A Thesis

For the Degree of Master of Veterinary Medicine

# Distribution of *Salmonella* spp. and *Clostridium perfringens* from equine feces in Jeju

GRADUATE SCHOOL

JEJU NATIONAL UNIVETSITY

Department of Veterinary Medicine

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# Abstract

A precise cause of diarrhea can be established in less than 50% of cases. Therefore, treatment of most diarrhea is similar in horses and thus allows supportive therapeutic management in spite of the lack of a precise diagnosis. Multiple bacterial agents have been reported as potential causes of acute diarrhea in horses, including *Salmonella*, *Neorickettsia risticii, Clostridium difficile,* and *C. perfringens*. Salmonellosis is one of the most commonly diagnosed infectious causes of diarrhea in adult horses. *C. perfringens* are common causes of enterocolitis in horses and foals. It is important to detect the presence of a toxin-producing strain. The purpose of this study is to know about distribution of *Salmonella* spp. and *C. perfringens* and toxin types of *C. perfringens* in fecal samples of normal horses in jeju. *C. perfringens* was found in 82 samples (79.6%) by selective media culture and all isolated bacteria were identified as *C. perfringens* by PCR amplification. Of 82 isolates, 26 (31.7%) *C. perfringens* were harboring only α-toxin and other toxins were not detected, indicating that all the bacteria were *C. perfringens* type A. *Salmonella* spp. were isolated only 2 samples (2.0%), including 6-10 age group and 11-18 age group. *C. perfringens* were found in 12 (100%) of 2-5 age group, 38 (74.5%) of 6-10 age group and 32 (80.0%) of 11-18 age group. Although *Salmonella* was estimated in 2%, it can not be underestimated. A long term, wide scale research and detection is required for a deeper understanding of *Salmonella* spp. in Jeju, because foal diarrhea is common in some locations of horse husbandries. Isolation rates of *C. perfringens* were much higher than other studies, in while only low toxigenic *C. perfringens* Type A were isolated.

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Keywords: *Clostridium perfringens* Type A, *Salmonella*, healthy horse

# I. Introduction

Enteritidis and enterocolitis with diarrhea and abdominal pain are important causes of high morbidity and mortality in foals and horses (2). Due to the large size of the colon and cecum, horses are more critically affected by colitis than other animal species (9). A precise cause of diarrhea can be established in less than 50% of cases. Therefore, treatment of most diarrhea is similar in horses and thus allows supportive therapeutic management in spite of the lack of a precise diagnosis. Multiple bacterial agents have been reported as potential causes of acute diarrhea in horses, including *Salmonella*, *Neorickettsia risticii, Clostridium difficile,* and *C. perfringens*. Although *Escherichia coli* is one of the most important agent of diarrhea in many animals, it is not common in a primary cause of horse diarrhea (9).

*Salmonella* is a non-spore-forming, rod-shaped facultative anaerobic bacteria of the Enterobacteriaceae family (23). They are facultative intracellular pathogens (15), finding all around the world, in both cold-blooded and warm-blooded animals, and in the environment (3). More than 2,500 *Salmonella* serovars have been documented. A large portion of the cause of infection is the consumption of contaminated foods. *Salmonella* serovars can be divided into two main groups; typhoidal and non-typhoidal *Salmonella*. Non typhoidal *Salmonella* are more common and refer to illnesses caused by all serotypes (15). *Salmonella* is a microorganism recognized as one of the most common agents of equine enteric disease and nosocomial infections of hospitalized horses (4). *Salmonella* serogroup B includes *S.* Typhimurium and *S.* Agona, two of the most frequently isolated serovars from horses with clinical disease.

*C. perfringens* is a spore-forming, rod-shaped obligatory anaerobic bacteria. This pathogen has been found all around the world. Five types of *C. perfringens* were classified by virulent toxins they produce. All *C. perfringens* types have α -toxin but every types have their major toxin with/without other toxins; *C. perfringens* type A, type B, type C, type D, and type E has α-toxin, β-toxin, β

-toxin, ε-toxin and ι-toxin, respectively as major toxin. *C. perfringens* type A and type C have been associated with intestinal diseases of foal and adult horse. All *C. perfringens* types found commonly in only intestine of warm-blooded animals, in while only type A habits in both soil and the intestine. Although *C. perfringens* type A is primarily associated with gas gangrene in humans and animals and with food poisoning in humans, it has also been implicated in necrotizing enterocolitis in suckling and feeder pigs, necrotic enteritis in broiler chickens, and canine haemorrhageic gastroenteritis. *C. perfringens* type C has also been associated with enteric diseases of many animals, such as an acute enterotoxaemia in adult sheep, sudden death in goats and feedlot cattle, necrotic enteritis in chickens, haemorrhagic enteritis in neonatal piglets. Although *C. perfringens* type A has been commonly, while *C. perfringens* type C rarely identified in the feces in healthy neonatal foals, both types are well known infectious agents in horse. *C. perfringens* type C infection has been associated with an acute, hemorrhagic diarrhea in young foals less than 10 days old and *C. perfringens* type A with or without β2 toxin gene has also been associated with typhlocolitis in horses (24).

Numerous studies on the etiology of bacterial diarrhea have been conducted for various animals including cattle, pigs, and chicken (14, 21, 26), however, there are no reports on the agents in horses because horse industry is inferior than other population animals in Korea. The purposes of this study were to identify and detect *Salmonella* and *C. perfringens* in fecal samples of normal horses currently inhabiting Jeju island, which can be used as a baseline data to further equine intestinal disease research.

# Ⅱ. Material and method

## Sample collection

Fecal samples were collected directly from rectum or fresh manure in local farms breeding only horses during 2015 and 2016. After the rectal exam, samples were added to 120 ml specimen cups and were transported to the veterinary bacteriology laboratory of Jeju National University within 4 hrs and maintained storage at –20°C in case of impossible to do continuous isolation works. Total 103 fecal samples were taken from 17 different horse farms, including 12 of 2-5 age group, 51 of 6-10 age group and 40 of 11-18 age group (Table 1).

Table 1. Information of samples collected in this study

| Horse farms | Age of horses | | | Total |
|---|---|---|---|---|
| | 2-5 | 6-10 | 11-18 | |
| A | 2 | 2 | 3 | 7 |
| B | 0 | 3 | 2 | 5 |
| C | 0 | 2 | 0 | 2 |
| D | 0 | 2 | 5 | 7 |
| E | 2 | 0 | 2 | 4 |
| F | 2 | 7 | 3 | 12 |
| G | 0 | 9 | 6 | 15 |
| H | 0 | 2 | 3 | 5 |
| I | 1 | 2 | 1 | 4 |
| J | 0 | 2 | 3 | 5 |
| K | 1 | 2 | 1 | 5 |
| L | 0 | 2 | 2 | 4 |
| M | 0 | 4 | 1 | 5 |
| N | 2 | 4 | 1 | 7 |
| O | 0 | 2 | 1 | 3 |
| P | 1 | 2 | 4 | 7 |
| Q | 1 | 4 | 2 | 7 |
| Total | 12 | 51 | 40 | 103 |

### Isolation and identification of *Salmonella*

Each sample was processed by enrichment and selective plating according to the method of Gohari *et al* (9). Enrichment broth was Selenite Cystine Broth (SCB) (Difco, Detroit, USA) and selective plate was Xylose Lysine Tergitol 4 agar (XLT4) (Oxoid Ltd, Hampshire, England). After incubation of 1 g of feces added to 10 mL of SCB, 1 loopful of SCB culture was spreaded onto XLT4 plates. Both cultures were aerobically incubated overnight at 37°C. Black colonies onto selective plates were considered to be *Salmonella* (Fig. 1). Isolates were stored in 1.5 ml collection tubes containing 25% glycerol and maintained storage temperature of -75°C until further use. *Salmonella* spp. were identified by API20E kit using the manufacturer's protocol.

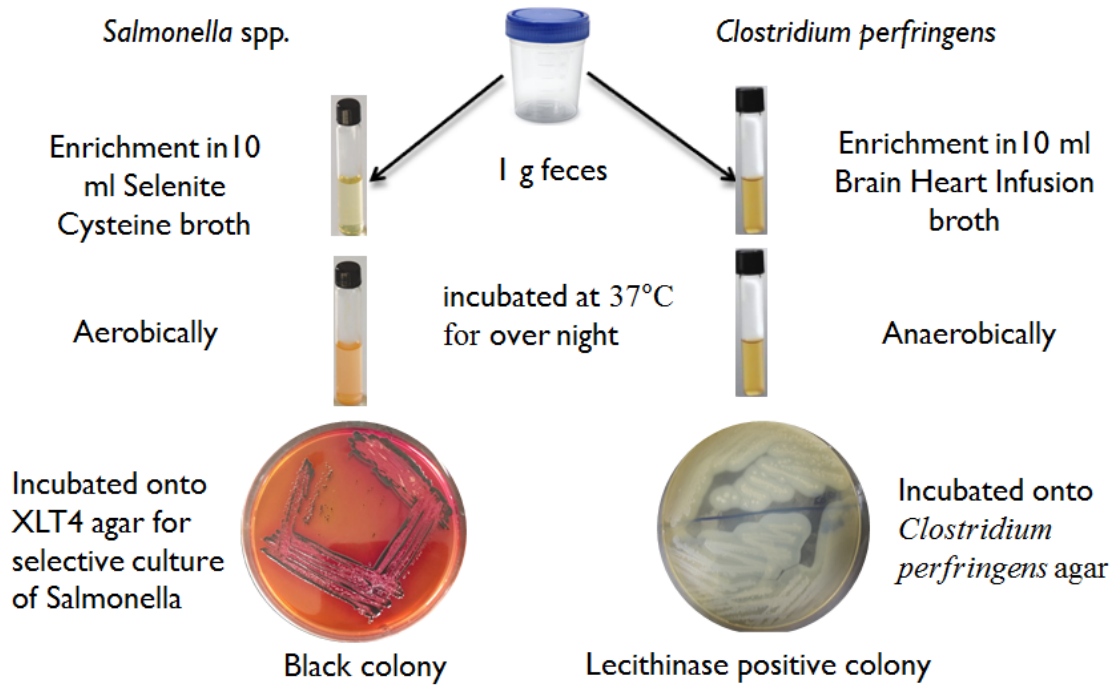


**Fig. 1. Diagram of the method of bacterial pathogens**

## Isolation of *Clostridium perfringens*

Each sample was processed by enrichment and selective plating modifying to the method of Gohari *et al* (9). Enrichiment borth was the Brain Heat Infusion (BHI) broth (Difco, Detroit, USA) and selective plate was *C. perfringens* agar (CPA) (Oxoid Ltd, Hampshire, England) containing 5% egg yolk emulsion and *C. perfringens* selective supplement (Oxoid). After incubation of 1 g of feces added to 10 mL of BHI broth, 1 loopful of the culture was streaked onto selective media. Both cultures were anaerobically incubated overnight at 37°C. Plates containing lecithinase-positive colonies were selected for bacterial enumeration (Fig. 1). To identify *C. perfringens*, 3 suspected lecithinase-positive colonies were sub-cultured onto blood agar plates. The colonies with large double hemolysis zone were considered to be *C. perfringens.* Three isolates of each of these were also stored at -75°C.

## Identification of *Clostridium perfringens*

DNA of lethicinase-positive bacteria was extracted by manufacturer's protocol using QIAamp DNA mini kit (Quiagen, germany) from BHI broth cultures. Extracted DNA was stored at –20℃ freezer until needed. Identification of *C. perfringens* was performed to polymerase chain reaction (PCR) using Cp16s primer set (32) specific to the 16S-rRNA of the bacterium (Table 2). PCR was carried out in a 20 μℓ Maxime PCR Premix (*i*-StatTaq) (Intron Biotechnology, Inc). The amplification was carried out in a thermal cycler (ASTEC PC-818, Japan) for 35 cycles, including initial denaturation for 5 min at 95℃ and final elongation for 4 min at 72℃. and 1 cycle consists of at 94℃ for 1 min, at 53℃ for 1 min, and at 72℃ for 1 min.

Table 2. PCR primer for detection of *Clostridium perfringens*

| Species and Target gene | Primer | sequences (5'-3') | Product |
|---|---|---|---|
| *Clostridium perfringens*/16SrRNA | Cp16SF | AAA GAT GGC ATC ATC ATT CAA C | 279bp |
| | Cp16SR | TAC CGT CAT TAT CTT CCC CAA A | |

Table 3. PCR primer for detection *Clostridium perfringens* toxin

| Toxin gene | Primers | Sequence(5`-3`) | product |
|---|---|---|---|
| cpa(α-toxin) | CPAlphaF | GCTAATGTTACTGCCGTTGA | 324bp |
| | CPAlphaR | CCTCTGATACATCGTGTAAG | |
| cpb(β-toxin) | CPBetaF3 | GCGAATATGCTGAATCATCTA | 195bp |
| | CPBetaR3 | GCAGGAACATTAGTATATCTTC | |
| cpb2(β2-toxin) | CPBeta2totalF2 | AAATATGATCCTAACCAAMaAA | 548bp |
| | CPBeta2totalR2 | CCAAATACTYbTAATYGATGC | |
| etx(ε-toxin) | CPEpsilonF | TGGGAACTTCGATACAAGCA | 376bp |
| | CPEpsilonR2 | AACTGCACTATAATTTCCTTTTCC | |
| iap(ι-toxin) | CPIotaF2 | AATGGTCCTTTAAATAATCC | 272bp |
| | CplotaR | TTAGCAAATGCACTCATATT | |
| cpe(enterotoxin) | CPEnteroF | TTCAGTTGGATTTACTTCTG | 485bp |
| | CPEnteroR | TGTCCAGTAGCTGTAATTGT | |

## Toxin typing of *Clostridium perfringens*

*C. perfringens* type was classified by multiplex PCR method to detect *cpa* (α -toxin), *cpb* (β-toxin), *cpb2* (β2-toxin), *ext* (ε-toxin), *iap* (ι-toxin) and *cpe* (enterotoxin) genes (10). PCR was carried out in a 20 μℓ Maxime PCR Premix (*i*-StatTaq) (Intron Biotechnology, Inc). The amplification was performed in a

thermal cycler (ASTEC PC-818, Japan) for 35 cycles (1 cycle consists of at 95℃ for 1 min, at 54℃ for 1 min, and at 72℃ for 1 min), including initial denaturation for 5 min at 95℃ and final elongation for 5 min at 72℃ (Table 3). PCR products to identify and classify were examined by gel electrophoresis using a 2.0% gel and examined after staining with 0.5 mg/ml of ethidium bromide.

# Ⅲ. Results

## Distribution of *Clostridium perfringens*

Presumptive bacterial pathogens were grown in 82 of 103 samples. Onto 82 CPA agar, 246 lethicinase-positive colonies were peaked and 182 colonies of them were shown the large double zone of hemolysis onto blood agar, indicating that some lethicinase colonies were not true lethicinase producing bacteria. All hemolytic colonies were identified as *C. perfringens* by PCR reaction amplifying 279 bp of *C. perfringens* 16S-rRNA specific gene fragment (Fig 2 and Table 4). Isolation rates of *C. perfringens* were 79.6% (82 of 102 samples) because the 182 hemolytic colonies were 1 to 3 organisms of 3 colonies selected from 82 plates with presumptive bacterial pathogens.

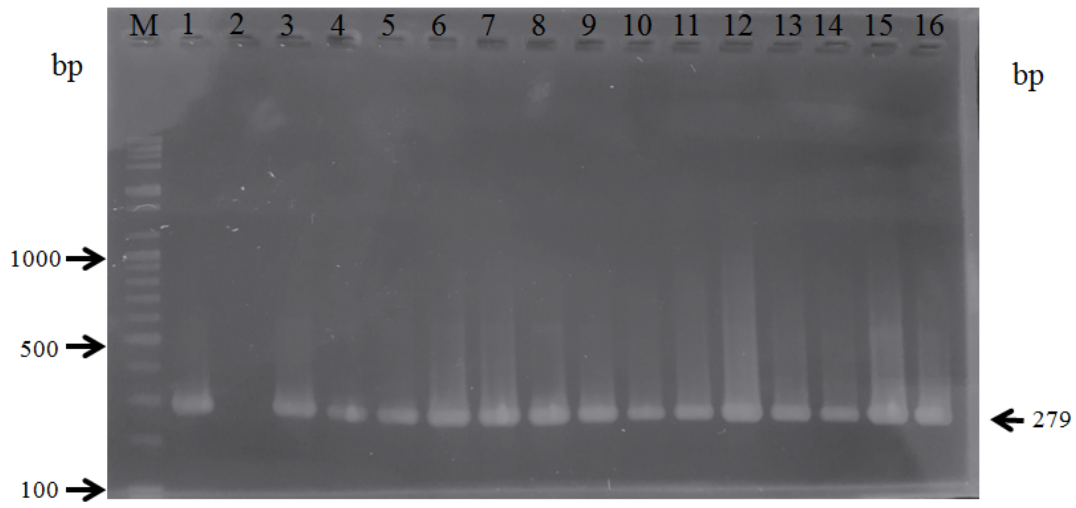


Fig 2 . Representative PCR products amplified by primers specific to 16SrRNA gene of *Clostridium perfringens*. Lane M, 100bp Plus DNA Ladder( Bioneer); Lane1, positive control; Lane 2, negative control; Lane 3, Cp1a1; Lane4, Cp1b1; Lane5, Cp3b2; Lane6, Cp8a2; Lane7, Cp10a1; Lane8, Cp20a1; Lane9, Cp26b1; Lane10, Cp29a1; Lane11, Cp30a1; Lane11, Cp30a2; Lane12, Cp32a1; Lane13, Cp34b1; Lane14, Cp35b2; Lane15, Cp36a1; Lane16, Cp40b2.

## Toxin typing of *Clostridium perfringens*

Only *cpa* gene was amplified by multiplex PCR and of 182 *C. perfringens* isolates, 55 were type A. None of the isolates was positive for *cpb, cpb2, ext, iap* and *cpe* (Fig 3). Type A isolates were included in 1 to 3 isolates from only 26 (31.7%) of 82 *C. perfringens*-positive horses (Table 4).

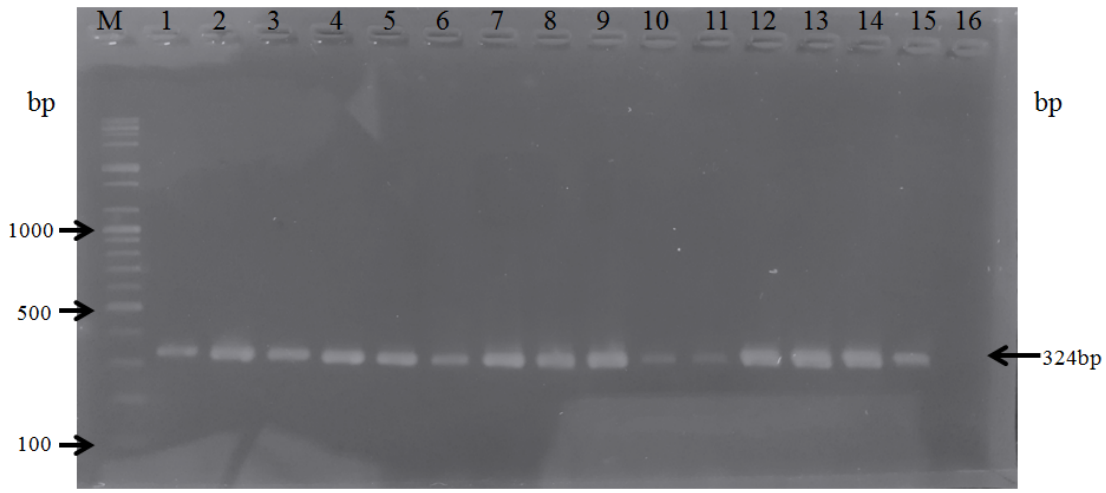


Fig 3. Representative PCR products amplified by the multiplex PCR assay for genotyping of *Clostridium perfringens* and detection of the genes encoding all alpha toxin. Lane M, 100bp Plus DNA Ladder (Bioneer); Lane1, Cp1a1; Lane2, Cp3b2; Lane3, Cp8a2; Lane4, Cp10a1; Lane5, Cp20a1; Lane6, Cp26b1; Lane7, Cp29a1; Lane8, Cp30a1; Lane9, Cp34b1; Lane10, Cp36b1; Lane11, Cp39a1; Lane12, Cp40b2; Lane13, Cp41b1; Lane14, Cp43b1; Lane 15, Cp50b1; Lane 16, negative control.

Table 4. Prevalence of *Clostridium perfringens* by culture, cp16sr PCR and toxin typing in sample.

| Sample | Culture | Cp16SR PCR | Toxin typing |
|---|---|---|---|
| 103 | 82 | 82 | 26 |
| Percentage (%) | 79.6 | 79.6 | 31.7 |

## Age distribution of *Salmonella* spp. and *Clostiridium perfringenes*

*Salmonella* spp. were isolated from 2 (2.0%) of 103 horses, including 1 (2.0%) of 51 horses with age range from 6 to 10 years old and from 1 (2.5%) of 40 horses with age range from 11 to 18 years old. *C. perfringenes* were found from 100% of 12 horses with age range from 2 to 5 years old, 38 (74.5%) of horses with age range from 6 to 10 years old and from 32 (80.0%) of horses with age range from 11 to 18 years old (Table 5).

Table 5. Age distributions of *Salmonella* spp. and *Clostridium perfringens*

| Age ranges of horses | No. of samples positive for | | Total |
|---|---|---|---|
| | *Salmonella* spp. | *C. perfringens* | |
| 2-5 | 0 | 12 (100) | 12 |
| 6-10 | 1 (2.0) | 38 (74.5) | 51 |
| 11-18 | 1 (2.5) | 32 (80.0) | 40 |
| Total | 2 (0.2) | 82 (79.6) | 103 |

# IV. Discussion

This study demonstrated that *Salmonella* spp. were found in 2.0% of adult healthy horses. *Salmonella* spp. is a microbe monitored for human and animal hygiene surveillance all around world. There are numerous studies investigating the prevalence of *Salmonella* shedding in horse populations. A United States Department of Agriculture, Animal Health Inspection Service examined for *Salmonella* fecal shedding from 8,417 horses in the general horse population by common bacterial culture method. The study found that overall prevalence was less than 1% (28). Others have reported that *S. Enteritidis* were isolated from 50 horse lymph nodes (71.4%) of 70 healthy and asymptomatic horses in slaughter establishments (22). However, another study found quite different results (13). *Salmonella* was isolated from only 2% of the mesenteric lymph nodes that presented for necropsy at a veterinary teaching hospital (13). Recently a study also demonstrated that the prevalence of *Salmonella* fecal shedding was 2.1% of 429 Thoroughbred horses housed at four racetracks in Louisiana (5). This study showed that *Salmonella* were isolated from 7 (1.6%) horses by either primary bacterial culture or delayed secondary enrichment and were detected from an additional 2 horses (0.5%) by PCR (5). Thus, the prevalence of *Salmonella* in healthy horses is quite varied and horses may have *Salmonella* in their body systems but rarely shed it. Although the result of this study does not qualify to fully represent the prevalence of *Salmonella* spp. in Jeju horse industry, it is believed that shedding of *Salmonella* rarely occurs in adult horses of Jeju. However, the prevalence of *Salmonella* in hospitalized patients has been reported that it is various but it is usually higher than that in healthy horses. In the early findings on horses submitted to veterinary hospitals, the prevalences of *Salmonella* was between 1.7 and 10% (5). Therefore, a long term and wide scale study is required for a deeper understanding on epidemiology of *Salmonella* spp.

Numerous studies on *C. perfringens* have been conducted for various animals such as chicken, cattle and pigs in Korea. The prevalencies of *C. perfringens* were 13.2%-50.4% and type A is popular than type C (17,18,20). However, it was difficult to find the prevalence of the organism in horse industry. This study firstly suggests that *C. perfringens* were isolated from 79.6% of adult healthy horses in Jeju, indicating much higher prevalence than previous studies by other countries. *C. perfringens* were found in 6 (8.0%) of 69 horses at the United Kingdoms (30), in 10 (13.2%) of 76 horses at Brazil (25) and in 22 (40.0%) of 55 horses at Canada (9).

*C. perfringens* type A produces α-toxin, enterotoxin and/or β2-toxin. This study found that of 82 *C. perfringens* culture-positive horses, 26 (31.7%) were *C. perfringens* type A with only α-toxin. This was consistent with earlier finding (9), which *Clostridium perfringens* α-toxin was detected in one-third of *C. perfringens* culture-positive horses. However, other types of *C. perfringens* did not isolate and enterotoxin (CPE) and β2-toxin (CPA2) were not detected in any *C. perfringens* type A isolate in this study. This implicates that the isolates is not considered a primary cause of enteric diseases (11).

There are variable previous investigations evaluating CPE in horses with diarrhea and enterocolitis. Frederick *et al*. have reported that *C. perfringens* was detected in 42 of 233 feces of foals with diarrhea, and all 24 isolates genotyped were type A but CPE was identified in only 3 (8). By contrast with the results, Weese *et al.* reported that CPE was detected in 19% of 47 adult horses and 29% of 28 foals with colitis and diarrhea using ELISA (31). However, Van Baelen and Devriese did not detect CPE in 9 adult horses with diarrhea (29). Interestingly, one report suggests that CPE has been detected in 15.8% of 57 diarrheic horses, but not in any of 57 healthy horses (7).

The finding that CPA2 did not detect in any isolate was inconsistent with previous investigations, which CPE2 were found in about 50% of the *C. perfringens*-positive cases (1, 12), and in 22% and 9% of healthy foals and adult horses, respectively (27). Geographical and seasonal variations, as well as

differences in detection methods might give to these differences from previous studies.

This study has some limitations, including the relatively small number of animals and farms, and the absence of diseased horses, it is concluded that healthy adult horses may not shed high virulent *C. perfringens* in Jeju horse industry. For all that, this study provides that future work may form with larger number of samples from foals and adult horses with/without enteric disease because foal diarrhea is common in some locations of horse husbandries, *C. perfringens* is major cause in equine diarrhea and a potential threat to other domestic animals.

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