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CASE REPORT

Fatal Case of *Clostridium difficile* Infection in a Neonatal Piglet in Korea

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There have been litters of neonatal piglets that died shortly after birth, with a fatality of nearly 100%. The submitted pig had pasty yellowish brown colonic contents and severe mesocolonic edema with distinct suppurative colitis and lymphadenitis. *Clostridium difficile* toxins were detected and heavy growth of toxigenic *C. difficile* was yielded in the colonic contents. This strain was characterized by ribotype 078 and had multidrug resistance. A fatal case of porcine enteritis associated with *C. difficile* in Korea has been described.

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INTRODUCTION

Clostridium difficile is a spore-forming, anaerobic bacterium that grows in the large intestine, before normal gut flora has been established, or when it has been altered in some way, such as by dietary changes or exposure to antimicrobials (Songer *et al*., 2000; Yaeger *et al*., 2002). *C. difficile* causes pseudomembranous colitis and antibiotic-associated diarrhea in both domestic animals and humans (Waters *et al*., 1998; Koene *et al*., 2012). The disease results from the toxins that *C. difficile* produces, including enterotoxin TcdA (toxin A), and enterotoxin/ cytotoxin TcdB (toxin B); binary toxin may also be involved in pathogenesis, but there is a lack of solid evidence for this (Koene *et al*., 2012). Isolating *C. difficile* may have little diagnostic relevance because *C. difficile* is commonly found in the colons of clinically normal animals (Songer and Uzal, 2005). The standard diagnostic method of *C. difficile* infection (CDI) is the detection of TcdA and TcdB in feces (Songer and Uzal, 2005). Toxigenic culture, however, has proven useful in the diagnosis of porcine *C. difficile*-associated disease. In pigs, *C. difficile* has emerged as an important agent of neonatal enteritis after a report of typhlocolitis caused by *C. difficile* in suckling piglets (Songer and Uzal, 2005; Hopman *et al*., 2011). Recently, we included CDI in the diagnostic panel of porcine neonatal diarrhea to increase the success rate of diagnosis. Here, we describe a fatal case of porcine neonatal enteritis caused by *C. difficile* in Korea.

Case history and diagnosis: A pig farm with 450 sows had a history of tremor, lack of appetite, and sudden death of neonatal piglets. These clinical signs had occurred sporadically in five litters at once, and the case fatality was nearly 100%. The piglets at the farm were not given any antimicrobials for treatment or preventive action. In December 2011, a five-day-old piglet was submitted to Animal and Plant Quarantine Agency. After necropsy, all organs were fixed in 10% neutral buffered formalin and processed routinely for histopathology. The small intestine contents were aseptically inoculated onto sheep blood agar (KisanBio, Korea) and BBL™ MacConkey agar (Becton, Dickinson and company, USA). To isolate *C. difficile*, colonic contents were shocked with an equal volume of 96% ethanol for 50 min, then plating on BBL™ *C. difficile* Selective Agar (Becton, Dickinson and company, USA). The plates were incubated for 48 h at 37°C in both aerobic and anaerobic atmospheres. PCRs for *tcdA, tcdB, cdtA*, and *cdtB* were performed, using colonies with compatible morphology, which produced L-proline-aminopeptidase (PRO Disc, Remel, USA) (Hopman *et al*., 2011; Koene *et al*., 2012). PCR ribotyping was performed as described previously (Stubbs *et al*., 1999). *C. difficile* toxins were detected in the colonic contents with a commercially-available enzyme immunoassay (EIA) kit (C. DIFFICILE TOX A/B II™, TechLab, USA), according to the manufacturer's instructions. For differential diagnosis, additional PCRs were performed to detect transmissible gastroenteritis

virus (TGEV), porcine epidemic diarrhea virus (PEDV) and porcine rotavirus. The reference agar dilution procedure recommended by the Clinical and Laboratory Standards Institute (CLSI) was performed to determine MICs of ampicillin, cefoxitin, ciprofloxacin, enrofloxacin, erythromycin, vancomycin, and metronidazole (CLSI, 2007).

Grossly, pasty yellowish brown colonic contents and marked edema of the mesocolon were observed (Fig. 1). The mesenteric lymph nodes were enlarged and the stomach was filled with a large amount of milk curd. Histopathologically, gram-positive bacilli were found in the colonic content in the lumen. Moderate to severe edema was observed in the mesocolon with infiltration of lymphocytes and neutrophils around the blood vessels (Fig. 2A). Furthermore, multifocal segmental erosions of mucosal epithelium and submucosal edema were observed in the colon (Fig. 2B). Suppurative inflammation was primarily observed in the upper part of lamina propria, with exfoliation of enterocytes (Fig. 2B; inset). In addition, few inflammatory cells infiltrated the cortex and medulla of the mesenteric lymph nodes. There were no remarkable lesions in the small intestine or other organs. *C. difficile* toxins were detected in the colonic contents analyzed by an EIA (OD_{450} = 0.247). Bacterial culture of colonic contents yielded heavy growth of *C. difficile* only, which was identified by a ground-glass appearance of colonies, horse stable-like odor, and PRO Disc test. PCR revealed these to be toxigenic strains, with positive results for *tcdA*, *tcdB*, *cdtA*, and *cdtB*. In addition, an isolate was typed as ribotype 078 (RT078). No other aerobic or anaerobic pathogens were detected. PCR results were also negative for TGEV, PEDV, and rotavirus. A *C. difficile* isolate from this case was susceptible to ampicillin, vancomycin, and metronidazole. On the other hand, the isolate was also resistant to cefoxitin, ciprofloxacin, enrofloxacin, and erythromycin. Therefore, this isolate was multidrug resistant (quinolones, cephems, and macrolides). After treatment with susceptible antimicrobials such as ampicillin and intensive disinfection with sporicides, there was no more piglet loss in this farm.

DISCUSSION

C. difficile is an important pathogen of porcine neonatal enteritis throughout the swine-producing areas of some countries (Songer *et al*., 2000; Yaeger *et al*., 2002; Hopman *et al*., 2011). Previous reports indicate that uncomplicated CDI is involved in 34.1% of porcine neonatal enteritis, mixed infections developed in a further 20–25% and *C. difficile* toxin was detected in the colonic contents of 0-97% of the piglets (Yaeger *et al*., 2002). Authors' unpublished data also show that *C. difficile* was isolated from 73.9% of farms (n=23) and *C. difficile* toxins were detected from 47.8% of farms. Therefore, we assume that this disease may affect the Korean pig industry, and *C. difficile* should be included in the differential diagnosis of porcine neonatal enteritis. Here, we confirm porcine neonatal CDI with the pathology in the present study.

Typical CDI targets piglets of 1-7 days in age that present with a history of diarrhea shortly after birth

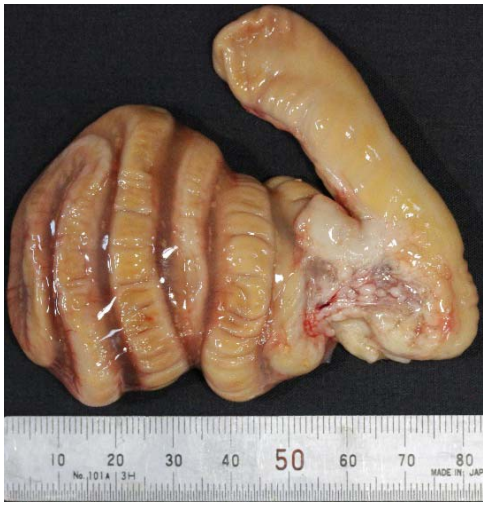

Fig. 1: Characteristic mesocolonic edema was observed in the spiral colon of a neonatal piglet with *C. difficile* infection.

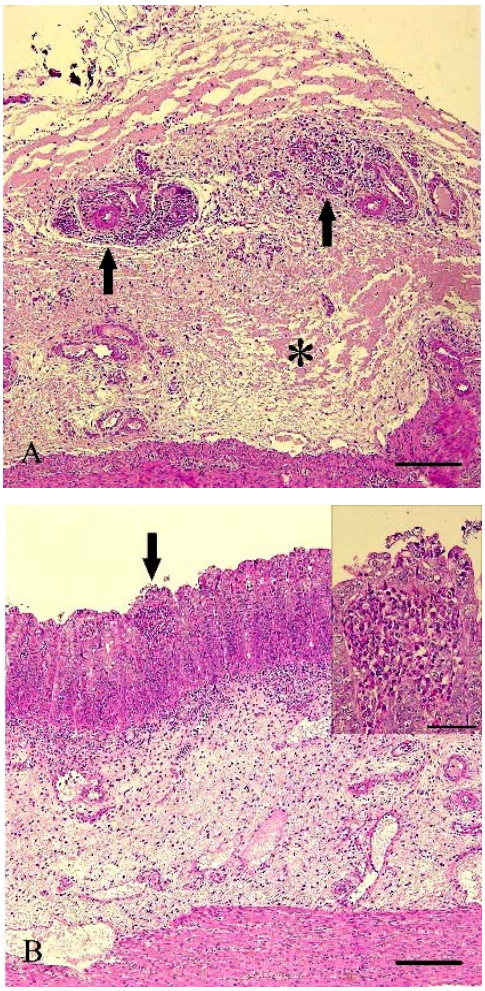

Figure 2: Microscopic lesions of *C. difficile* infection in a neonatal piglet. (A) Mesocolon. Severe mesocolonic edema (asterisk) with infiltration of mononuclear and polymorphonuclear cells around the blood vessels (arrows). H&E. Scale bar = 200 µm; (B) Severe submucosal edema with infiltration of mononuclear cells. Note the focal suppurative inflammation in the colonic mucosa (arrow). H&E. Scale bar = 200 µm. Inset: Higher magnification of the mucosal epithelium. Weak exfoliation of enterocytes, and neutrophil infiltration in the colonic lamina propria. H&E. Scale bar = 50 µm.

(Songer *et al*., 2000; Songer and Uzal, 2005; Hopman *et al*., 2011). Pathology includes moderate to severe mesocolonic edema, pasty to watery intestinal contents (Songer *et al*., 2000; Songer and Uzal, 2005). Even though these gross findings are not pathognomonic, severe mesocolonic edema could be a predictor of the presence of *C. difficile* toxins. And the presence of colitis is strongly dependent on toxin level in neonatal pigs (Yaeger *et al*., 2002). Careful examination is needed to find histopathological lesions, since characteristic gross lesions are not always observed and histopathological lesions were limited to the large intestine (Songer *et al*., 2000). In the present case, suppurative colitis and characteristic mesocolonic edema may be unique indicators of infection with *C. difficile*. In addition, the small intestines were normal.

A diagnosis of CDI is established by culture of the organism or detection of toxins. Culture may be a more sensitive method, but is not valid for diagnosis because both toxigenic and nontoxigenic strains may be recovered from intestinal contents (Yaeger *et al*., 2002). A preferred method for rapid diagnosis of CDI is toxin detection with EIA (Yaeger *et al*., 2002). A high concentration of *C. difficile* toxins was identified in the colonic contents and the *C. difficile* isolate encoded *tcdA*, *tcdB*, and binary toxin genes. Therefore, *C. difficile* was apparently responsible for the disease in this piglet.

C. difficile RT078 has been reported as an emerging hypervirulent strain that is more frequently community-acquired in humans, though its source has not been clearly defined (Debast *et al*., 2009). In pigs, *C. difficile* RT078 is the most predominant worldwide, and porcine strains are genetically indistinguishable from human strains (Debast *et al*., 2009; Koene *et al*., 2012). The relationship between porcine and human RT078 strains has suggested that animals may act as reservoirs for *C. difficile* (Koene *et al*., 2012). Further research is needed to investigate the phenotypic and genotypic characterization of *C. difficile* due to its zoonotic nature and potential for human exposure.

There are few data available on the antimicrobial resistance of porcine *C. difficile* (Thakur *et al*., 2010). The finding of this study is in accordance with other reports, which have noted that *C. difficile* has a high frequency of resistance to the older fluoroquinolones (Thakur *et al*., 2010). In general, reports of vancomycin and metronidazole resistance in *C. difficile* have been infrequent (Thakur *et al*., 2010). Any resistance to these drugs is a significant public health concern as these antimicrobials are preferred treatments for *C. difficile* disease in humans. The isolate was also found to be susceptible to both antimicrobials in this study. Further study is needed to determine MICs of representative strains against various antimicrobials used in the Korean pig industry.

Conclusion: Based on the variable results, this piglet was diagnosed with CDI. The actual incidence of CDI in Korea is probably much greater than is currently recognized, due to the inherent difficulties in testing for the presence of *C. difficile* and its toxins. CDI should be included in the differential diagnosis of neonatal enteritis in pigs. Further investigation is needed to assess the national prevalence of CDI in the Korean pig industry.

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