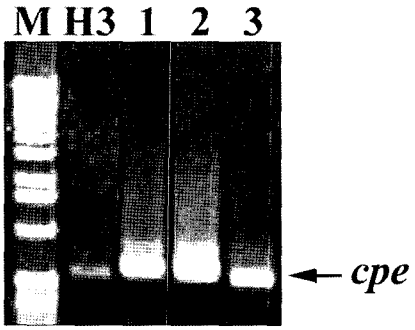

Fig. 1. Detection of enterotoxigenic *C. pefringens* strains from swine diarrhea by PCR. PCR bands representing the *cpe* gene were detected in three strains (lanes 1, 2, and 3) among nineteen *C. perfringens* isolates. Lanes M and H3 indicate molecular size marker (1-kb DNA ladder) and *cpe* band from enterotoxigenic strain (H3) used as a control, respectively. The arrow indicates the position of the PCR product of the *cpe* gene (599 bp).

Nineteen *C. perfringens* strains were isolated and maintained in the cooked meat medium (Difco Laboratories, Detroit, MI, U.S.A.). The isolates were analyzed by PCR for the presence of the enterotoxin gene (*cpe*) using *C. perfringens* type A strain NCTC 8239 [Hobb's serotype 3 (H3)] carrying *cpe* as a control strain [14]. GeneReleaser kit (Bio Ventures, Inc., Murfreesboro, TN) was used for rapid and easier extraction of genomic DNA. The primers used in the PCR were designed based on the published sequences of the *cpe*, as shown in Table 1 [3]. PCR amplification reactions were performed in a GeneAmp PCR System 9600 (Perkin-Elmer, U.S.A.). Amplification of DNA was performed in a total volume of 50 μl with 30 amplification cycles of a three-step PCR (94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min).

Three strains among nineteen isolates were found to be enterotoxigenic *C. perfringens* (Fig. 1). The ratio of three enterotoxigenic strains among nineteen isolates was 15.8%, and the ratio was nearly three times higher than the previous reports that about 6% of *C. perfringens* strains in nature carry the *cpe* gene encoding enterotoxin [15, 33]. The particularly high prevalence of enterotoxigenic *C. perfringens* strains suggests the possibility that *C.*

Table 1. Sequences of the primers used for PCR in this study

Toxin/gene	Primer sequence (5' to 3')	Product length (bp)
Enterotoxin/*cpe*	ACTTAGAGTATCTATAAACTTGATACTC TAAATTGTTACTAAGCATATTATAATTAACATC	599
α-Toxin/*cpa*	GTTAGCATGCTGTTTTCTAAGTTAAAACC TCCCCTTTCTAGATAACGATTAACAC	343
β-Toxin/*cpb*	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	197
ε-Toxin/*etx*	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	656
ι-Toxin/*iap*	TTTTAACTAGTTCATTTCCTAGTTA TTTTTGTATTCTTTTTCTCTAGGATT	299

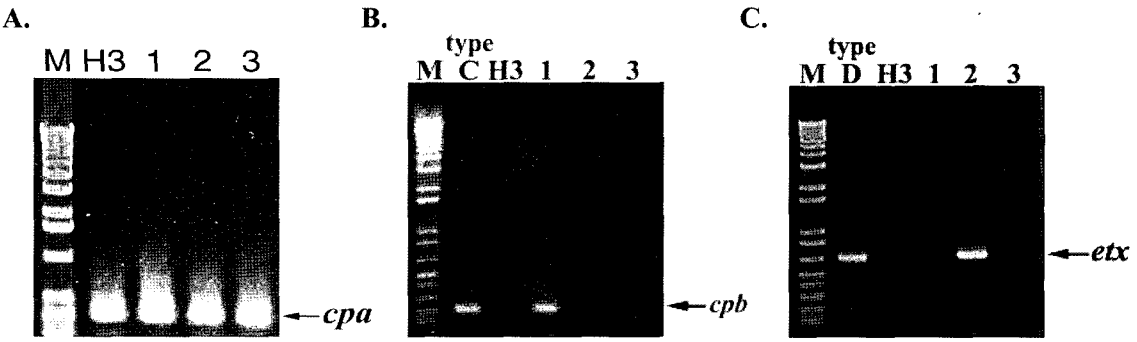

Fig. 2. Amplification of major toxin genes for genotyping of the three enterotoxigenic *C. perfringens* isolates from swine diarrhea. A. Amplification of α-toxin gene (343 bp of *plc* gene). B. β-Toxin gene (197 bp of *cpb* gene). C. ε-Toxin gene (656 bp of *etx* gene). Lanes 1 to 3 indicate the three enterotoxigenic *C. perfringens* isolates. Lanes M and H3 indicate molecular size marker (1 kb DNA ladder) and enterotoxigenic type A strain (H3) used as a control, respectively. The two lanes named type C and type D indicate β- and ε-toxin genes from *C. perfringens* type C and type D strains, respectively.

perfringens enterotoxin can be a causative agent for swine diarrhea, and therefore, the incidence of food poisoning by *C. perfringens* can be high.

Typing of Three Enterotoxigenic Isolates by PCR

For rapid and convenient classification of toxin types of three enterotoxigenic isolates carrying the *cpe* gene, PCR-based toxin typing was done with four primer sets specifically designed to detect four major toxin (α-, β-, ε-, ι-toxin) genes (Table 1) [7, 9, 10, 27, 32]. Three isolates were classified into types A, C, and D. The α-toxin gene was detected in all isolates tested (Fig. 2A). However, the β-toxin gene and ε-toxin gene were detected only in the first and second isolates, respectively (Figs. 2B and 2C). The ι-toxin gene was not detected in all three isolates (data not shown). These results suggest that the two isolates are type C and type D, respectively, and the other one is type A strain expressing only α-toxin.

The pathogenicity of *C. perfringens* is associated with the production of major lethal toxins, and each *C. perfringens* strain produces its own set of toxin related to a particular pathology. The β-toxin produced by type C plays a major role in necrotic enteritis, and the ε-toxin produced by type D is involved in enterotoxemia of livestock [20, 21]. These toxins together with enterotoxin influence swine diarrhea by *C. perfringens*. It is interesting to note that we detected enterotoxigenic *C. perfringens* type C and type D strains, even though Yoo *et al.* [34] have reported that type A of *C. perfringens* was the most prevalent type in livestock of Korea. It is not clearly understood why only type A isolates are strongly associated with *C. perfringens* food poisoning. One possible explanation is that the enterotoxigenic type A isolates are more common than other types of enterotoxigenic *C. perfringens* present in environments that are conducive to food poisoning [22]. Because some cases of *C. perfringens* type A food poisoning may be caused by ingesting meats contaminated with *C. perfringens* carrying the *cpe* gene, it is important from a food safety point of view to detect in the livestock, such as swine, enterotoxigenic *C. perfringens* strains of various types, which are capable of causing food poisoning. The role of enterotoxigenic type C and D isolates of *C. perfringens* in food poisoning needs to be further studied in order to improve our understanding of the mechanism of food poisoning by *C. perfringens*.

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