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# Prevalence of *Clostridium difficile* Infections in Pigs in Jeju

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**Abstract :** *Clostridium* (*C.*) *difficile* has been recognized as an important emerging pathogen in both humans and animals. The prevalence of *C. difficile* in rectal feces and frozen colons of 132 pigs with diarrhea from the Jeju Island was investigated by polymerase chain reaction (PCR) to detect *C. difficile* toxin A and B genes. PCR findings revealed toxin A and B in 5 pigs (3.8%), including 2 suckling pigs, 2 weaned pigs and 1 growing pig. The result of PCR was closely matched histopathologic lesions of *C. difficile* in large intestines of pigs. Histopathologically, the cecum and colons of *C. difficile* toxin-positive pigs had severe submucosal and mesocolonic edema. Mucosal lesions ranged from random single cell necrosis and exfoliation to segmental, transmural necrosis of the cecum and colon. According to bacteriology, 4 *C. difficile*-positive pigs (80%) were co-infected with *Salmonella typhimurium*.

***Key words*** **:** *Clostridium difficile*, diarrhea, PCR, pig, toxin.

## Introduction

*Clostridium* (*C.*) *difficile* is a gram-positive, anaerobic, spore-forming bacillus and ubiquitous in the environment. It was first isolated from feces and meconium of asymptomatic newborn infants, and was originally named *Bacillus difficilis* because of its morphology and the difficulties encountered in cultivation (5,7). This bacteria has been commonly associated with diarrhea and colitis in humans and other animals including cats, dogs, guinea pigs, hamsters, horses, ostriches, pigs, and rabbits (5,14). *C. difficile* is the most important cause of antimicrobial-associated and hospital-associated diarrhea in humans (7,10).

Some virulence factors, including flagella and hydrolytic enzymes produced by the *C. difficile*, have been related with the development of disease (7,14). The most essential virulence factors of the bacteria are toxin A and toxin B. Toxin A is called an enterotoxin because it causes fluid accumulation in the intestine. Toxin B, a cytotoxin, is cytopathic for tissue cultured cells (7,12). The toxins act synergistically; toxin A induces widespread damage to the mucosa, permitting toxin B to affect epithelial cells (6). Both toxins induce the production of a variety of inflammatory mediators, including tumor necrosis factor-alpha and interleukins, which contribute to the associated inflammatory response and pseudomembrane development in humans (14). However toxin A is thought to play a more critical role than toxin B in the pathogenesis of *C. difficile* diarrhea.

The isolation of *C. difficile* from pigs was first reported in 1983 in UK (3). Several cases of severe morbidity and mortality associated with infection in young pigs have been described in Canada and US (12,13). In Korea, *C. difficile* infections have been widely reported in humans as a common nosocomial infection (8,9). There are no available data concerning *C. difficile* in animals. This study reports on the results of a survey of *C. difficile* infection in suckling and weaned pigs in Jeju Island, Korea.

## Materials and Methods

### Study subjects

A total of 132 diarrheic pigs ranging from 4- to 90-days old, submitted to the College of Veterinary Medicine, Jeju National University for diagnosis from 2006 to July 2008, were included in this study. Rectal feces were obtained from 132 pigs: 36 from 4- to 30-day-old suckling pigs, 74 from 1- to 2-month-old weaned pigs, and 22 from 2- to 3-month old growing pigs. The pigs were from 45 private farms throughout Jeju Island, Korea.

### Histopathology and special stain

After necropsy, all major parenchymal organs were fixed in 10% phosphate-buffered formalin, routinely processed, embedded in paraffin, and stained with hematoxlyin and eosin for light microscopy examination. During necropsy, portions of the colons and rectal feces were also collected aseptically for microbiological analysis. Replicate sections of the large intestines

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were used for detection of bacterial colony with Gram staining method.

**Polymerase chain reaction (PCR) analyses**

Rectal feces and frozen colons were analyzed by PCR for the presence of *C. difficile* toxin A and toxin B genes. Total DNA from rectal feces and colon were extracted using a G-spin DNA extraction kit (iNtRON Biotechnology, Korea). After the preparation of 30 mg of colon and rectal feces, add 400 μl of G-buffer and homogenized them using homogenizer. Incubate at 70°C for 10 min and added 400 μl of binding buffer. Centrifuge tissue or feces lysates for 5 min at 13,000 rpm at 4°C and carefully transfer the supernatant to a new 1.5 ml tube by pipetting. Apply the sample to G-spin™ columns and centrifuge for 1 min. To wash, add 500 μl of washing buffer to columns and centrifuge for 1 min. Discard filtrate and centrifuge the column for 1 min at 13,000 rpm. Place the column into a fresh 1.5 ml tube. To elute DNA, add 200 μl elution buffer to the column and incubate at room temperature for 1 min, and then centrifuge for 1 min. The supernatant containing DNA was stored at −70°C until use.

PCR amplification of total DNA extracted from rectal feces for the detection of toxin A and B genes was performed as previously described (8,9). The sequences of the specific primers for toxin A and B genes of *C. difficile* were NK3 (5'-GGAA-GAAAAGAACTTCTGGCTCACTCA GGT-3'), NK2 (5'-CCC-AATAGATTCAATATTAAGCTT-3'); NK104 (5'-GTGTAG-CAATGA AAGTCCAAGTTTACGC-3') and NK105 (5'-CACT-TAGCTCTTTGATTGCTGCACC-3'), respectively. All analyses were carried out using a Dice TP600 PCR thermal cycler (TaKaRa, Japan). The amplified products were visualized by electrophoresis on a 1.2% agarose gel containing ethidium bromide. DNA from *C. difficile* was achieved from the Sanggye Paik Hospital of Inje University and used as positive control. Negative controls included of DNase RNase free water.

**Bacteriology**

If necessary, both aerobic and anaerobic cultures were performed using colon and aseptically collected rectal feces. Isolated bacteria were identified using a Vitek system automatic identification apparatus (bioMerieux Vitek, USA).

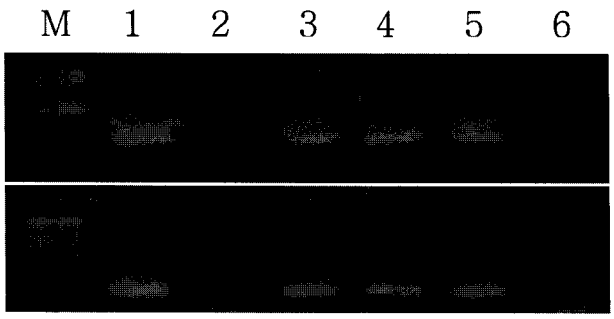


**Fig 1.** PCR products of *Clostridium difficile* toxin A (up) and toxin B (down). Lane M: 100 bp DNA ladder; lane 1-4 field samples; lane 5: 252 bp toxin A positive control (up) and 204 bp toxin B positive control (down); lane 6: negative control.

## Results

**PCR analyses**

PCR analyses yield specific amplification of 252 bp for the toxin A gene and 204 bp for the toxin B gene, respectively (Fig 1). Five out of 132 (3.8%) pigs were both positive for toxin A and toxin B genes. The ages of *C. difficile* positive pigs were 14, 30, 35, 55, and 72 days, respectively. Among them, 14- and 30-day-old piglets were categorized in suckling pig, 35- and 55-day-old pigs were in weaned pig, and 72-day-old pig was in growing pig.

**Gross and histopathologic findings**

At necropsy, five pigs had yellow or yellowish green diarrhea. Small intestines were empty or contained scant amounts of ingesta. Marked edema of the ascending mesocolon was present in 14-day-old piglet (Fig 2). Semifluid dark green feces were existed in the colon of this pig. Severe dendritic expansions of serosal blood vessels were observed in other 4 pigs. The contents in the large intestines of these pigs were yellowish watery or semifluid yellowish green in color. The mucosa of the cecum and colon was covered with diphtheric membrane in a 35-day-old pig.

Microscopically, sections of colon had severe submucosal and mesocolonic edema. Multifocal exudations of mucus, fibrin, degenerated neutrophils, and exfoliated enterocytes into the lumen were observed the cecum and colon of 14-day-old piglet (Fig 3). Diffuse congestion, infiltration of chronic inflammatory cells in lamina propria, and mutifocal cyrptitis were commonly observed in four other pigs. Mucosal lesions ranged from random single cell necrosis and exfoliation to segmental, transmural necrosis of the cecum and colon. Severe multifocal ulcerative colitis with intra-lesional *Balantidium coli* is observed in a 35-day-old pig. Typical volcano lesions, exudation of neu-

**Fig 2.** Fourteen-day-old piglet. Note edema of ascending colon (arrow).

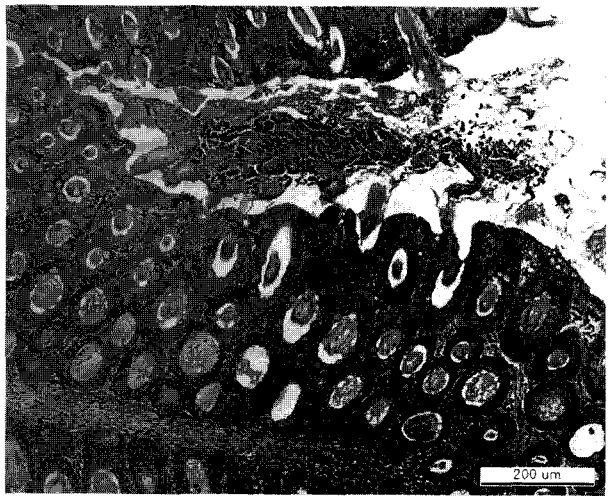


**Fig 3.** Colon of pig. Note exudation of mucus and neutrophils into the colonic lumen. H&E, Bar = 200 μm.

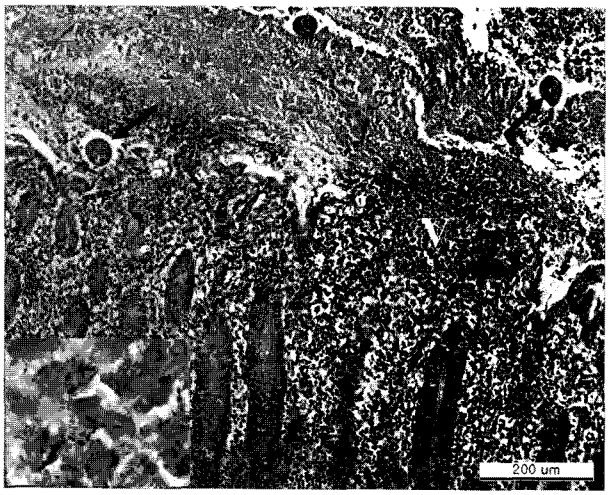


**Fig 4.** Colon of pig. Note volcano lesion (V) characterized by the focal surface erosion, infiltration and exudation of neutrophils. *Balatidium coli* (arrows) also infected. H&E, Bar = 200 μm. Insert: Gram positive rod bacteria in the luminal exudate. Gram stain, × 1,000.

trophils and fibrin into lumen, and segmental erosion were also evident in this case (Fig 4). Large numbers of Gram positive rod bacteria were revealed in histologic sections of Gram staining (Fig 4, insert).

### Bacteriology

Among 5 pigs infected with *C. difficile*, *Salmonella* (*S.*) *typhimurium* was isolated from the large intestines of 4 pigs except a 14-day-old piglet. Pathogenic *Escherichia coli*, transmissible gastroenteritis virus, porcine epidemic diarrhea virus and rotavirus were not detected in any of the cases.

## Discussion

Based on the PCR, special staining and histopathology, only five pigs (3.8%) on Jeju Island with diarrhea were infected with *C. difficile*. Two of the infected pigs were from suckling stage, other two pigs were from weaned stage, and last one was from growing stage. Four pigs (80%) were co-infected with *S. typhimurium*. Among 127 *C. difficile*-negative pigs, other enteric pathogens were detected from 93 pigs (73.2%, data not shown). The most commonly detected pathogens were *S. typhimurium* followed by *Escherichia coli*, coccidiosis, *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, and *Clostridium perfringens*.

Laboratory diagnosis for *C. difficile* infection is contingent on isolation of the pathogen from fecal samples or detection of toxins, or both (1,5,7,11,13). Because the isolation method is difficult and do not distinguish between toxigenic and non-toxigenic isolates, culture for *C. difficile* can be somewhat challenging (5,11,13). In addition, this microorganism can be found in normal healthy pigs (1,14). Therefore various assays such as cell culture cytotoxicity assays, latex agglutination test, enzyme immunoassays have been developed for the detection of *C. difficile* toxin (5,7,8). The cytotoxin assay is specific and sensitive, but requires intensive labor and long time. Enzyme immunoassay has been widely used in many clinical laboratories in human, but its low sensitivity this method has a limitation in use. Recently the PCR with primers specific for toxin A and B gene sequences can be used to identify isolates and formalin-fixed, paraffin-embedded tissues (4). In the present study, we applied PCR to detect toxigenic *C. difficile* in fecal samples and colonic tissues. And the result of PCR was closely matched histopathologic lesions of *C. difficile* in large intestines of pigs. The PCR method is sensitive and rapid, and consistently detected both toxin A and B (4,8). In addition, this method can be applied in fresh or frozen tissues of pig instead of fecal samples.

*C. difficile* has been reported to cause diarrhea and death in suckling and nursing pigs (13). According to the data from US and Canada, *C. difficile*-associated disease affects piglets 1-7 days of age born to gilts or multiparous sows (12,14). One study in Spain demonstrated that this bacteria was not found in any of the 239 rectal swabs taken from 1- to 2-month-old pigs (1). However, there is a case report of pseudomembranous typhlocolitis associated with *S. typhimurium* and *C. difficile* in 8-week-old pigs in England (3). Furthermore, outbreaks of postparturient sow losses have also been described in Croatia (6). In this study, *S. typhimurium* was co-infected in 80% (4/5) *C. difficile* positive pigs. Unlike human, *C. difficile* associated disease in pigs is not necessarily related with antibiotic usage (14). Colonization of *C. difficile* usually is prevented by an established colonic microflora. If the normal flora is disrupted, however, the likelihood that *C. difficile* will multiply and produce toxins is increased (12). Therefore underlying salmonella associated necrotic lesions in the cecum and colon may enhance the colonization of *C. difficile* in the pigs of this study. Mesocolonic serosal edema and suppuration of colonic lamina propria in this study is the most common microscopic lesions in *C. difficile* infected pigs (11,12). Mucosal lesions range from single cell necrosis to segmental erosion of the cecum and

colon, and restrict in superficial area, but ulceration is not typical. Therefore ulcerative colitis in this study is associated with *S. typhimurium* than *C. difficile*.

Recently, *C. difficile* has been isolated from uncooked and ready-to-eat retail meats, and many of the strains are of ribotypes also associated with the infection in humans and food animals (10). Hence food has been hypothesized as a possible source of *C. difficile* in community setting (2). Although the overall prevalence (3.8%) of *C. difficile* in pigs from 45 herds in Jeju Island is lower than other countries, it is clear that *C. difficile* infection in pigs occurred in Jeju. Further investigation for larger scale survey for *C. difficile* in neonatal piglets and clarification of the pathogenesis of *C. difficile* caused necrotic enteritis in young pigs is warranted.

## Acknowledgments

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# 제주도 돼지에서 *Clostridium difficile* 감염 양상

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**요 약** : *Clostridium* (*C.*) *difficile* 은 사람뿐만 아니라 동물에서도 새롭게 출현한 중요한 병원체로 인식되어 왔다. 제주도에서 설사 증상이 있는 132두의 돼지 직장 분변 및 냉동 결장을 대상으로 *C. difficile* 균체의 독소 A, B 유전자를 검출하기 위한 중합효소연쇄반응을 실시하였다. 그 결과 포유자돈 2두, 이유자돈 2두 및 육성단계 돼지 1두 등 총 5두의 돼지에서 독소 A 및 B에 대하여 양성반응을 나타내었다. PCR 결과는 돼지 대장의 조직학적 병변과 일치하였다. 병리조직학적 소견으로 독소 양성 돼지의 맹장과 결장에서는 점막하직 및 장막의 부종이 관찰되었다. 점막의 병변은 국소적인 상피세포의 괴사, 부분적인 탈락 및 장벽의 괴사까지 다양하였다. 세균 검사 결과 4두 (80%)는 *Salmonella typhimurium*과 혼합감염되어 있었다.

**주요어** : *Clostridium difficile*, 설사, 중합효소연쇄반응, 돼지, 독소