

Genetic and Histopathological Identification of *Cystoisospora suis* in a Post-weaned Piglet with Watery Diarrhea

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Abstract

Cystoisospora suis causes neonatal diarrhea in piglets worldwide. Although histopathological identification of this parasite is difficult owing to its similarity with other enteric coccidian parasites, including *Eimeria* spp., information regarding its molecular epidemiology in Japan is lacking. In this study, a 66-day-old post-weaned piglet reared in Shizuoka Prefecture, Japan, showed diarrhea and was examined using parasitological, histopathological, ultrastructural, and molecular methods to characterize the pathogens of the disease. Gross lesions were characterized by emaciation, thinning of the small intestinal wall, and lack of pulmonary collapse. Histopathologically, severe villous atrophy was detected in the jejunum, and *Cystoisospora*-like parasites were found in the cytoplasm of the epithelium. Interstitial pneumonia, purulent meningitis, pericarditis, and lymphatic abscesses were also noted. A microbiological and immunohistochemical analysis revealed that the piglet was also infected with porcine circovirus type 2, *Escherichia coli* serogroup O8, and group A rotavirus. Coccidial oocysts were recovered from diarrheic feces. PCR analysis targeting the rRNA internal transcribed spacer 1 (ITS1) and cytochrome c oxidase subunit 1 (COX1) genes of *C. suis* and sequencing revealed 100% similarity to those of *C. suis* in Kumamoto Prefecture, Japan. This study is the first report on the molecular identification of *C. suis* COX1 genes in Japan.

Discipline: Animal health

Additional key words: cytochrome c oxidase subunit 1, *Escherichia coli* serogroup O8, group A rotavirus, internal transcribed spacer 1, porcine circovirus type 2

Introduction

Parasites of the genera *Cystoisospora* and *Eimeria* are distributed worldwide, and some species infect the small intestine in pigs. *Cystoisospora suis* causes diarrhea and dehydration mainly in piglets 2-4 weeks of age (Mundt et al. 2005). Although *Eimeria* species are less pathogenic (Rommel 1992), *Eimeria suis*, *E. polita*, and *E. spinosa* are implicated in causing clinical symptoms including fever, diarrhea,

and weight loss in weaned pigs (Jones et al. 1985, Lindsay et al. 2012). The species are morphologically identified using oocysts by direct smear or flotation techniques. *C. suis* has four sporozoites in two sporocysts, and *Eimeria* species have two sporozoites in four sporocysts. Conversely, intracellular developmental zoites of these species are morphologically indistinguishable from each other. A polymerase chain reaction (PCR) and sequencing-based molecular identification method was reported as a sensitive and reliable method for diagnosis

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of *C. suis* infection (Rutkowski et al. 2001, Samarasinghe et al. 2008, Matsubayashi et al. 2016). Recently, we clarified the molecular characteristics of the rRNA internal transcribed spacer 1 (ITS1) gene of *C. suis* detected in piglets in the Kumamoto Prefecture of Japan, located in Kyushu Island (Matsubayashi et al. 2016). Surveillance in 15 prefectures in Japan, based on oocyst morphologies, indicated that the prevalence rate of *C. suis* is about 80% and 12-17% in farms and pigs, respectively (Saitoh & Hattori 2007). However, there is little information regarding the molecular analysis and epidemiology of this pathogen in Japan (Matsubayashi et al. 2009).

In this study, we examined three diseased piglets reared in the Shizuoka Prefecture of Japan, located in central Honshu Island, and detected *C. suis* in one of them. This report describes the histopathological, ultrastructural, and molecular characteristics of this *C. suis* isolate. In addition to the ITS1 gene, the mitochondrial cytochrome c oxidase subunit 1 (COX1) genes were examined. This is the first report on the molecular identification of the COX1 genes of *C. suis* in Japan. We discuss the genetic similarity between isolates of *C. suis* in Shizuoka and Kumamoto Prefectures.

Material and methods

Three-way crosses of Landrace, Large white, and Duroc pigs (approximately 40 sows) were raised on a farm in Shizuoka Prefecture. On April 6, 2016, 60-day-old piglets were observed to have gray watery diarrhea, emaciation, a rough coat, labored breathing, and difficulty standing. Two pooled samples of diarrheal content (six animals/pool) were examined for group A, B and C rotavirus [GAR (Chinsangaram et al. 1993), GBR (Kuga et al. 2009), GCR (Tsunemitsu et al. 1996)], porcine epidemic diarrhea (PED) virus (Ishikawa et al. 1997), and transmissible gastroenteritis (TGE) virus (Paton et al. 1997) by PCR. One pooled sample tested positive for only GAR. After testing, some piglets with similar clinical symptoms died. Three 66-day-old piglets (Nos. 1-3) were observed to have similar clinical symptoms on April 12, 2016 (Table 1). To confirm the cause of disease, necropsy was performed on the animals at the Shizuoka Chubu Livestock Disease Diagnostic Center.

Tissue samples of the intestines, liver, spleen, kidney, heart, lung, brain, and lymph nodes were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Tissue sections (approximately 3- μ m thick) were stained with hematoxylin and eosin (H&E) for histological examination.

For immunohistochemistry (IHC) analysis, intestines of the three piglets were stained with rabbit anti-GAR (provided by Dr. H. Tsunemitsu, National Institute of Animal Health, Japan), anti-PED (provided by Dr. A. Miyazaki, National Institute of Animal Health, Japan, and anti-TGE (provided

by Dr. A. Miyazaki, National Institute of Animal Health, Japan) sera, as previously described (Shibahara et al. 2001, Matsubayashi et al. 2016). The lungs, intestines, tonsils, and lymph nodes of the three piglets were also stained with anti-porcine circovirus type 2 (PCV2) rabbit serum (provided by Dr. T. Suzuki, National Institute of Animal Health, Japan). The jejunum (Piglet No. 2), ileum (Piglet Nos. 1 and 3), heart (Piglet No. 2), and cerebrum (Piglet No. 2) were stained with anti-*Escherichia coli* serogroup O8 rabbit serum (Denka Seiken Co., Ltd. Tokyo, Japan). The N-Histofine Simple Stain MAX PO kit (Multi, Nichirei Bioscience Inc., Tokyo, Japan) was used for immunohistochemical detection according to the manufacturer's instructions. The sections were counterstained with hematoxylin and examined using light microscopy.

Upon identifying the parasitological stages of *Cystoisospora*-like developmental parasites in H&E-stained jejunal sections of Piglet No. 2, a paraffin-embedded tissue section was processed to examine using transmission electron microscopy (TEM). The jejunal tissue was deparaffinized and dehydrated with xylene and ethanol, respectively, post-fixed with 1% osmium tetroxide, and dehydrated with ethanol before embedding in the resin mixture. Ultra-thin sections were obtained using an ultratome and stained with uranyl acetate (TAAB Laboratories Equipment, Ltd., Aldermaston, U.K.) and lead citrate. The sections were examined by TEM (H-7500, Hitachi, Tokyo, Japan).

Genomic DNA was purified from the sections of two different jejunal tissues of Piglet No. 2 by using a QIAamp DNA FFPE Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. To identify *Cystoisospora suis*, PCR was performed to target the rRNA ITS1 gene (amplicon size ~ 440 bp), using the primer set ITS1F-ITS2R (Samarasinghe et al. 2008), and the COX1 genes of *C. suis*. The primer set for the latter was designed in the present study based on sequences of *C. suis* deposited in GenBank (KF854262-5), Cyscytc-F1: 5'-GTC TTA TGA CCT TGA ATA CGG AAT- 3' and Cyscytc-R1: 5'-CAA TCC ACC TAG AAT AGA TAT ACA ACC- 3' (amplicon size ~ 450 bp). Each PCR amplification was conducted in a reaction volume of 25 μ l. A negative control containing reagents without a template was included in each PCR batch. PCR products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen GmbH) and sequenced using the PCR primers, and then aligned, while homology searches using the obtained partial gene sequences were performed using the FASTA program (<http://www.ddbj.nig.ac.jp/search/fasta-j.html>) (Matsubayashi et al. 2016). Simultaneously, the ileal samples of the Kumamoto case (Matsubayashi et al. 2016) were also examined to compare with the Shizuoka *C. suis* sequences.

Parasitological detection in the contents of the small intestines of all three animals was also conducted using the saturated salt flotation method.

Tissue samples of major organs (liver, spleen, kidney, heart, lung, and brain) and superficial lymph nodes were inoculated in 5% sheep blood agar medium for 48 h at 37°C anaerobically and with carbon dioxide gas. In addition, the small intestine contents were quantitatively cultured for *E. coli* and *Salmonella*. The O serogroup of isolated *E. coli* was determined by the slide agglutination method using an anti-serum of pathogenic *E. coli* immune serum (Denka Seiken). Genes encoding virulence factors, including toxins (LT, STa, STb, Stx1, and Stx2) and adhesion factors (F4, F5, F6, F18, F41, and eae), were detected by PCR in these isolates (Vu-Khac et al. 2007).

Results

At necropsy, all three piglets showed the same degree of emaciation and a rough coat (Fig. 1). Table 1 shows summary of the gross, histopathological, parasitological, bacteriological, and virological findings. Macroscopically, thinning of the entire small intestinal wall (Fig. 2), intussusception in the jejunum, and collapsing failure in the lung were detected in Piglet No. 2.

Histological examination of Piglet No. 2 revealed villous atrophy in the jejunum (Fig. 3) and the presence of numerous coccoidal parasites inside the endothelial cells. The parasites formed parasitophorous vacuoles or meronts containing one or more merozoites (Figs. 4 and 5). Two nuclei were detected in a few merozoites. Other microscopic lesions included interstitial pneumonia with polynucleated giant cells, mild purulent meningitis, pericarditis, and abscesses in the parotid and mandibular lymph nodes. Immunohistochemically, PCV2 antigen was detected in all the organs examined in the three



Fig. 1. Emaciation, rough coat, and difficulty standing in Piglet No. 2.

piglets. In Piglet No. 2, GAR antigen was detected in the jejunum, and *E. coli* O8 antigen was observed in the cerebral meninges.

Using a *Cystoisospora*-specific PCR, the predicted 440 bp product of the ITS1 gene was successfully amplified in DNAs from the jejunal sections of Piglet No. 2. The sequence of this product (Accession No. LC212985) was identical to that of *C. suis* (Accession No. LC085519, 100.0% identity), which was isolated in Kumamoto Prefecture (Matsubayashi et al. 2016). In addition, the predicted 420-440 bp product of the COX1 gene was also successfully amplified from the same sample (Fig. 6), and the sequence of this product (Accession No. LC212987) was identical to that of *C. suis* (Accession No. LC212986, Accession No. KF854265), (100.0% identity) isolated in Kumamoto (Matsubayashi et al. 2016) and Canada. The small intestinal content of Piglet No. 2 had 8,800 oocysts per gram. Notably, *C. suis* was detected only in Piglet No. 2.

Bacteriologically, *E. coli* serogroup O8 with the ST gene was isolated from the spleen, and the *E. coli* untypable serogroup without the ST gene was also detected in the brain, liver, kidney, and intestines in Piglet No. 2. Using immunohistochemical analysis, *E. coli* O8 antigens were detected in the cerebral meninges of Piglet No. 2.

Discussion

All three piglets were infected with PCV2 and *E. coli* O8, and these pathogens were most likely the cause of the observed symptoms. Lymphocyte depletion due to PCV2 in the piglets supports the previous finding that severely PCV2-infected pigs develop immunosuppression, leading to enhanced susceptibility to other pathogenic agents (Opriessnig et al. 2007). The present findings indicate a mixed infection of PCV2, *E. coli* O8, *C. suis*, and GAR in Piglet No. 2. PCV2 and *E. coli* O8 were associated with lesions in systematic organs, and *C. suis* and GAR were associated with intestinal lesions. Isolation of *E. coli* O8 from the systemic organs in Piglet No. 2 was considered to be due to postdiarrheal septicemia, as previously reported (Fairbrother et al. 1994, Berberov et al. 2004). Similar septicemic infection was also detected in the Kumamoto case with *E. coli* serogroup O149 and *C. suis* (Matsubayashi et al. 2016).

In Piglet No. 2, the thinning of the small intestine at necropsy was thought to be due to villous atrophy, which was associated with *E. coli* O8, *C. suis*, and GAR. *E. coli* O8 is one of the serogroups most frequently isolated from pigs, along with O138, O139, O141, O147, O149, and O157 (Fairbrother et al. 2005). ST toxins are associated with lethal diarrhea in pigs. The meronts or gamonts, the pathogenic stages of *C. suis*, damage the epithelial lining,

Table 1. Summary of diagnostic methods and results for piglets

Pathology					
Methods	Gross lesions		Histopathology		
			Jejunum	Ileum	Other organs
No. 1	Thickening of ileac wall, Enlarged intestinal lymph nodes		Villous atrophy	Villous atrophy, Proliferative enteropathy	Lymphocyte depletion in the lymph nodes
No. 2	Thinning of entire small intestinal wall, Jejunal interssusception, Collapsing failure in the lung		Villous atrophy	ND	Lymphocyte depletion and abscess in the lymph nodes
No. 3	Renal white foci		Villous atrophy	Villous atrophy	Lymphocyte depletion in the lymph nodes

Parasites			
Methods	Histopathology and PCR		Fecal examination
	<i>Cystoisospora suis</i>	Others	
No. 1	–	–	–
No. 2	+	–	Coccidian oocyst (OPG8,800)
No. 3	–	–	–

Bacteria					
Methods	Culture		O-serogrouping using anti- <i>E. coli</i> antisera	Immunocytochemistry for <i>E. coli</i> O8	PCR for genes encoding virulence factors
	SBA (type of hemolysis)	DHL (number of bacteria)*			
No. 1	Not detected	<i>E. coli</i> :Small intestine (1.5×10 ⁸ CFU/g)*	O8	+ (Ileum)	ST
No. 2	<i>E. coli</i> :brain, liver, spleen, and kidney (–)	<i>E. coli</i> :Small intestine (2.0×10 ⁸ CFU/g)*	O8 (Isolate from spleen) Untypable (Small intestine, brain, liver, and kidney)	+ (Cerebrum)	ST (Isolate from spleen) – (Small intestine, brain, liver, and kidney)
No. 3	Not detected	<i>E. coli</i> :Small intestine (1.5×10 ⁷ CFU/g)*	O8	–	ST

* The number of *Escherichia coli* was calculated in small intestinal contents.

Virus				
Methods	Immunocytochemistry			
	PCV2	GAR	PEDV	TGEV
No. 1	+	–	–	–
No. 2	+	+	–	–
No. 3	+	–	–	–

Abbreviations: CFU, colony-forming units; DHL, deoxycholate-hydrogen sulfide-lactose; GAR, group A rotavirus; ND, not done; OPG, oocysts per gram; PCR, polymerase chain reaction; PCV2, porcine corona virus type 2; PEDV, porcine epidemic diarrhea virus; SBA, sheep blood agar; TGEV, transmissible gastroenteritis virus

causing villous atrophy (Mundt et al. 2006, Nestrath et al. 2002). Villous atrophy leads to non-hemorrhagic diarrhea, dehydration, and poor nutrition absorption, resulting in reduced weight gain and emaciation (Lindsey et al. 2012). Piglets, from neonates to those approximately 56 days

old, can be infected by rotavirus (Dewey et al. 2003). The predominant histological lesions of porcine rotavirus infection are villous atrophy in the jejunum and ileum (Shaw et al. 1989). Therefore, the findings of this study, including characteristics of the detected pathogens, strongly indicate



Fig. 2. Thinning of small intestinal wall in Piglet No. 2

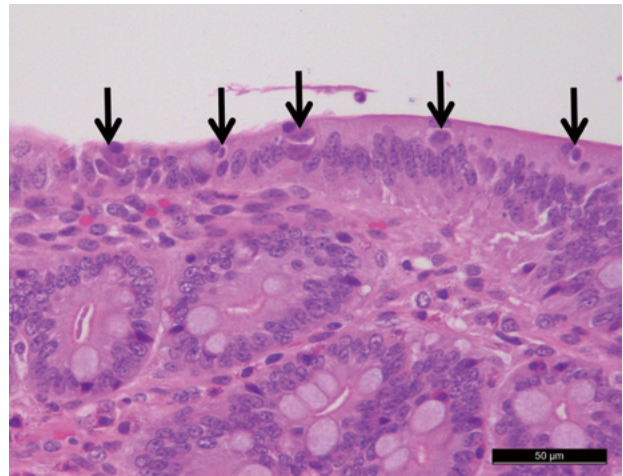


Fig. 3. Villous atrophy with several parasites (arrows) in the jejunum in Piglet No. 2. H&E. Bar = 50 µm.

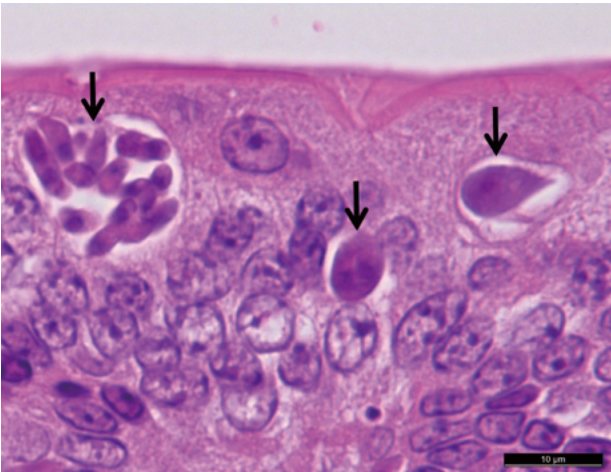


Fig. 4. Several merozoites in the meronts (arrows) in jejunal villous epithelial cells of Piglet No. 2. H&E. Bar = 10 µm.

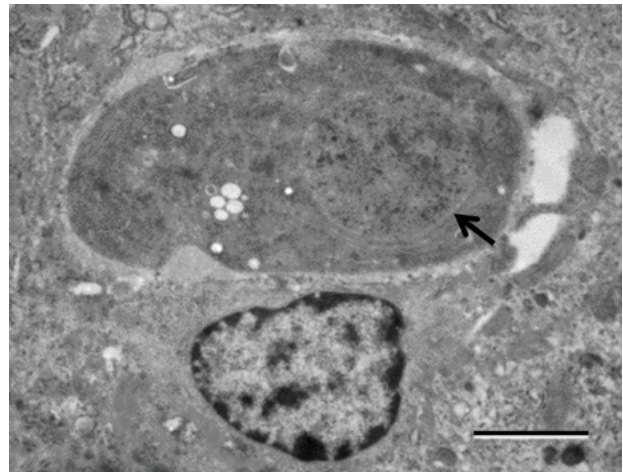


Fig. 5. *Cystoisospora suis* meronts. An immature merozoite located in the cytoplasm of an epithelial cell in the jejunum in Piglet No. 2. The arrow indicates nuclei in the zoite. TEM. Bar = 2 µm.

that *E. coli* O8, *C. suis*, and GAR cause diarrhea, and that PCV2 exacerbates the disease. However, these interactions are not fully understood. Further study is needed to define the role of these pathogens and determine the pathogenicity of their interactions.

Using a *Cystoisospora*-specific PCR, the predicted products of the ITS1 and COX1 genes were successfully amplified in this study. The 440 bp sequence of the ITS1 region of the present Shizuoka isolate shows 100% similarity with those obtained in Kumamoto Prefecture (Matsubayashi et al. 2016). The COX1 (450 bp) sequence of Shizuoka is also identical to that of *C. suis* in Canada and Kumamoto Prefecture. This study is the first report on a molecular identification of the COX1 gene of *C. suis*

in Japan. In the present study, we identified *Cystoisospora* isolates and demonstrated the usefulness of diagnostic amplification from affected tissue sections. Only a few gene sequences of *C. suis* isolated in Asia, including Japan, are available in GenBank (Matsubayashi et al. 2016). Therefore, epidemiological and molecular investigations using many isolates in Asian countries are needed to reveal the diversity and pathogenicity of *C. suis*.

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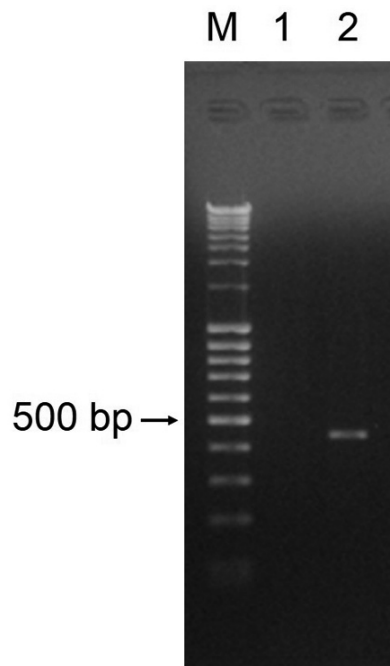


Fig. 6. Agarose gel electrophoresis of PCR products for cytochrome c oxidase subunit 1 gene (approximately 450 bp).

Lanes: M, molecular marker; 1 and 2, samples using genomic DNA purified from jejunal tissue sections of Piglet No. 2. The molecular weight marker was a 100-bp ladder. The specific band can be successfully amplified in sample No. 2.

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