

First report of molecular identification of *Cystoisospora suis* in piglets with lethal diarrhea in Japan

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Abstract

Cystoisospora suis is a pathogen that causes diarrhea in pigs and can lead to serious disease. Species identification, especially by histopathological examination, is often difficult because of morphologically similar parasites such as *Eimeria* species. In this study, we used histopathological, bacteriological, virological, and parasitological methods to identify the cause of the disease in two piglets with severe diarrhea. Villous atrophy, diffuse necrosis, and flattening of mucosal epithelial cells were found in the ilea of examined piglets, and coccidian parasites were found in the cytoplasm of the epithelial cells. In some merozoites in the meronts, the presence of two nuclei indicated type 1 merozoites, characteristic of *C. suis*. According to *Cystoisospora*-specific PCR targeting the rRNA internal transcribed spacer 1 (ITS1) gene, the sequences of the products were 98.5% similar to those of *C. suis*. *Escherichia coli* (O149 serogroup) exhibiting a virulence factor profile (LT, STb, and EAST1 as toxins and F4 as a colonization factor) was detected in one piglet. No other bacteria or significant enteric viruses were found. Co-infection with *C. suis* and *E. coli* could imply aggravation of the disease, although further study is needed to assess the pathogenicity of this interaction. This study is the first to clarify by molecular analysis the sequences of *C. suis* detected in piglets in Japan.

Keywords

Cystoisospora suis, *Escherichia coli*, ITS1 gene, piglet

Introduction

Many pathogens cause diarrhea in suckling or growing piglets. These include bacteria such as *Escherichia coli* or *Salmonella enterica*, parasites such as coccidian protozoa, and viruses such as porcine epidemic diarrhea (PED) virus. Infection is often closely related to poor farm management, including inappropriate hygiene.

Cystoisospora suis and *Eimeria* species are common protozoan parasites in pigs. *C. suis* in particular leads to serious disease in suckling piglets, causing diarrhea and dehydration mainly in animals 2–4 weeks of age (Mundt *et al.* 2005). *Eimeria* species are thought to be less pathogenic (Rommel 1992), although some species, including *Eimeria suis*, *Eimeria polita*, and *Eimeria spinosa*, may cause clinical signs such

as fever, diarrhea, and weight loss in young weaned pigs (Jones *et al.* 1985; Lindsay *et al.* 2002). Parasites of both genera infect intestinal mucosa, mostly of the small intestine, and generate asexually and sexually at the infected sites; oocysts are then shed in the feces. Until now, morphological characterization of oocysts by direct smear or flotation techniques has been used to detect parasites and identify their species (four sporozoites in two sporocysts in *C. suis*, and two sporozoites in four sporocysts in *Eimeria* species). However, it is impossible to identify the species of intracellular developmental zoites based only on histopathological examinations of intestinal tissue sections.

Recently, differentiation of *C. suis* and *Eimeria* species has been accomplished with molecular diagnostic methods such as polymerase chain reaction (PCR) with species-specific

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primers or subsequent sequencing (Ruttkowski *et al.* 2001; Samarasinghe *et al.* 2008). PCR is reportedly more sensitive than microscopic examination (Joachim *et al.* 2004) and is not complicated by morphological ambiguity in species identification. Identification of coccidial isolates is important for determining the pathological agents causing disease and for evaluating pathogenicity. However, surveys of these parasites in Japan have been limited, and their occurrence has not been well documented. In this study, we used histopathological, bacteriological, virological, and genetic methods to identify the causes of disease in piglets with severe diarrhea. This study is the first to clarify by molecular analysis the sequences of *C. suis* detected in piglets in Japan.

Materials and Methods

Animals

Six piglets (12–15 days old) at a farm rearing 130 sows in the Kumamoto prefecture of Japan, located in eastern Kyushu Island, exhibited diarrhea and died in November 2014. Two piglets (No. 1, 15 days old; No. 2, 12 days old) underwent histopathological, bacteriological, virological, and molecular analysis. At this farm, chronic diarrhea was not seen in piglets and intestinal lesions were not found in the examinations at slaughter houses. However, loose stools were shed in some piglets for a week before their death.

Diagnostic examinations

Following necropsy, the stomach, small intestine (duodenum, jejunum, and ileum), colon, lung, spleen, heart, kidney, liver, and central nerves of each of the two animals were fixed in 10% buffered formalin at room temperature and then embedded in paraffin or examined by electron microscopy. Parts of intestine were cryopreserved for additional analyses. Tissue embedded blocks were cut into 3- μ m thick and the sections were stained with hematoxylin and eosin (H & E) or periodic acid-Schiff (PAS).

Bacterial strains were isolated from the contents of the duodenum and ileum of the piglets using sheep blood agar (SBA) and deoxycholate-hydrogen sulfide-lactose agar (DHL) as the culture media. SBA and DHL are used to identify hemolytic bacteria and Enterobacteriaceae (such as *E. coli*, *S. enterica*, or *Brachyspira hyodysenteriae*), respectively; these bacteria are closely associated with porcine diarrhea. These intestinal contents were further examined by culture for *Clostridium perfringens* on Clostridium welchii agar with 10% egg yolk (YCW agar) (Eiken Chemical, Tokyo, Japan) for 18 hours at 37° C. The agar was incubated anaerobically. The bacterial strains isolated were identified by biochemical tests using API20E (Sysmex-bioMerieux, Tokyo, Japan).

O serogroups of the *E. coli* isolates were determined by agglutination using commercially available antisera (Statens

Serum Institut, Copenhagen, Denmark) according to the manufacturer's instructions. From these isolates, genes encoding virulence factors including toxins (LT, STa, STb, EAST1, Stx1, and Stx2) and colonization factors (F4, F5, F6, F18, F41, and intimin) were examined by PCR as described by Vu-Khac *et al.* (2007).

All sections were stained with anti-*E. coli* O149 antibody (Statens Serum Institut). Sections of jejunum and ileum were also stained with antibodies to transmissible gastroenteritis (TGE) virus and PED virus (both provided by Dr. A. Miyazaki, National Institute of Animal Health, Japan). Immunolabeling and visualization were performed with the universal immunoenzyme polymer method using a Histofine Simple Stain MAX-PO® kit (Nichirei Co., Tokyo, Japan). The sections were lightly counterstained with hematoxylin and assessed using light microscopy.

For virological examination, contents of the small intestines of the two piglets were inspected by species-specific PCR using PED and TGE viruses (Kim *et al.* 2001). Colon contents were tested for rotavirus using RapidTesta Rota Adeno (Sekisui Medical, Tokyo, Japan).

Transmission electron microscopy

Following identification of *Cystoisospora* parasites in piglet No. 2 by histopathological examination, the formalin-fixed ileum tissue was examined by transmission electron microscopy (TEM) as described previously (Matsubayashi *et al.* 2014). The sample was rinsed with 0.1 M phosphate buffer (pH 7.4), post-fixed with 1% OsO₄, dehydrated in an ethanol series, and embedded in Epon resin. Ultra-thin sections were stained with saturated uranyl acetate (TAAB Laboratories Equipment, Ltd., Aldermaston, United Kingdom) and examined with an electron microscope (H-7500; Hitachi, Tokyo, Japan).

Parasitological identification

The presence of parasites in the ileum was confirmed by microscopy, and DNA was purified from serial sections using a TaKaRa DXPAT (Takara Bio, Shiga, Japan) for identification of *Cystoisospora* species. To obtain more DNAs of the parasites, DNA was also purified from the cryopreserved ilea of two piglets using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. To identify *Cystoisospora* species, PCR was performed to target the rRNA internal transcribed spacer 1 (ITS1) gene (approximately 440 base pair [bp] primer set ITS1G-ITS2) (Samarasinghe *et al.* 2008). 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 in

both directions on an ABI 3130 automated sequencer (Applied Biosystems Inc., CA, USA). The obtained sequences were aligned with the representative nucleotide sequences of *Entamoeba* species or genotypes using ClustalW with initial fixed parameter values (DNA Data Bank of Japan [DDBJ], <http://clustalw.ddbj.nig.ac.jp/top-j.html>). Homology searches using the obtained partial gene sequences were performed using the FASTA program (<http://www.ddbj.nig.ac.jp/search/fasta-j.html>). A phylogenetic tree was constructed using the neighbor joining algorithm with evolutionary distances computed using the Tamura-Nei model and 1,000 bootstrap replicates. The resulting tree was generated using the MEGA software package (version 5; Tamura *et al.* 2011).

Ethics statement

Veterinarians or civil servants who were employed by prefectural governments and belonged to the Livestock Hygiene Service Center conducted the examination of the animals or field hygienic surveys of pig farms. All examinations in this study were permitted by the farm owners and conducted as part of governmental affairs. Ethical approval of animal experimentation was not necessary. Human participants were not involved in this study.

Results and Discussion

Necropsy and histopathological findings

Necropsy of piglet No. 1 revealed no gross findings; but thinning of the small intestine was seen in piglet No. 2. Histopathological findings in the jejunum included villous atrophy, formation of vacuoles, and adhesions of bacilli at the surface. Epithelial cells with adherent bacilli were removed into the lumen. Villous atrophy, diffuse necrosis, and flattening of mucosal epithelial cells were found in the ilea of both piglets (Fig. 1A). In these regions, coccidian parasites were found in the epithelial cells and intestinal gland cells of Peyer's patch (Table I). These parasites were located in the cytoplasm of epithelial cells and stained positively with PAS. Most of them formed parasitophorous vacuoles or meronts containing one or more merozoites (Figs. 1B and 2). The presence of two nuclei in a few meronts indicated that these were type 1 meronts, as is characteristic of *C. suis* (Lindsay *et al.* 2012).

Bacteriological and virological findings

Bacteriological and virological examinations of intestinal contents are summarized in Table I. Strains of beta-hemolytic bacteria were isolated by SBA agar culture from the contents of the duodenum and ileum of piglet No. 2. All of the strains formed red colonies on DHL agar and were identified as *E. coli* by biochemical tests using API20E. *C. perfringens*, by contrast, was not detected by culture on YCW agar. All 10

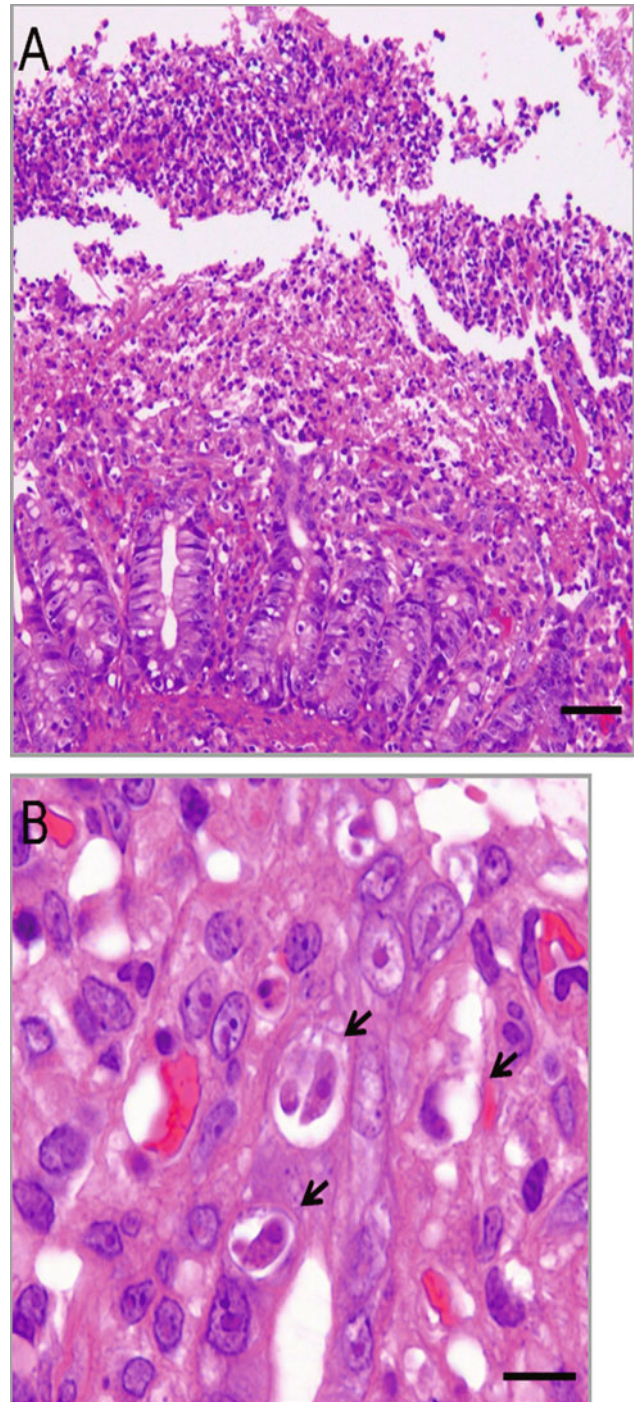


Fig. 1. **A** – Histopathological photomicrographs of ileum infected with *Cystoisospora suis* (hematoxylin and eosin). The necrotic lesion was seen on the surface mucosa. **B** – High-power magnification of *C. suis* meronts (arrows). One or two merozoites are present in the meronts. Scale bars: 50 µm (A) and 10 µm (B)

strains isolated with DHL agar belonged to the O149 serogroup and exhibited the same virulence factor profile (LT, STb, and EAST1 as toxins and F4 as a colonization factor). By immunohistochemical analysis of the sections, *E. coli*

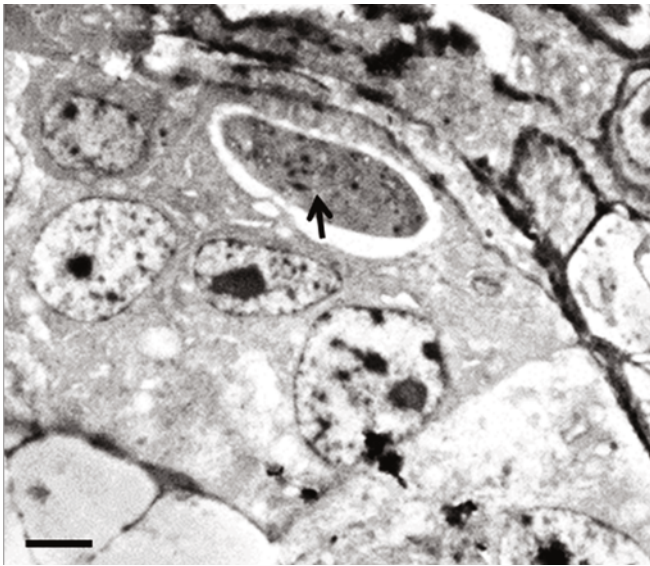


Fig. 2. Transmission electron microscopy of *Cystoisospora suis* meronts. Immature merozoite parasites are present in the cytoplasm of an epithelial cell. The arrow indicates nuclei in the zoite. Scale bar: 5 μ m

O149 antigens were also detected in the lumen of the stomach, duodenum, jejunum, ileum, colon, liver, and cerebrum of piglet No. 2. The detection of the *E. coli* from the internal organs was considered as the result of postdiarrheal septicemia as previously reported (Fairbrother *et al.* 1994; Berberov *et al.*

2004). The thinning of the small intestine at necropsy was thought to be due to villous atrophy, which was caused by the *E. coli* O149. PED virus, TGE virus, and rotavirus were not detected in either animal.

E. coli O149 is one of the serogroups most frequently isolated from pigs, along with O8, O138, O139, O141, O147, and O157 (Fairbrother *et al.* 2005). Enterotoxins (LT and ST) are associated with lethal diarrhea in pigs. Therefore, the findings of this study, including characteristics of the isolated bacteria, strongly indicate that coccidian parasites cause diarrhea and imply that *E. coli* worsens the disease.

Genetic identification of *Cystoisospora* species

By using *Cystoisospora*-specific PCR, the predicted 440-bp product of the ITS1 gene was successfully amplified in DNAs from the cryopreserved ileum and serial sections only of the No. 1 piglet. The reason why the product could not be amplified from DNA sample of No. 2 remained unknown. The sequence of this product (Accession No. LC085519) was almost identical to that of *C. suis* (EU124685), except for 6 bp nucleotide substitutions (98.5% identity). It was clearly different from those of *Cystoisospora anthochaerae* from a bird (KF766051) (94.5% identity, with 21 bp nucleotide substitutions) and *Cystoisospora belli* (HM630354) (84.6% identity, with 67 bp nucleotide substitutions). We constructed a phylogenetic tree of the ITS1 gene (Fig. 3), although only a small number of *Cystoisospora* species (seven species from five hosts) have been

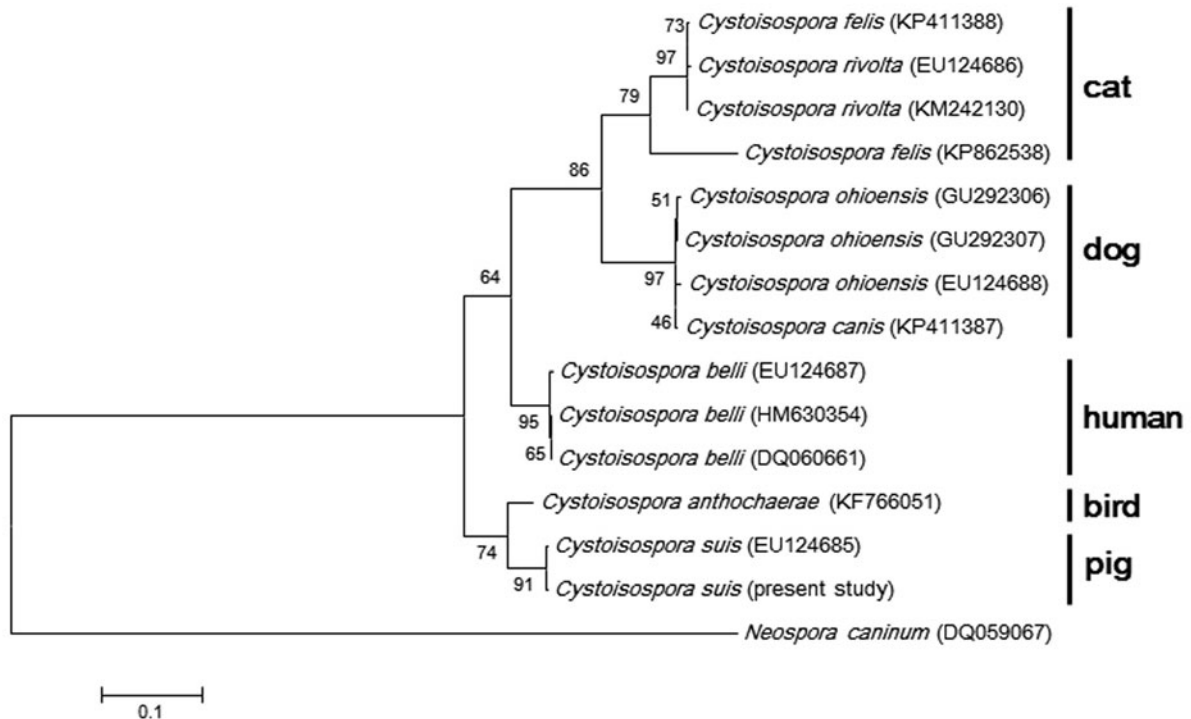


Fig. 3. Phylogram of *Cystoisospora suis* and other *Cystoisospora* species inferred from the neighbor-joining method using partial ITS1 gene sequences. Accession numbers and derived hosts are shown in parentheses, and hosts of the isolates are listed on the right. The scale bar represents substitutions per nucleotide, and bootstrap values are indicated (>1000)

Table I. Summary of diagnostic methods and results for piglets No. 1 and No. 2

Parasites							
Methods	Histopathology and PCR						
	<i>Cystoisospora suis</i>	Others					
No. 1	+	-					
No. 2	+	-					
Bacteria							
Methods	Culture			O-serogrouping using anti- <i>E. coli</i> antisera	Immunocytochemistry for <i>E. coli</i> O149	PCR for genes encoding virulence factors	
	SBA	DHL	YCW				
(type of hemolysis)							
No. 1	-	-	-	ND	-	ND	
No. 2	+	<i>E. coli</i>	-	O149	+	LT, STb, and EAST1 (toxins) and F4 (colonization factor)	
(beta)							
Virus							
Methods	RT-PCR		Immunocytochemistry		Antigen-antibody reaction		
	PED		TGE	Rotavirus	Adenovirus		
No. 1	-	-	-	-	-	-	
No. 2	-	-	-	-	-	-	

Abbreviations: CFU, colony-forming units; DHL, deoxycholate-hydrogen sulfide-lactose; ND, not done; PCR, polymerase chain reaction; PED, porcine epidemic diarrhea virus; RT-PCR, reverse transcription polymerase chain reaction; SBA, sheep blood agar; TGE, transmissible gastroenteritis coronavirus; YCW, *Clostridium welchii* with egg yolk.

deposited in GenBank. Consequently, the isolate in the present study clustered with *C. suis*, and *C. anthochaerae* from *Anthochaera carunculata* was closely related as a sister clade. The other species formed each host-specific clade. It can be taken as a valid result that the isolate detected are *C. suis*.

We amplified the ITS region of *Cystoisospora* species using a method whose efficacy for identification has been previously reported (Samarasinghe *et al.* 2008). Although primers targeting the 18S rRNA gene for detection or identification of *Cystoisospora* species are available (Ruttkowski *et al.* 2001), some primers can amplify other organisms in addition to *Cystoisospora* species (Clark and Diamond 1991). In these cases, isolation of a single oocyst is needed for amplification of the target gene (Matsubayashi *et al.* 2011). In the present study, we successfully identified *Cystoisospora* isolates and demonstrated the potential for diagnostic amplification from tissue sections of lesions. The effectiveness of this method of targeting ITS1 is in accordance with that of previous reports (Samarasinghe *et al.* 2008).

Infection with *C. suis* is common in piglets worldwide. Farms in Japan are reported to have a high positive rate of *C. suis* (about 80%), with a prevalence in pigs of 12–17% (Saitoh and Hattori 2007); however, only one report has been published. It is possible that this parasite is widespread in Japan. Although the age of the piglets at the infection has been previously shown to be crucial for the outcome like diarrhea and mortality (Stuart *et al.* 1982; Mundt *et al.* 2003), disturbances in the intestinal flora caused by the infection are also considered to play a role for pathogenesis such as clostridial overgrowth (Mundt *et al.* 2008; Mengel *et al.* 2012). Until now, interactions with other pathogens such as bacteria and viruses have not been fully investigated. Here we describe co-infection with *E. coli*, which possesses genes encoding toxin

and colonization factors, and indicate the implication for aggravation of disease. Further study is needed to assess the pathogenicity of this interaction.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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