

Distribution of *Clostridium perfringens* Isolates from Piglets in South Korea

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ABSTRACT. *Clostridium perfringens* causes various digestive system disease symptoms in pigs. In the present study, 11 *C. perfringens* isolates were obtained from diarrheic piglets and 18 from healthy piglets. All of the *C. perfringens* isolates were shown to be type A using a multiplex PCR assay. The $\beta 2$ toxin gene was detected in 27/29 *C. perfringens* isolates, i.e., 81% (9/11) of diarrheic piglets and 100% (18/18) of healthy piglets, and all of the genes had the same sequence. In conclusion, the $\beta 2$ toxin gene of *C. perfringens* was distributed widely in Korean piglets regardless of the incidence of diarrhea, and there was no clear relationship with enteric disease. A pulsed-field gel electrophoresis analysis of DNA digested using *Sma*I demonstrated the non-clonal spread of *C. perfringens* isolates from piglets.

KEY WORDS: *Clostridium perfringens*, multiplex PCR, piglet, pulsed-field gel electrophoresis, toxin.

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Clostridium perfringens is abundant in the soil and in the intestinal contents of animals and humans [6, 10, 15, 23]. However, *C. perfringens* is a pathogen that induces digestive diseases, such as diarrhea and hemorrhagic and necrotic enteritis in pigs. The pathogenicity of *C. perfringens* is associated with its major toxins, and *C. perfringens* isolates are divided into five types (A, B, C, D and E) based on their major toxins (alpha, beta, epsilon and iota toxins) [12, 24]. In South Korea, *C. perfringens* type A (alpha toxin) is a common isolate from pigs, whereas *C. perfringens* type C (alpha and beta toxins) is isolated occasionally from pigs with diarrhea and hemorrhagic and necrotic enteritis [27, 28]. However, *C. perfringens* type A is a ubiquitous pathogen, which can also cause necrotic enteritis and malignant edema [21]. A toxin produced by *C. perfringens* types A and C, which is referred to as $\beta 2$ toxin, has a role in the causation of digestive system diseases in horses, pigs, dogs and calves [4, 11]. The $\beta 2$ toxin sequence shares no significant amino acid sequence homology with *C. perfringens* β toxin (only 15% identity) or any other known toxin [16]. The $\beta 2$ toxin is plasmid-borne, at least in some strains, which suggests that there is the potential for mobility and the subsequent transfer of the $\beta 2$ toxin gene among *C. perfringens* strains [11, 21, 26]. A high percentage of diarrheic pigs with *C. perfringens* type A produces the $\beta 2$ toxin [17, 25]. However, several recent studies have demonstrated the widespread distribution of $\beta 2$ -toxigenic *C. perfringens* isolates in diseased poultry and healthy calves and pigs [1, 8, 22].

Epidemiological studies of *C. perfringens* have also

been conducted using random amplified polymorphic DNA (RAPD), multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) [2, 5, 14, 18–20]. PFGE was used for subtyping to provide information on the molecular relatedness of isolates for comparison with other analytical methods [19]. *C. perfringens* has been isolated from many pigs in South Korea, but no major studies have been performed to characterize the strains or their epidemiology.

Thus, the present study investigated the distribution of alpha (cpa gene), beta (cpb gene), epsilon (etx gene), iota (iap gene), $\beta 2$ (cpb2 gene) toxins and enterotoxin (cpe gene) in *C. perfringens* isolates from diarrheic and healthy piglets in South Korea. The genetic characteristics of *C. perfringens* isolates from diarrheic piglets were also compared using a PFGE assay.

Diarrheal fecal samples were collected from pigs with diarrheal diseases by the Optifarm Solution Co. (Optifarm Co., Osong, Korea) of the Animal Appraisal Organization. Four-hundred diarrheal fecal samples were obtained from seven provinces (Gyeonggi, Chungbuk, Chungnam, Jeonbuk, Jeonnam, Gyeongbuk and Gyeongnam) throughout South Korea between 2009 and 2011. The Animal and Plant Quarantine Agency also collected 136 fecal samples from healthy pigs in six provinces (Gyeonggi, Gyeongnam, Chungnam, Gangwon, Jeonbuk and Jeju) for diarrheal disease monitoring during 2010.

All of the fecal samples were collected aseptically, inoculated into brain heart infusion (BHI) agar (Difco, Beckton, Dickinson and Co., Sparks, MD, U.S.A.) containing 5% sheep blood and incubated anaerobically at 37°C for 24 hr. Two to three presumptive colonies with characteristic dual hemolytic zones were identified by biochemical tests and 16S rRNA sequencing. The genomic DNA was extracted from *C. perfringens* using a Genomic DNA Extraction Kit (Intron Co., Seongnam, South Korea) and stored at –20°C until use.

Multiplex PCR was performed to detect the toxin genes,

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Table 1. Results of the multiplex PCR analysis of the toxin genes* from 29 *Clostridium perfringens* isolates from piglets

Clinical sign	Strain No.	Province	Date	Major toxin genes				Other toxin genes	
				<i>cpa</i>	<i>cpb</i>	<i>etx</i>	<i>iap</i>	<i>cpb2</i>	<i>cpe</i>
Diarrhea	KVCC-BA1200001	Gyeonggi	Jan. 2009	+	-	-	-	+	-
	KVCC-BA1200002	Gyeongnam	Feb. 2009	+	-	-	-	-	-
	KVCC-BA1200010	Gyeonggi	Dec. 2009	+	-	-	-	+	-
	KVCC-BA1200015	Gyeongnam	Oct. 2010	+	-	-	-	+	-
	KVCC-BA1200017	Chungnam	Jul. 2011	+	-	-	-	+	-
	KVCC-BA1200018	Chungnam	Aug. 2011	+	-	-	-	+	-
	KVCC-BA1200020	Gyeonggi	Aug. 2011	+	-	-	-	+	-
	KVCC-BA1200021	Chungnam	Sep. 2011	+	-	-	-	+	-
	KVCC-BA1200022	Chungnam	Sep. 2011	+	-	-	-	+	-
	KVCC-BA1200024	Chungnam	Sep. 2011	+	-	-	-	+	-
	KVCC-BA1200025	Chungnam	Oct. 2011	+	-	-	-	-	-
No diarrhea	KVCC-BA1200285	Gyeongnam	Mar. 2010	+	-	-	-	+	-
	KVCC-BA1200286	Gyeongnam	Mar. 2010	+	-	-	-	+	-
	KVCC-BA1200288	Gangwon	Mar. 2010	+	-	-	-	+	-
	KVCC-BA1200289	Gyeongnam	Mar. 2010	+	-	-	-	+	-
	KVCC-BA1200290	Gyeonggi	Mar. 2010	+	-	-	-	+	-
	KVCC-BA1200291	Gyeonggi	Mar. 2010	+	-	-	-	+	-
	KVCC-BA1200292	Jeju	Mar. 2010	+	-	-	-	+	-
	KVCC-BA1200293	Jeju	Mar. 2010	+	-	-	-	+	-
	KVCC-BA1200295	Jeju	Mar. 2010	+	-	-	-	+	-
	KVCC-BA1200297	Chungnam	Apr. 2010	+	-	-	-	+	-
	KVCC-BA1200298	Chungnam	Apr. 2010	+	-	-	-	+	-
	KVCC-BA1200299	Chungnam	Apr. 2010	+	-	-	-	+	-
	KVCC-BA1200300	Chungnam	Apr. 2010	+	-	-	-	+	-
	KVCC-BA1200303	Chungnam	Apr. 2010	+	-	-	-	+	-
	KVCC-BA1200306	Chungnam	Apr. 2010	+	-	-	-	+	-
	KVCC-BA1200308	Jeonbuk	Apr. 2010	+	-	-	-	+	-
	KVCC-BA1200325	Jeonbuk	Apr. 2010	+	-	-	-	+	-
	KVCC-BA1200326	Jeonbuk	Apr. 2010	+	-	-	-	+	-

*The *cpa*, *cpb*, *etx*, *iap*, *cpb2* and *cpe* genes encode the alpha, beta, epsilon, iota, $\beta 2$ and enterotoxin toxins, respectively.

as described previously [3]. The $\beta 2$ toxin gene (*cpb2* gene) of *C. perfringens* was amplified using a primer set (5'-GAAAGGTAATGGAGAA-3' and 5'-GCAGAATCAGGATTTT-3'), as described previously [13]. After confirmation of the DNA fragments on a 1% agarose gel, they were extracted from the gel and purified using a Gel Extraction kit (Qiaquick, Qiagen, Valencia, CA, U.S.A.), before sequencing by the Sanger method using an ABI Prism 373 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The *cpb2* gene nucleotide sequence data generated in the present study and data obtained from NCBI GenBank were analyzed using CLUSTALW 1.8X, which generated .aln and .nxs files. The .aln files were converted into .meg files using MEGA 4.1. A neighbor-joining (NJ) tree was constructed using Kimura's two-parameter model, assuming uniform rates of change among sites.

PFGE was performed according to a published method [19] with a small modification using 40U *Sma*I (MBI Fermentas, Hanover, MD, U.S.A.), and the digested genomic DNA fragments were separated using a CHEF-DR III system (Bio-Rad, Richmond, CA, U.S.A.) at 12°C for 20 hr with an initial pulse for 5 sec and a final pulse for 40 sec at 6 V/cm at an angle of 120°, where a lambda DNA marker (Sigma) was

employed as a DNA molecular weight marker. The DNA bands were stained with ethidium bromide, and the DNA banding patterns were analyzed using BioNumerics software (Applied Maths, Kortrijk, Belgium) to calculate the Dice correlation coefficients and to generate a dendrogram, where clustering was performed using the unweighted pair-group method with arithmetic means (UPGMA).

Among the 400 diarrheic fecal samples, *C. perfringens* alone was isolated from 11 samples without other pathogens (i.e., enterotoxigenic *E. coli*, *Salmonella* spp., transmissible gastroenteritis virus, porcine epidemic diarrhoea virus and rotavirus). *C. perfringens* isolates (n=11) were obtained from three regions, as follows: Gyeonggi (n=3), Gyeongnam (n=2) and Chungnam (n=6) (Table 1). Eighteen *C. perfringens* isolates were obtained from healthy piglets without diarrhoea. These isolates (n=18) were obtained from six regions, as follows: Gyeonggi (n=2), Gyeongnam (n=3), Chungnam (n=6), Gangwon (n=1), Jeonbuk (n=3) and Jeju (n=3) (Table 1). Table 1 shows the distributions of the six toxin genes (*cpa*, *cpb*, *etx*, *iap*, *cpb2* and *cpe*) among the *C. perfringens* isolates (n=29). All of the *C. perfringens* isolates (n=11) from diarrheic samples were confirmed as type A, and the *cpb2* gene was present in 81% of the isolates

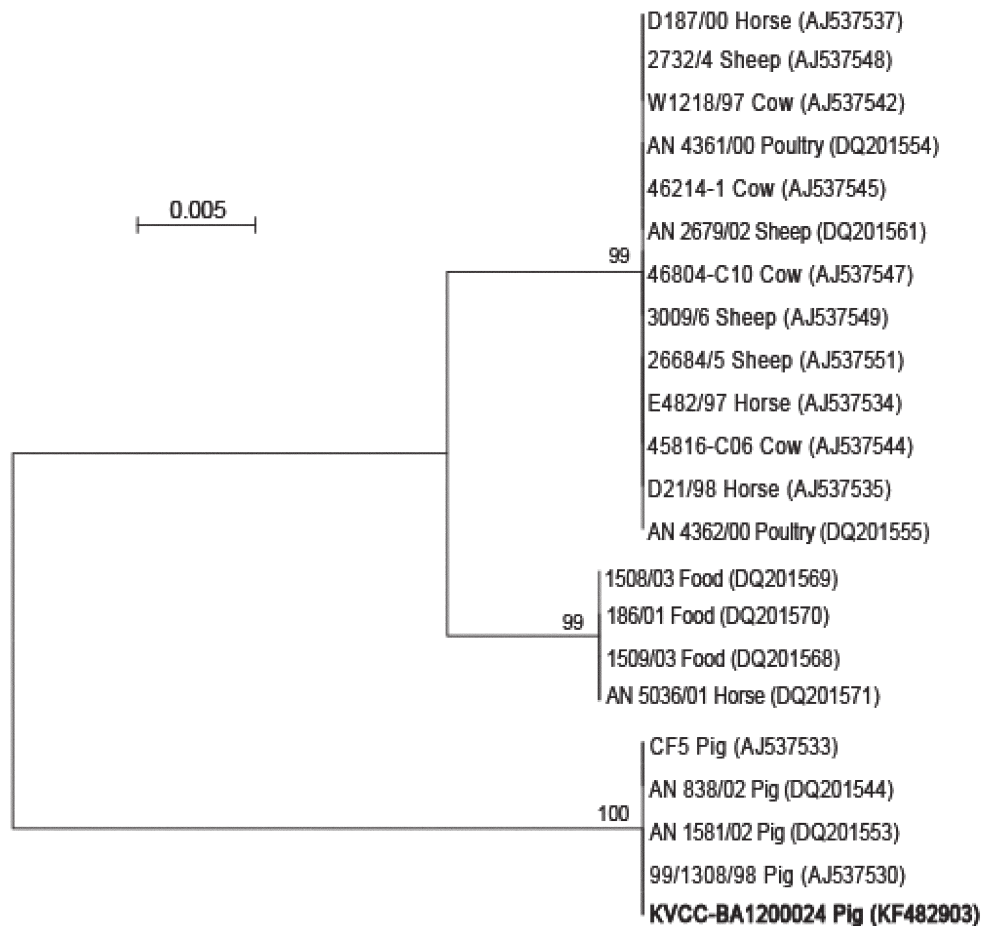


Fig. 1. Phylogenetic relationships among the β_2 toxin gene sequences. The trees were constructed using the neighbor-joining method with Mega 4.1 based on 1,000 bootstrap replicates. The *cpb2* gene (KF482903) identified in Korean piglet isolates is indicated in bold.

(9/11), whereas the *cpe* gene was not detected in any of the strains. The 18 *C. perfringens* isolates from healthy piglets were all confirmed as type A, and they possessed the *cpb2* gene.

Sequence analysis of the 478 nucleotides of the *cpb2* gene showed that the 27 *cpb2* genes from Korean isolates all shared the same sequence. To determine the level of homology, the *cpb2* genes were compared with those of isolates from animals (cow, horse, pig, poultry and sheep) and food deposited in the NCBI GenBank, which showed that the *cpb2* gene (KVCC-BA1200024; GenBank accession no. KF482903) of the Korean piglet isolates clustered with the European enteritis pig group in the phylogenetic tree (Fig. 1). Figure 2 shows the DNA banding patterns of the *C. perfringens* isolates obtained by PFGE analysis. PFGE of the *C. perfringens* isolates generated 7–10 DNA fragments, which ranged in size from 48.5 to 485 kb. There was low similarity between the *C. perfringens* isolates from healthy and diarrheic piglets, which indicates that *C. perfringens* exhibits genetic variation in pig farms.

The alpha and beta toxins produced by *C. perfringens*

cause porcine digestive system diseases, such as diarrhea or necrotizing enterocolitis [8]. The β_2 toxin, which is comparable to β toxin, is also known to cause these diseases [9]. Therefore, the β_2 toxin gene has been investigated actively in European countries, the USA and African countries [14]. Bueschel *et al.* reported that β_2 was present in 11.1% of *C. perfringens* isolates from healthy pigs, whereas the majority of isolates (90.9%) from pigs with enteritis or diarrhea were positive for β_2 , which strongly suggests that this gene has a significant association with diarrhea in piglets [4].

In the present study, 29 *C. perfringens* isolates (11 from diarrheic and 18 from healthy piglets) were detected where only *cpa* was present as a major toxin. Nine positive isolates for the *cpb2* gene were also obtained from diarrheic piglets and 18 from healthy piglets. Interestingly, β_2 -toxigenic *C. perfringens* was isolated frequently from healthy piglets in South Korea, which contrasts with previous reports from the U.S.A. and Europe. Recently, Farzan *et al.* reported that the presence of the *cpb2* gene in the intestinal contents of normal and diarrheic piglets in the USA did not differ significantly, although further work is required to confirm whether

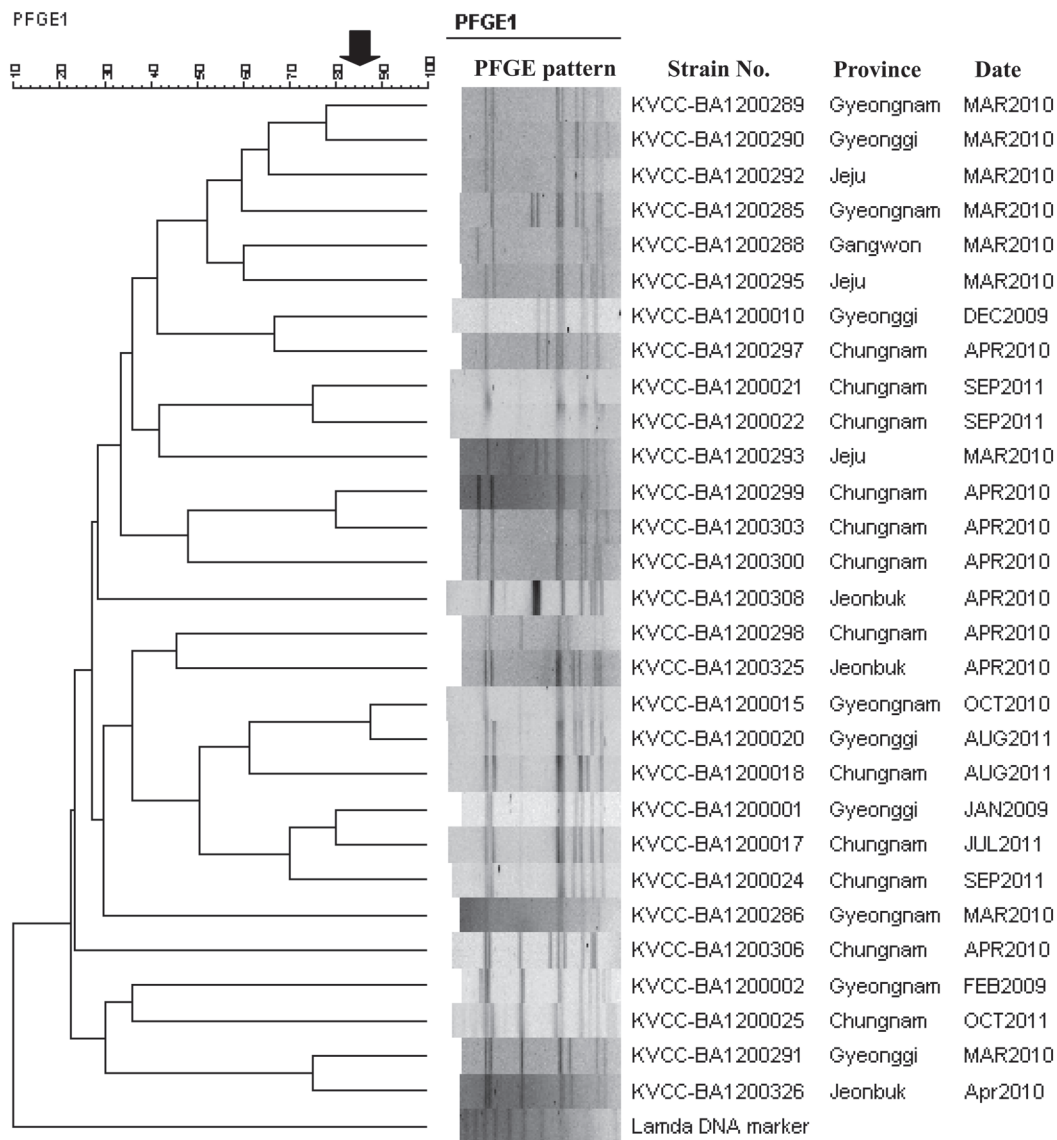


Fig. 2. Pulsed-field gel electrophoresis profiles of *Clostridium perfringens* isolates from piglets, which were analyzed using the BioNumerics program (Applied Maths) to generate a dendrogram based on the Dice coefficients.

the presence of the *cpb2* gene in *C. perfringens* has a pathogenic role during enteric infections of piglets [7].

In conclusion, the $\beta 2$ toxin gene of *C. perfringens* did not have a clear relationship with enteric disease. However, the $\beta 2$ toxin gene of *C. perfringens* might be toxigenic in healthy piglets, and it could cause enteric pathogenicity. The PFGE analysis of 29 *C. perfringens* isolates from piglets showed that they grouped into 28 separate clusters based on a criterion of 85% similarity, which suggests that *C. perfringens* is not geographically restricted and that it is widely distributed.

The present study indicates that careful monitoring of the distribution and toxin patterns of *C. perfringens* will be necessary to develop preventive measures to facilitate disease control.

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