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Molecular Typing and Epidemiological Survey of Prevalence of *Clostridium perfringens* Types by Multiplex PCR

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Clostridium perfringens **has been classified into five toxigenic types (A through E) on the basis of its capability to produce major lethal toxins (alpha, beta, epsilon, and iota toxins). Seroneutralization with mice or guinea pigs has been used to type each toxin, but this conventional method has some disadvantages. Therefore, we used a molecular biological technique to type the bacterium in the present study. A multiplex PCR was developed for this purpose. This method has several advantages in comparison with seroneutralization with mice or guinea pigs. By this method, we also investigated the most prevalent type(s) of the organism in Korean calves, piglets, and chickens showing clinical symptoms such as diarrhea, enterotoxemia, and necrotic enteritis. Only type A was isolated from calves and chickens, while type C (2 of 14 isolates), in addition to type A, was isolated from piglets. These results suggested that seroneutralization could be replaced by our new method and that type A of *C. perfringens* is the most prevalent type in livestock in Korea.**

Since *Clostridium perfringens* was first described as *Bacillus aerogenes capsulatus* in 1892 (46), the bacterium has been identified as an anaerobe responsible for a wide range of diseases in humans and animals (31). The pathogenicity of the organism is associated with several toxins. The alpha, beta, epsilon, and iota toxins are the major lethal toxins produced by the organism and are closely related to its virulence, even though they produce several minor extracellular toxins (12). Usually, *C. perfringens* has been classified into five toxigenic types (A through E) on the basis of its ability to produce the major lethal toxins (5, 12).

Alpha toxin is commonly produced by all five types and is a phospholipase C that can hydrolyze lecithin into phosphorylcholine and diglyceride and is believed to be a major factor responsible for the organism's tissue pathology (2, 19, 20). This is the predominant product of *C. perfringens* type A. Therefore, type A exhibits several powerful toxicities, and infection with type A may result in myonecrosis, hemolysis, an increase in vascular permeability, and platelet aggregation (10, 12, 20, 33, 43). The major lethal effects associated with this toxin are gas gangrene in humans and necrotic enteritis and enterotoxemia in animals (10, 12).

Beta toxin is a major lethal toxin produced by type B and C strains of *C. perfringens* and is a single-chain polypeptide of approximately 40 kDa which is highly sensitive to trypsin (14). The beta toxin is known to play a major role in the pathogenesis of necrotic enteritis in humans and animals (23, 25, 47). In humans, the disease has been termed pig-bel which is caused by *C. perfringens* type C infection and which shows clinical signs of vomiting, abdominal pain, and bloody diarrhea (18, 21, 23). Also, necrotic enteritis associated with toxigenic *C. perfringens* has been reported in calves, lambs, and piglets (21, 32). In experimental infection and clinical studies (22, 27), the presence of trypsin or protease inhibitors in the gut has been shown to be the most important cofactor in beta toxin-induced necrotic enteritis. However, it remains unclear whether the presence of dietary trypsin inhibitors is a prerequisite for the disease. Also, type B of this organism was identified as a causative agent of enterotoxemia or necrotic enteritis in foals, lambs, sheep, and goats (14, 16, 32, 42).

Epsilon toxin is produced by types B and D of *C. perfringens* and is responsible for a rapid fatal enterotoxemia in economically important livestock (12, 15). This toxin is secreted as a relatively inactive protoxin and is activated to a potent heat-labile toxin with the loss of an N-terminal peptide by a proteolytic enzyme produced by the organism (4). The activated protein is highly toxic and can have lethal, dermonecrotic, and edematic activities (3, 15). The effect on the brain is the most critical since the effect can induce death by cerebral edema and necrosis of brain tissue.

Iota toxin is produced only by type E of this bacterium and is also produced as a protoxin (12, 41). The protoxin can permeate the vascular wall as a result of proteolytic activation (9, 36, 38, 41). This toxin consists of two independent polypeptides: Ia, which is an ADP transferase, and Ib, which is involved in the binding and internalization of this toxin into the cell (36, 41). Although they can be distinguished immunologically and biochemically, their activities assist each other to produce toxicity such as dermonecrosis in mice (22, 38, 41). This toxin has also been implicated in calf and lamb enterotoxemia (12).

Seroneutralization with mice or guinea pigs has been performed to type *C. perfringens* (11, 13, 40) since it is almost impossible to classify the isolates into toxigenic types by colony morphology, biochemical properties, and analyses of fatty acid and organic acid end products of metabolism by gas-liquid chromatography (5). However, the method has some disadvantages since it is time-consuming, requires antitoxin antibodies, etc.

PCR has been applied in several areas since the late 1980s. This method has been highlighted as a rapid and accurate method for the detection of low copy numbers of genes. Also, the sensitivity and specificity of this method were confirmed by amplification of specific target DNA under a unique conditions. This method is more accurate and faster than seroneutralization with mice or guinea pigs (10). Therefore, we developed a specific method with primers to type *C. perfringens* by

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multiplex PCR. Using this method, we also typed field isolates from calves, piglets, and chickens with critical symptoms in Korea. These results indicated that the PCR method can be used to type *C. perfringens* isolates and the prevalent types of the organism existing in Korea.

MATERIALS AND METHODS

Bacterial strains. *C. perfringens* type A, B, C, D, and E reference strains were used to set up unique conditions for the multiplex PCR. Reference strains of the bacteria were obtained from the National Animal Disease Center (Ames, Iowa) and were kept in our laboratory. In order to confirm the specificity of our PCR conditions, *Clostridium chauvoei*, *Clostridium tetani*, and *Clostridium septicum*, obtained from the National Animal Disease Center, and enteric bacteria including *Salmonella typhimurium* isolated in our laboratory were also used as negative controls. Fifty-one isolates of *C. perfringens* from calves, piglets, and chickens with clinical symptoms were also used to investigate the prevalent toxin type(s) in Korea.

Identification of *C. perfringens*. Samples were smeared onto blood agar plates containing 5% defibrinated sheep blood, and the plates were incubated anaerobically at 37°C for 24 h. To compare the PCR results with biochemical identification results, colonies showing characteristic dual hemolytic zones were picked up and identified by biochemical tests as described in Bergey's manual (5).

Isolation of total DNA. In order to develop unique PCR conditions, *C. perfringens* type A, B, C, D, and E reference strains were cultured in cooked meat medium (Difco, Detroit, Mich.), and total DNA was purified as described by Murray and Thompson (26). Briefly, the reference strains were cultured in cooked meat medium overnight. The bacteria were harvested by centrifugation at 6,000 × *g* for 10 min. The harvested bacteria were resuspended in 567 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and the solution was incubated at 37°C for 1 h after the addition of 30 μl of 10% sodium dodecyl sulfate and 3 μl of proteinase K (20 mg/ml in distilled water). After incubation of the reaction mixture with 100 μl of 5 M NaCl and 80 μl of CTAB-NaCl solution (10% cetyl trimethyl ammonium bromide in 0.7 M NaCl) at 65°C for 10 min, the solution was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) solution and DNA was precipitated with absolute ethanol by incubation at −20°C overnight. The DNA was harvested by centrifugation at 12,000 × *g* for 20 min, washed with 70% ethanol, and resuspended in 100 μl of TE buffer after it was vacuum dried. RNA was removed by incubation of the solution with RNase (1 μg/ml) at 37°C for 1 h. The concentration of the DNA was measured at 260 nm with a spectrophotometer (Pharmacia Biotech., Piscataway, N.J.). The DNA solution was kept at −20°C until use. Those DNAs were used to investigate the sensitivity and specificity of this PCR system. Total DNA from field isolates of *C. perfringens* was purified by the boiling method (34). Briefly, four to five colonies of *C. perfringens* grown on a blood agar plate were suspended in 0.5 ml of distilled water and the mixture was boiled for 10 min. The pellets were removed by centrifugation at 12,000 × *g* for 10 min, and the supernatant was used as template DNA in the PCR.

Primers. Specific primers corresponding to each toxin were designed by using the sequence data obtained from GenBank (National Institutes of Health) and were synthesized with a DNA synthesizer (Expedite 8905; Perseptive Co.) (Table 1).

PCR. The PCRs were performed in a Pharamacia-LKB Gene ATAQ controller (Pharmacia Biotech.). The PCR mixture contained 5 μl of 10× PCR buffer (Gibco/BRL, Grand Island, N.Y.), 4 μl of 25 mM $MgCl_2$, 1 μl of 10 mM deoxynucleoside triphosphate mixture, 100 pmol of primers, 100 ng of template DNA, 1 μl of *Taq* polymerase (Gibco/BRL), and up to 50 μl of distilled water. The following program was used in this experiment: 5 min at 94°C, followed by 30 cycles consisting of 1 min at 55°C, 1 min at 72°C, and 1 min at 94°C. Ten microliters of the amplified products was then analyzed by electrophoresis in a 1.5% agarose gel.

Cloning and sequencing of PCR products. The amplified PCR products were cloned by using a TA cloning kit (Invitrogen Co., San Diego, Calif.). The cloned products were sequenced by Sanger's dideoxynucleotide chain termination method with the Sequenase, version 2.0, kit (United States Biochemical Co., Cleveland, Ohio). The cloning and sequencing procedures were performed as described by the manufacturer. The identities of the products were confirmed by comparison of the sequence with previous reports obtained from GenBank.

Typing of field isolates. Samples of stools or intestinal contents from calves, piglets, and chickens with diarrhea or showing necrotic enteritis at autopsy were plated onto blood agar, and the plates were incubated anaerobically overnight at 37°C. Typical colonies showing dual hemolysis were picked up and subcultured. The subcultured colonies were identified by Gram staining, urease test, lecithinase test, reverse CAMP (Christie Atkins and Munch-Peterson) test (48), and other biochemical tests described in Bergey's manual (5). Isolation of total DNA and PCR were performed as described above.

TABLE 1. Nucleotide sequences of primers used in this study

Primer (direction)	Nucleotide sequence	Location	Size (bp) of amplified products
CPA (alpha toxin)			
Forward	5′-GTTGATAGCGCAGGACATGTTAAG-3′	511–535	402
Reverse	5′-CATGTAGTCATCTGTTCCAGCATC-3′	913–889	
CPB (beta toxin)			
Forward	5′-ACTATACAGACAGATCATTCAACC-3′	589–613	236
Reverse	5′-TTAGGAGCAGTTAGAACTACAGAC-3′	824–801	
CPE (epsilon toxin)			
Forward	5′-ACTGCAACTACTACTCATACTGTG-3′	436–459	541
Reverse	5′-CTGGTGCCTTAATAGAAAGACTCC-3′	976–953	
CPI (iota toxin)			
Forward	5′-GCGATGAAAAGCCTACACCACTAC-3′	563–586	317
Reverse	5′-GGTATATCCTCCACGCATATAGTC-3′	879–856	

RESULTS

From the stool or intestinal contents of animals with diarrhea or showing necrotic enteritis, 51 field isolates of *C. perfringens* were isolated and identified by biochemical tests as described in Bergey's manual (5). The characteristics of the isolates were positive fermentation of glucose, lactose, sucrose, and maltose; hydrolysis of gelatin; production of lecithinase; and a positive reverse CAMP test result. Also, some other biochemical properties of the isolates were identical to those of the reference strains. To develop the multiplex PCR, five reference strains of each toxin type were selected. After growth at 37°C in cooked meat medium, total DNA was isolated by the method described in Materials and Methods. Then, a PCR was performed with the primer pairs listed in Table 1. Toxin genes were amplified with a single pair of primers (Fig. 1) or a specific combination of multiplex pairs of primers based on the toxin types of the reference strains defined by the seroneutralization test with mice (6, 7, 48) (Fig. 2). As shown in Fig. 2, the alpha toxin gene from all types, the beta toxin gene from types B and C, the epsilon toxin gene from types B and D, and the iota toxin gene from type E were amplified by multiplex PCR with the primer pairs listed in Table 1 under unique conditions. The specificities of the PCRs were confirmed by specific amplification of the toxin genes from only *C. perfringens* (Fig. 3). The PCR could detect up to 10 pg of template DNA in each toxin gene (Fig. 4). In a comparison of DNA extraction methods, there was no difference in the PCR results, regardless of the culture method. The amplified PCR products were confirmed by analyses of nucleotide sequences and comparison of the sequences with previously reported sequences. In the application of the PCR to field isolates from piglets, calves, and chickens in Korea (Table 2), only the alpha toxin gene was amplified from all isolates from calves and chicken. The results suggested that toxin type A of *C. perfringens* was the most prevalent causative agent in samples collected from calves and chickens in Korea. However, the alpha and beta toxin genes were amplified from 2 of 14 isolates from piglets, while only the alpha toxin gene was amplified from the rest of the isolates. The results for isolates from piglets indicated that type C of *C.*

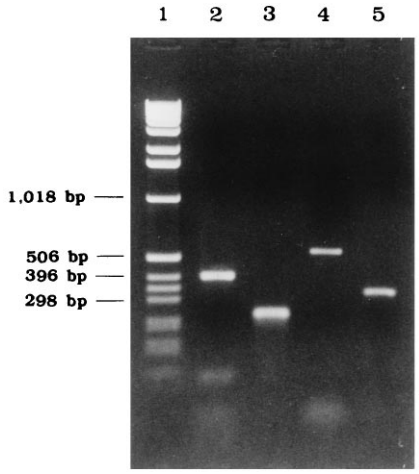

FIG. 1. Detection of *C. perfringens* toxin genes amplified by a PCR. *C. perfringens* reference strains were cultured in cooked meat medium, and genomic DNA of the bacterium was purified as described in Materials and Methods. Genomic DNAs from *C. perfringens* types A, B, and C were used as template DNA. Each toxin gene was amplified with each primer set listed in Table 1 by PCR under the condition described in Materials and Methods. The amplified products were detected by electrophoresis on a 1.5% agarose gel. Lane 1, DNA size marker (1-kb ladder); lane 2, alpha toxin gene; lane 3, beta toxin gene; lane 4, epsilon toxin gene; lane 5, iota toxin gene.

perfringens could be one of the important types of this bacterium in Korea.

DISCUSSION

The pathogenicity of *C. perfringens* is closely related to the production of major lethal toxins (alpha, beta, epsilon, and iota toxins) and other toxins, including enterotoxin (12, 42). The patterns of production of the toxins are different, depending on the *C. perfringens* type. Therefore, the patterns have been used to type the bacterium into types A, B, C, D, and E. There were geographical differences in the prevalent types of the bacterium. Also, the type could be different depending on the animals species in the area. For example, enterotoxemia caused by types D and E of the organism has never been reported in Korea, since sheep and goats are not popular livestock for breeding in Korea, while types A and C of *C. perfringens*, which cause enterotoxemia and necrotic enteritis in calves and piglets, respectively, were reported to be prevalent types in Korea (6, 7, 48). Toxigenic typing of the organism has been done by seroneutralization with mice and antitoxin antibodies. The presence of toxin was detected by several methods such as

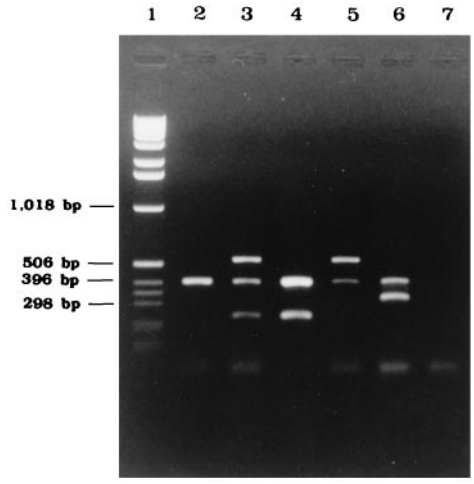

FIG. 2. Electrophoretic analysis of *C. perfringens* toxin genes amplified by a multiplex PCR. *C. perfringens* reference strains were cultured in cooked meat medium, and genomic DNA from the bacterium was purified as described in Materials and Methods. All toxin genes in *C. perfringens* reference strains (types A through E) were amplified with all primers listed in Table 1 under the conditions described in Materials and Methods. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel. Lane 1, DNA size marker (1-kb ladder); lane 2, *C. perfringens* type A (alpha toxin); lane 3, *C. perfringens* type B (alpha, beta, and epsilon toxins); lane 4, *C. perfringens* type C (alpha and beta toxins); lane 5, *C. perfringens* type D (alpha and epsilon toxins); lane 6, *C. perfringens* type E (alpha and iota toxins); lane 7, negative control (no template DNA).

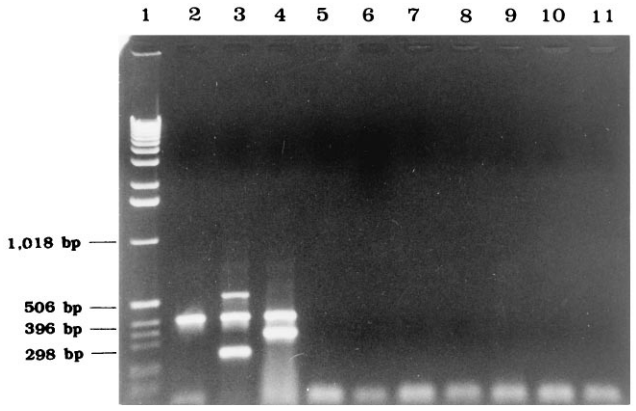

FIG. 3. Specific amplification of *C. perfringens* toxin genes by the multiplex PCR. Chromosomal DNA was isolated from the bacteria listed in Materials and Methods, and multiplex PCR was performed with template DNA under the conditions described in Materials and Methods. Amplified PCR products were analyzed by electrophoresis on 1.5% agarose gel. Lane 1, DNA size marker (1-kb ladder); lane 2, *C. perfringens* type A (alpha toxin); lane 3, *C. perfringens* type B (alpha, beta, and epsilon toxins); lane 4, *C. perfringens* type E (alpha and iota toxins); lane 5, *C. chauvoei*; lane 6, *C. septicum*; lane 7, *C. tetani*; lane 8, *Escherichia coli* (heat-labile toxin positive); lane 9, *Salmonella typhimurium*; lane 10, *Yersinia enterocolitica*; lane 11, *Proteus vulgaris*.

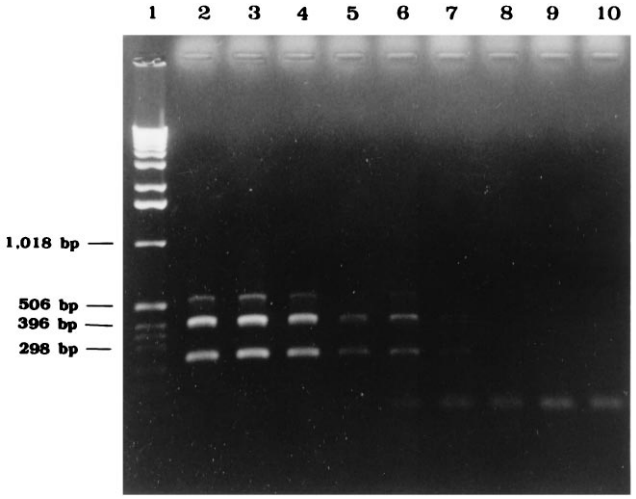

FIG. 4. Sensitivity of the multiplex PCR for the detection of *C. perfringens* toxin genes. Chromosomal DNA was isolated from *C. perfringens* type B, and the DNA was diluted 10-fold. The diluted DNA was used as template DNA. The PCR was performed with the primers listed in Table 1 as described in Materials and Methods. Lane 1, DNA size marker (1-kb ladder); lanes 2 to 10, 10-fold serial dilutions of chromosomal DNA from 1 μg to 10 fg.

TABLE 2. Toxigenic types of *C. perfringens* isolates from calves, piglets, and chickens in Korea

Species	No. of isolates	No. of isolates of the following toxin type:					Clinical symptoms
		A	B	C	D	E	
Calves	28	28					Enterotoxemia
Piglets	14	12		2			Enterotoxemia or necrotic enteritis
Chickens	9	9					Necrotic enteritis

seroneutralization with mice and an enzyme-linked immunosorbent assay (28, 29). However, development of molecular biological tools allowed for an easier in vitro test, and the PCR method of typing *C. perfringens* was developed by Daube et al. (10). The enterotoxin gene instead of the iota toxin gene was amplified along with other major lethal toxin genes (alpha, beta, and epsilon toxin genes) by a multiplex PCR in their experiment. Therefore, it was not enough to complete typing of the bacterium by their method since the iota toxin is one of the important toxins for typing *C. perfringens* isolates. To develop a new typing method that uses multiplex PCR, specific primers were designed for each toxin gene by using nucleotide sequences of the toxin genes obtained from GenBank, and each toxin gene was amplified in reference strains in this study (Fig. 1). Also, all toxin genes were simultaneously amplified by a multiplex PCR method with combinations of the primers (Fig. 2). The specificity of the PCR method was confirmed by detection of only toxin genes from *C. perfringens* without nonspecific amplification of several clostridia and enteric bacteria (Fig. 3). The identity of the amplified PCR products was confirmed by sequencing and by comparison of the sequence data obtained from GenBank. These results suggested that the PCR instead of seroneutralization could be used to type the bacterium. Also, the PCR method has several performance advantages for typing the organisms. Types A and C of *C. perfringens* were reported to be the most prevalent types in calves and piglets, respectively, in Korea when prevalence had been tested by seroneutralization with mice (6, 7, 48). The *C. perfringens* types prevalent in calves and chicken were similar to those found in other studies (1, 6, 30, 39, 45). *C. perfringens* type C infection in pigs has been reported as a cause of necrotic enteritis worldwide (7, 17, 24, 44, 48). However, type A was the most prevalent type in piglets with diarrhea, and in this experiment, only 2 of 14 isolates (14.3%) were *C. perfringens* type C. Recently, an association of *C. perfringens* type A has been reported in suckling and feeder pigs with mild symptoms (8, 35, 37). So, the difference in the prevalence of a particular type in Korea might be due to changes in the disease pattern in piglets. In conclusion, our multiplex PCR method could be used as a new method for typing *C. perfringens*. Furthermore, epidemiological studies of the prevalent types of *C. perfringens* in Korea are possible by this technique, and it will be of help in developing new measures for preventing this bacterial infection in animals.

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