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Retrospective and Histopathological Studies of *Entamoeba* spp. and Other Pathogens Associated with Diarrhea and Wasting in Pigs in Aichi Prefecture, Japan

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Abstract

Postweaning diarrhea and wasting are a major concern in pig farms' management. Although hemolytic enterotoxigenic *Escherichia coli*, porcine epidemic diarrhea virus, porcine circovirus type 2, and *Salmonella* spp. are the most frequent etiological agents of these diseases, *Entamoeba suis* and *E. polecki* were recently reported to be associated with diarrhea in pigs. Since the infection rate of *Entamoeba* in pigs and its relationship with other pathogens are unknown, we examined 206 pigs exhibiting diarrhea and/or wasting in Aichi Prefecture, Japan to determine the prevalence of porcine *Entamoeba* spp. *E. sui*s- and *E. polecki*-like trophozoites were detected by histopathology in 53 pigs, mainly in the lumen of the large intestine. Ulcerative colitis with infiltrating trophozoites was observed in 16 pigs, and most of these trophozoites were identified as *E. polecki* subtype 3 by PCR and sequence analysis. Tissue-invasive *Entamoeba* spp. were prevalent in pigs exhibiting diarrhea and wasting, and most samples were also positive for either *Salmonella* spp. or *Lawsonia intracellularis* by immunohistochemistry. These results suggested that *Entamoeba* was widespread in farms in Aichi Prefecture, and in most cases, enteritis was caused by coinfection by *Entamoeba* with *Salmonella* spp. or *L. intracellularis*, which causes wasting by exacerbating the original mucosal lesions.

Discipline: Animal health
Additional key words: *Entamoeba polecki*, *Entamoeba suis*, pig, diarrhea, wasting

Introduction

The control of diarrhea is a critical issue in farm management. Diarrhea in young animals is typically caused by hemolytic enterotoxigenic *Escherichia coli* (ETEC) and rotavirus (Tzipori et al. 1980), as well as porcine epidemic diarrhea virus (PEDV) (Bertolini et al. 2017). Postweaning multisystemic wasting syndrome (PMWS) has contributed to significant financial losses in the pig industry (Alarcon et al. 2013). In pigs, porcine circovirus type 2 (PCV2) is the major cause of wasting, whereas *E. coli* and *Lawsonia intracellularis* (*Li*) are known to be involved in postweaning wasting (Ellis et al. 1998, Järveots et al. 2016). Salmonellosis, particularly that associated with *Salmonella* Typhimurium and *Salmonella* Choleraesuis, can occur concurrently with PMWS (Ha et al. 2005).

The enteric protozoan parasites *Entamoeba* spp., especially *E. histolytica*, *E. polecki* (Matsubayashi et al. 2015a, 2015b, 2016), and *E. suis* (Matsubayashi et al. 2014), were recently identified as the causative agent of diarrhea in pigs. *E. polecki* can be classified into four subtypes (ST1–ST4) by polymerase chain reaction (PCR) and nucleotide sequencing of the small-subunit ribosomal

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RNA (SSU rRNA) gene (Stensvold et al. 2011). It was previously thought that most *Entamoeba* spp. are nonpathogenic to pigs; however, both *E. suis* and *E. polecki* have been found to cause severe enteritis. *E. suis* is thought to induce hemorrhagic colitis by invading the lamina propria (Matsubayashi et al. 2014). *E. polecki* ST1 and ST3 are associated with proliferative enteritis caused by *Li* (Matsubayashi et al. 2015a, 2015b, 2016), although the ST2 and ST4 subtypes, which have been detected in humans and nonhuman primates (Jirků-Pomajbíková et al. 2016, Tuda et al. 2016), have not been reported in pigs. *E. histolytica* infection has only been observed in miniature pigs in an experimental setting, but never in farmed animals (He et al. 2012). *Entamoeba* spp. have been detected in moist soils around sources of drinking water or puddles (Hirashima et al. 2017), and infection occurs via the oral intake of food and water containing mature cysts and excysted trophozoites that subsequently proliferate in the intestines.

Although there have been case reports of *Entamoeba* infection in pigs, the prevalence and relationship with other pathogens besides *Li* are not known. It is also unclear whether *Entamoeba* can cause diarrhea and/or wasting, because no experimental studies using porcine *Entamoeba* isolates have ever been performed. In this study, we retrospectively conducted histopathological, virological, bacteriological, and parasitological examinations on pigs exhibiting diarrhea and/or wasting in order to assess mucosal or lamina propria invasion and to elucidate the pathogenicity of *Entamoeba* spp. We detected *Entamoeba* spp. by PCR and sequence analysis in several samples in this study.

Material and methods

1. Necropsy, histopathological, and immunohistochemical examinations

A total of 206 pigs (dead or euthanized) (numbered 1-206) exhibiting diarrhea and/or wasting from 53 out of 204 farms in Aichi Prefecture, located on Honshu Island (the main island of Japan), were necropsied at the Aichi Prefectural Central Livestock Hygiene Service Center between April 2011 and March 2016. The pigs were between 3 and 150 days old and were from farms throughout Aichi Prefecture. Following necropsy, the small intestine (duodenum, jejunum, and ileum), cecum, colon, and rectum were fixed in 10% neutral-buffered formalin at room temperature and then embedded in paraffin. Tissue sections were cut at a thickness of 3 µm and stained with hematoxylin and eosin (H&E) for histopathological examination.

In order to investigate the association between *Entamoeba* spp. and tissue-invading bacteria, *Li* and *Salmonella* spp. antigens were detected by immunohistochemistry, and *Entamoeba*-like trophozoites in the lamina propria from the jejunum to the colon were detected by H&E staining. Formalin-fixed tissue samples were cut into 3 µm thick sections that were first treated with 3% hydrogen peroxide in methanol (to suppress endogenous peroxidase activity), followed by treatment with 0.1% actinase E solution and incubation at 37°C for 30 min for antigen retrieval. The sections were then incubated with mouse anti-*Li* serum (Bio-X Diagnostics, Rochefort, Belgium) or rabbit anti-*Salmonella* spp. polyvalent O-grouping serum (Denka Seiken Co., Tokyo, Japan) as the primary antibody for 60 min at room temperature and then with a secondary antibody (Histofine Simple Stain MAX PO; Nichirei Bioscience, Tokyo, Japan). After rinsing with phosphate-buffered saline (pH 7.4), the sections were incubated with Histofine Simple Stain aminoethyl carbazole solution (Nichirei Bioscience) at room temperature for 5 min and then counterstained with hematoxylin.

2. Examination of pathogenic viruses and bacteria

We screened for the presence of viruses and bacteria associated with diarrhea or wasting as described below. The contents of the small intestine and organs including the heart, lungs, liver, spleen, kidneys, and brain were cultured in normal blood agar and deoxycholate–hydrogen sulfide–lactose agar at 37°C under 5% CO_2 in aerobic or anaerobic conditions. Genomic DNA was extracted from the isolated *E. coli* using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The DNA was used as a template for PCR amplification of heat-labile and heat-stable enterotoxins to identify ETEC (Stacy-Phipps et al. 1995). DNA from ileal and lung tissues was used for PCR detection of *Li* and *Mycoplasma hyorhinis*, respectively, as previously described (Jones et al. 1993, Møller et al. 1998, Kobayashi et al. 1996). Viral DNA and RNA were extracted from the lungs, tonsils, and sera using the QIAamp MinElute Virus Spin kit (Qiagen GmbH, Hilden, Germany) and used for PCR detection of porcine reproductive and respiratory syndrome virus (PRRSV) (Christopher-Hennings et al. 1995) and PCV2 (Ellis et al. 1999). Viral RNA was extracted from the contents of the small intestine using the QIAamp Viral RNA Mini kit (Qiagen GmbH) and used for PCR detection of transmissible gastroenteritis virus (Paton et al. 1997), PEDV (Kim et al. 2001), and rotavirus (Matthijnssens et al. 2008). All amplicons were electrophoresed on a 1.5% agarose/Tris-borate-EDTA (TBE) gel that was stained with ethidium bromide and

visualized using under ultraviolet (UV) transillumination. Infection was determined when the bacteria or virus was identified in the sample by a relevant isolation method, PCR, and/or immunohistopathology.

3. Molecular identification of *Entamoeba* spp.

We performed a molecular survey of *Entamoeba* using 16 paraffin sections of intestinal tract tissue from pigs harboring *Entamoeba* trophozoites. Genomic DNA was extracted from fixed intestinal tissue sections using the QIAamp DNA FFPE Tissue kit (Qiagen GmbH) according to the manufacturer's instructions.

Using the extracted DNA, PCR was performed to detect four *Entamoeba* spp. (*E. histolytica*, *E. dispar*, *E. suis*, and *E. polecki*) and *E. polecki* subtypes (ST1–ST4). The following primers were used: EhL-EhR multiplex primer sets targeting the SSU rRNA gene of *E. histolytica* (or EdL-EdR for *E. dispar*) (Evangelopoulos et al. 2000), 764-RD3 and 764-765 for nested PCR detection of *E. suis* (Clark et al. 2006), and Epolec F6-R6 primers to distinguish among *E. polecki* subtypes (Matsubayashi et al. 2015b). DNA from a human isolate of *E. histolytica* (unpublished) and from *E. suis* and *E. polecki* isolates previously reported by Matsubayashi et al. (2014, 2015a, 2015b) was used as a positive control.

PCR assays were conducted using a Taq PCR Core kit (Qiagen GmbH) or Takara Ex Taq (Takara Bio, Kusatsu, Japan). Amplicons were stained with GelRed (Wako Pure Chemical Industries, Osaka, Japan) or ethidium bromide, electrophoresed on 1.5% agarose/TBE gels, and visualized using a UV transilluminator. PCR products were excised from the gel, purified using a QIAquick Gel Extraction kit (Qiagen GmbH), and sequenced. Alignments and homology searches were performed as previously reported (Matsubayashi et al. 2014).

4. Determination of *Entamoeba* spp. infection

Entamoeba infection and tissue distribution were identified by histopathological observations, and the rate of infection was determined histopathologically on the basis of the number of pigs with trophozoites. In this study, clinical infection (amoebiasis) and subclinical infection (subclinical amoebiasis) were determined for pigs with membrane-invading *Entamoeba* trophozoites and with trophozoites only in the lumen, respectively. *Entamoeba* infection in this study included both amoebiasis and subclinical amoebiasis.

5. Statistics analysis

The frequencies of *Entamoeba* spp., *Salmonella* spp., and *Li* infections were compared in order to determine the relationship between infections. Differences between groups were analyzed using the χ^2 test and residual analysis (Benjamini & Hochberg 1995). A p-value < 0.05 was considered statistically significant.

6. Ethics statement

All experiments were carried out according to the ethical policy for animal experimentation of the Aichi Prefectural Chuo Livestock Hygiene Service Center. Veterinarians who were employed as civil servants by the prefectural government and affiliated with the Livestock Hygiene Service Center examined the animals or conducted field hygiene surveys at the pig farms. Farm owners provided their consent for all examinations in this study, which were conducted as part of the government affairs. There were no human participants in this study. The authors have no conflicts of interest to declare.

Results

1. Clinical and subclinical *Entamoeba* infections and tissue distribution

Entamoeba trophozoites were found in the lumen and/or intestinal lesions in 53/206 (25.7%) pigs from 28/53 (52.8%) farms. The trophozoites were morphologically distinguished as *E. suis*-like (with the cytoplasm containing one nucleus and ingested host red blood cells visible as small dark bodies) (Matsubayashi et al. 2014) or *E. polecki*-like (with the cytoplasm containing one nucleus and some vacuoles) (Matsubayashi et al. 2015a, 2015b, 2016). Most of the pigs with trophozoites were approximately 60 days old (average: 60.8 days; median: 60.0 days). Although trophozoites were present in the lumen in most samples (indicating subclinical amoebiasis), they were detected in tissues with trophozoites invading the membrane epithelium, especially the lamina propria (indicating amoebiasis), in 16 samples (from 12 farms) harboring mild (nos. 1, 5, 12, 16, 41, 52, and 53) to severe (nos. 2, 19, 22, 36, 37, 38, 40, 42, and 50) lesions in the intestinal mucosa (Figs. 1a–c). Coinfection was detected in all 16 samples. The frequency of *Entamoeba* invasion into membrane lesions was not different among these pigs regardless of the *Entamoeba* species.

Although most lesions and invading trophozoites were found in the large intestine, pig no. 42 had numerous trophozoites and severe ulceration in the mucosa of the small intestine. No extraintestinal lesions, such as abscesses, were observed in any of the samples.

The tissue distribution of *Entamoeba*-like trophozoites is shown in Figure 2. Most *Entamoeba* trophozoites were present in the large intestine, with few

or none in the duodenum and rectum (Fig. 2, red and blue graphs). Tissue-invading trophozoites were also observed in the large intestine (Fig. 2, blue graph).

2. Clinical symptoms of amoebiasis and coinfection with other pathogens

The major coinfecting bacteria, viruses, and parasites associated with diarrhea and/or wasting are shown in Table 1. PEDV was the most frequently detected pathogen in pigs with diarrhea (22/73, 30.1%), followed by ETEC (14/73, 19.2%). In contrast, PRRSV (1/73, 1.4%), *Li* (1/73, 1.4%), and *Salmonella* spp. (1/73, 1.4%) were detected at low rates in these pigs. In pigs with wasting, PRRSV (30/118, 25.4%) and *Li* (16/118, 13.6%) were found at the highest frequencies, whereas PEDV (0/118, 0%) and ETEC (3/118, 2.5%) were the least common. In pigs exhibiting both diarrhea and wasting, *Entamoeba* spp. (5/15, 33.3%) and *Salmonella* spp. (4/15, 26.7%) were the most prevalent, whereas PEDV (0/15, 0%) was absent. Furthermore, coinfection of *Entamoeba* trophozoites with a coccidian species, such as *Cystoisospora suis*, was not detected. There was no difference in the clinical symptoms between subclinical amoebiasis and non-amoebiasis pigs. Amoebiasis was

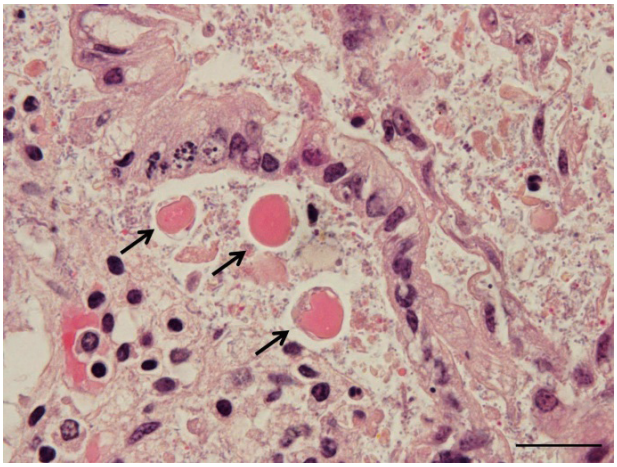

Fig. 1a. Numerous *E. suis*-like trophozoites (arrows) are observed in the lumen and lamina propria of the cecum in pig no. 41.
H&E stain. Bar = 20 μm.

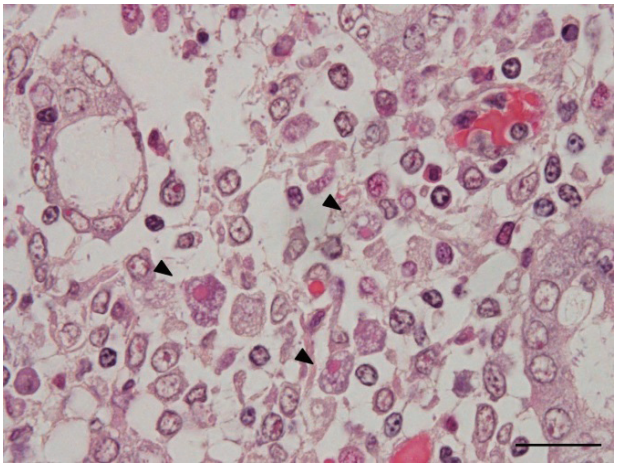

Fig. 1b. Infiltration of *E. polecki*-like trophozoites (arrowheads) into the lamina propria of the colon in pig no. 36.
H&E stain. Bar = 20 μm.

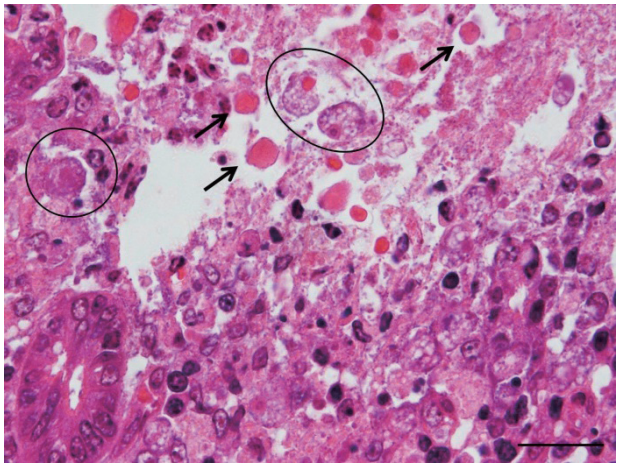

Fig. 1c. Coinfection by *E. suis* and *E. polecki*. *E. suis*-like trophozoites (arrows) are present in the lumen, and *E. polecki*-like trophozoites (circles) infiltrate into the lamina propria of the colon in pig no. 40.
H&E stain. Bar = 20 μm.

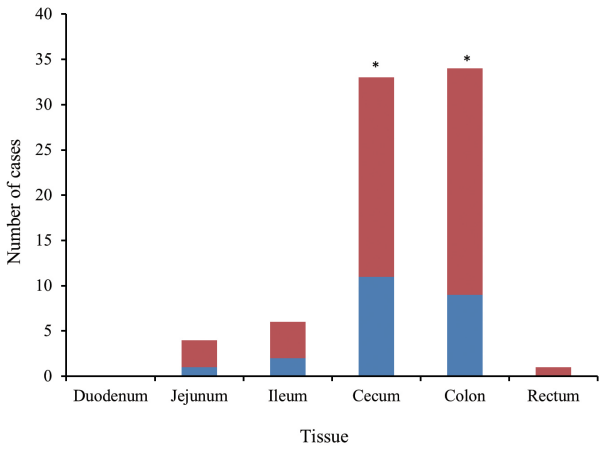

Fig. 2. Tissue distribution of samples harboring *Entamoeba*-like trophozoites in the intestine.
The red areas represent the number of samples with trophozoites only in the lumen, and the blue areas show the number of samples with membrane-invading trophozoites. $^{*}p < 0.01$.

common in pigs exhibiting both diarrhea and wasting in this population (Table 1), but significantly less common in pigs with diarrhea only.

3. Infection rate of *Entamoeba* spp.

The infection rates by *Entamoeba* trophozoites are shown in Table 2. The number of pigs infected with *E. suis*-like or *E. polecki*-like trophozoites was 20 (9.7%) and 16 (7.8%), respectively. Different types of *Entamoeba* trophozoites were detected in approximately one-quarter of all animals (25.7%), mostly in the lumen of the digestive tract (*E. suis*-like: 9.2%; *E. polecki*-like: 4.9%). *E. suis*-like trophozoites invaded into the membrane lesions in one pig (0.5%), whereas *E. polecki*-like trophozoites invaded the lamina propria in six pigs (2.9%) (Table 2). The presence of both *E. suis*-like and *E. polecki*-like trophozoites was observed in 17 pigs (8.3%).

In 16 pigs with membrane-invading *Entamoeba*, the infection of *E. suis*-like and *E. polecki*-like trophozoites was observed in nine pigs (nos. 1, 2, 12, 16, 19, 38, 40, 50, and 52; diarrhea: 2; wasting: 6; diarrhea and wasting: 1), including three pigs with membrane-invading *Entamoeba* (nos. 1, 2, and 40; wasting: 3) (Table 3). Tissue invasion by *E. polecki* appeared to be more common in pigs with diarrhea and wasting (26.7%) than in the other pig groups.

4. PCR and sequence analysis of *Entamoeba*

The results of the PCR and sequence analysis are shown in Table 3. Of the 16 samples subjected to the analyses, 13 were positive for *E. polecki* ST3 (Table 3). Although *E. suis*-like trophozoites were observed in four samples, *E. suis*-specific PCR was unsuccessful. *E. histolytica* and *E. dispar* were not detected in this study.

5. Immunohistochemical analysis

We performed an immunohistochemical analysis to detect *Li* and *Salmonella* spp. in samples harboring membrane-invading *Entamoeba* trophozoites (Table 3). Positive immunoreactivity against the *Salmonella* antigen (Fig. 3a) was observed in nine samples (nos. 12, 16, 19, 22, 38, 40, 41, 52, and 53), although bacterial isolation was negative except for sample no. 22. Immunopositivity for the *Li* antigen (Fig. 3b) was observed in five samples (nos. 1, 2, 5, 42, and 50); however, two samples (nos. 36 and 37) were negative, even though *Li* was detected by PCR in these samples.

6. Association between *Entamoeba* infection and other microbes

We examined samples with *Entamoeba* spp. coinfection with representative pathogens (Table 4). The infection rate by *Salmonella* spp. was high in pigs infected with *Entamoeba* spp. (9/16 pigs), followed by coinfection with *Li* (7/16 pigs) and PCV2 (5/16 pigs).

Salmonella spp. and *Li* were found at a significantly higher rate in pigs with amoebiasis than in those with subclinical amoebiasis or without amoebiasis (Table 5). However, their prevalence in pigs with subclinical amoebiasis was not significantly different from that in pigs with no *Entamoeba* infection.

Discussion

In this study, we carried out a histopathological analysis of pigs exhibiting diarrhea and/or wasting and determined whether they harbored *Entamoeba* trophozoites. Approximately a quarter of the pigs (25.7%) were positive for *Entamoeba* trophozoites.

Table 1. Prevalence of amoebiasis (tissue-invasive *Entamoeba*) and non-amoebiasis and main pathogens detected by microbial isolation, PCR, or immunohistochemistry in pigs with diarrhea and/or wasting in the present study

	Amoebiasis (tissue-invasive *Entamoeba*) (16)	Non-amoebiasis (except for amoebiasis) (190)	PEDV	Rotavirus	ETEC	*Salmonella* spp.	*Lawsonia intracellularis*	*Cryptosporidium* spp.	*Balantidium* spp.	PRRSV	PCV2
Diarrhea (73)	2 *[1] (2.7%)	71*[2]	22 (30.1%)	4 (5.5%)	14 (19.2%)	1 (1.4%)	1 (1.4%)	2 (2.7%)	1 (1.4%)	1 (1.4%)	4 (5.5%)
Wasting (118)	9 (7.6%)	109	0 (0.0%)	2 (1.7%)	3 (2.5%)	6 (5.1%)	16 (13.6%)	6 (5.1%)	5 (4.2%)	30 (25.4%)	12 (10.2%)
Diarrhea and wasting (15)	5 *[3] (33.3%)	10*[4]	0 (0.0%)	2 (13.3%)	1 (6.7%)	4 (26.7%)	3 (20.0%)	1 (6.7%)	1 (6.7%)	3 (20.0%)	6 (40.0%)

PEDV, porcine epidemic diarrhea virus; ETEC, enterotoxigenic *Escherichia coli*; PRRSV, porcine reproductive and respiratory syndrome virus; PCV, porcine circovirus.

*[1] The prevalence of amoebiasis was significantly low in pigs with diarrhea ($p < 0.05$).

*[2] The prevalence of non-amoebiasis was significantly high in pigs with diarrhea ($p < 0.05$).

*[3] The prevalence of amoebiasis was significantly high in pigs with diarrhea and wasting ($p < 0.01$).

*[4] The prevalence of non-amoebiasis was significantly low in pigs with both diarrhea and wasting ($p < 0.01$).

Table 2. Rate of infection with *E. suis*-like, *E. polecki*-like, or coinfection with *E. suis*- and *E. polecki*-like trophozoites in the intestinal lumen and mucosa of pigs with diarrhea and/or wasting in this study

***E. suis*-like**	**Total**	**Lumen*[1]**	**Mucosa*[2]**
Diarrhea	6/73 (8.2%)	6/73 (8.2%)	0/73 (0.0%)
Wasting	14/118 (11.9%)	13/118 (11.0%)	1/118 (0.8%)
Diarrhea and wasting	0/15 (0.0%)	0/15 (0.0%)	0/15 (0.0%)
Total	20/206 (9.7%)	19/206 (9.2%)	1/206 (0.5%)
***E. polecki*-like**	**Total**	**Lumen*[1]**	**Mucosa*[2]**
Diarrhea	6/73 (8.2%)	6/73 (8.2%)	0/73 (0.0%)
Wasting	4/118 (3.4%)	2/118 (1.7%)	2/118 (1.7%)
Diarrhea and wasting	6/15 (40.0%)	2/15 (13.3%)	4/15 (26.7%)
Total	16/206 (7.8%)	10/206 (4.9%)	6/206 (2.9%)

Coinfection of *E. suis*- and *E. polecki*-like	**Total**	***E. suis***		***E. polecki***	
		Lumen*[1]	**Mucosa*[2]**	**Lumen*[1]**	**Mucosa*[2]**
Diarrhea	5/73 (6.8%)	5/73 (6.8%)	0/73 (0.0%)	3/73 (4.1%)	2/73 (2.7%)
Wasting	11/118 (9.3%)	8/118 (6.8%)	3/118 (2.5%)	5/118 (4.2%)	6/118 (5.1%)
Diarrhea and wasting	1/15 (6.7%)	0/15 (0.0%)	1/15 (6.7%)	1/15 (6.7%)	0/15 (0.0%)
Total	17/206 (8.3%)	13/206 (6.3%)	4/206 (1.9%)	9/206 (4.4%)	8/206 (3.9%)

*[1] Subclinical amoebiasis (trophozoites infecting only the lumen).
*[2] Amoebiasis (enteritis or colitis with trophozoites invading the membrane).

Table 3. Molecular identification of *Entamoeba* spp. and coinfecting pathogens in amoebiasis pigs with *Entamoeba* spp. morphologically detected in membrane lesions

Sample no.	**Morphologically observed trophozoite**	**Molecular analysis results**	**Coinfection**	**Immunohistochemistry results**
1	*E. polecki*, *E. suis*	*E. polecki* ST 3	*Lawsonia intracellularis*[*1]	*Lawsonia*
2	*E. polecki*, *E. suis*	*E. polecki* ST 3	*Lawsonia intracellularis*[*1]	*Lawsonia*
5	*E. polecki*	N.D.	*Lawsonia intracellularis*[*1]	*Lawsonia*
12	*E. polecki*	*E. polecki* ST 3	ETEC	*Salmonella*
16	*E. polecki*	N.D.	PRRSV[*2], *Mycoplasma hyorhinis*[*3]	*Salmonella*
19	*E. polecki*	*E. polecki* ST 3	PCV2[*4]	*Salmonella*
22	*E. polecki*	*E. polecki* ST 3	PCV2[*4], *Salmonella* Choleraesuis	*Salmonella*
36	*E. polecki*	*E. polecki* ST 3	*Lawsonia intracellularis*[*1], *Balantidium* spp., *Trichuris suis*	−
37	*E. polecki*	*E. polecki* ST 3	*Lawsonia intracellularis*[*1], *Trichomonas* spp.	−
38	*E. polecki*	*E. polecki* ST 3	PCV2[*4], Rotavirus	*Salmonella*
40	*E. suis*, *E. polecki*	*E. polecki* ST 3	PRRSV[*2], *Pneumocystis carinii, Balantidium* spp.	*Salmonella*
41	*E. suis*	N.D.	PCV2[*4], PRRSV[*2], *Haemophilus parasuis*, *Balantidium* spp.	*Salmonella*
42	*E. polecki*	*E. polecki* ST 3	*Lawsonia intracellularis*[*1], *Balantidium* spp.	*Lawsonia*
50	*E. polecki*	*E. polecki* ST 3	PCV2[*4], *Lawsonia intracellularis*[*1]	*Lawsonia*
52	*E. polecki*	*E. polecki* ST 3	PRRSV[*2], *Haemophilus parasuis, Mycoplasma hyorhinis*[*3]	*Salmonella*
53	*E. polecki*	*E. polecki* ST 3	N.D.	*Salmonella*

*[1] *Lawsonia intracellularis* was detected by PCR in small intestinal mucosa.
*[2] Porcine reproductive and respiratory syndrome virus (PRRSV) was detected by PCR.
*[3] *Mycoplasma hyorhinis* was detected by PCR.
*[4] Porcine circovirus (PCV) 2 was detected by PCR.
N.D., not detected
−: No positive reaction was obtained.

E. suis-like, *E. polecki*-like, and mixed *E. suis*- and *E. polecki*-like trophozoites were detected in 20 (9.7%), 16 (7.8%), and 17 (8.3%) pigs, respectively. Tissue invasion by trophozoites was detected in 16 animals. *E. polecki* infection was observed in approximately 1.1% of the children in Cambodia (Park et al. 2004) and approximately 25% of nonhuman primates at a zoo in the United Kingdom (Regan et al. 2014). *E. polecki* (17%) and *E. suis* (8%) have also been detected in wild boar (Solaymani-Mohammadi et al. 2004). Although farmed pigs are typically slaughtered at approximately six months of age, *Entamoeba* spp. infection rates in farmed pigs were comparable to those of wild boars, although the rate of *E. polecki* appeared to be lower in pigs than in nonhuman primates in captivity. Additionally, *Entamoeba* trophozoites were present in 52.8% of the farms sampled throughout Aichi Prefecture in Japan, albeit at varying frequencies. Most *Entamoeba* trophozoites in this study were found only in the lumen and were not associated with membrane lesions. Typically, infection with either

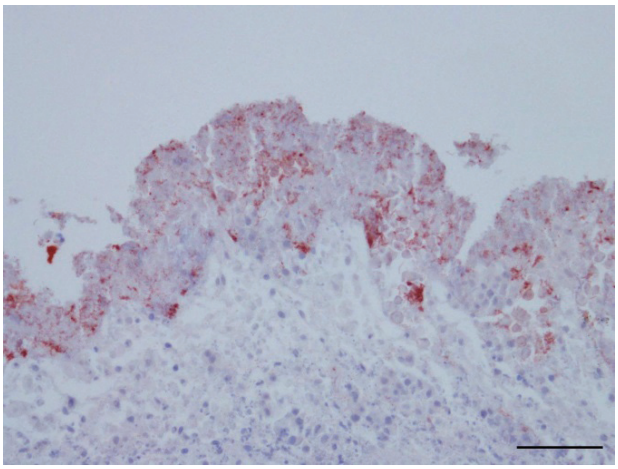

Fig. 3a. Immunohistochemical detection of *Salmonella* polyvalent O-grouping antigen.
Strong positive immunoreactivity is observed in the necrotic membrane of the colon in pig no. 38. Bar = 50 μm.

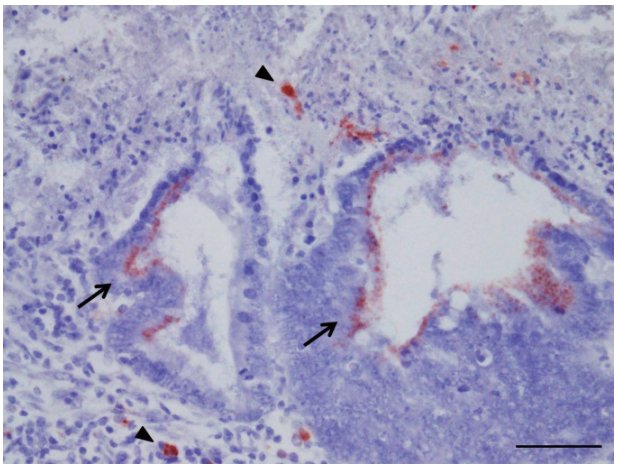

Fig. 3b. Immunohistochemical detection of *L. intracellularis* antigen.
Strong positive immunoreactivity is observed in the colon crypt epithelium (arrows) and cytoplasm of macrophages (arrowheads) in pig no. 42. Bar = 50 μm.

Table 4. Coinfecting pathogens in amoebiasis and non-amoebiasis pigs in this study

	Total	PEDV	Rotavirus	ETEC	*Salmonella* spp.	*Lawsonia intracellularis*	*Cryptosporidium* spp.	*Balantidium* spp.	PRRSV	PCV2
Amoebiasis	16	0	1	1	9	7	0	4	4	5
Non-amoebiasis	190	22	7	17	2	13	9	3	30	17

PEDV, porcine epidemic diarrhea virus; ETEC, enterotoxigenic *Escherichia coli*; PRRSV, porcine reproductive and respiratory syndrome virus; PCV, porcine circovirus.

Table 5. Frequency of *Entamoeba* spp. coinfection with *Salmonella* spp. or *Lawsonia intracellularis*

	Amoebiasis (16)	Subclinical amoebiasis (37)	Non-amoebiasis (153)
Salmonella spp. (11)	9 (56.3%)*[1]	0 (0.0%)	2 (1.3%)
Lawsonia intracellularis (20)	7 (43.8%)*[1]	4 (10.8%)	9 (5.9%)
Not infected with either *Salmonella* spp. or *Lawsonia intracellularis* (175)	0 (0.0%)*[2]	33 (89.2%)	142 (92.8%)

*[1] *Salmonella* spp. and *Lawsonia intracellularis* infections were significantly more common in pigs with amoebiasis than in pigs with subclinical amoebiasis or without *Entamoeba* spp. infection ($p < 0.01$).

*[2] The absence of both *Salmonella* spp. and *Lawsonia intracellularis* was uncommon in pigs with amoebiasis than in pigs with subclinical amoebiasis or without *Entamoeba* spp. infection ($p < 0.01$).

E. polecki or *E. suis* alone does not cause membrane lesions. Because *Salmonella* spp. and *Li* infections were associated with amoebiasis but not with subclinical amoebiasis in this study, most pigs with amoebiasis might have been subclinically infected with *Entamoeba* spp. and displayed membrane lesions due to *Salmonella* spp. or *Li*.

Although *E. suis*- and *E. polecki*-like trophozoites mainly parasitized the large intestine, some were also detected in the small intestine. For instance, we observed *E. polecki* ST3 invasion into small intestinal membrane lesions (from the jejunum to the ileum) and the large intestine in pig no. 42, which displayed severe enteritis and colitis. In humans, *E. histolytica* was found to cause amoebiasis in the small intestine (Mannan et al. 2008), although this has not been reported for other *Entamoeba* spp. The presence of trophozoites in the small intestine suggested that *Entamoeba* can cause not only colitis but also enteritis in pigs. Although most *E. polecki* PCR reactions were successfully performed in this study, we were not able to amplify *E. suis* by PCR (Table 3). Protein–protein, nucleic acid–nucleic acid, and/or protein–nucleic acid cross-linking in formalin-fixed paraffin-embedded tissues and DNA fragmentation were shown to influence gene analysis (Kennedy-Darling & Smith 2014, Do & Dobrovic 2015). In this study, the *E. suis* DNA may have been affected by these factors, although we were not able to definitively determine the cause of PCR failure and sensitivity.

Li genes have been detected in pigs infected with *Entamoeba* spp. (Matsubayashi et al. 2015b), and it has been suggested that enteritis caused by *Entamoeba* spp. can enhance *Li* lesions (Matsubayashi et al. 2015a, 2016). In our study, *Li* was isolated from six pigs and *Salmonella* Choleraesuis was isolated from one pig. In addition, *Salmonella* antigen was detected in nine pigs by immunohistochemistry. Furthermore, coinfection of *Entamoeba* spp. with *Salmonella* or *Li* was observed at a significantly high rate. These results demonstrate that amoebiasis is associated with salmonellosis and *Li* infection. Coincubation of *E. histolytica* with *E. coli* or *Shigella dysenteriae* enhanced amebic adherence and cytotoxicity against Madin-Darby canine kidney cell monolayers (Galván-Moroyoqui et al. 2008). The virulence of *Salmonella* Typhimurium was found to depend on fucose and sialic acid (Ng et al. 2013), whereas glucose starvation enhanced *E. histolytica* virulence, motility, and lectin expression through upstream regulatory element 3 binding protein (Tovy et al. 2011, Gilchrist et al. 2008), suggesting mutual enhancement of the virulence of certain bacteria and amebae in the presence of a sugar source and by degradation of available carbohydrates (Nakada-Tsukui & Nozaki 2016). Thus, *Li* and other *Salmonella* spp. may influence the virulence of *Entamoeba* in pigs.

In this study, amoebiasis was observed in two pigs (2.7%) with diarrhea, nine (7.6%) with wasting, and five (33.3%) with both diarrhea and wasting. Although acute infectious diseases such as PEDV and ETEC appeared to be frequently detected in pigs exhibiting diarrhea, in pigs exhibiting wasting, enteritis appeared to be most often caused by chronic infectious bacteria such as *Li*, followed by *Entamoeba* spp. and *Salmonella* spp. In pigs with both diarrhea and wasting, *Entamoeba* spp. were the most commonly observed, whereas *Salmonella* spp. and *Li* occurred at comparable rates. These results suggest that *Entamoeba* infection can enhance intestinal lesions caused by *Salmonella* or *Li* and prolong the recovery time, resulting in persistent wasting.

In summary, this is the first report on the infection rates of *E. suis* and *E. polecki* and the incidence of severe enteritis in pigs. We found that *Salmonella* spp. and *Li* were important for *Entamoeba* spp. invasion into the membrane and proposed that *Entamoeba* contributes to wasting by exacerbating *Salmonella* spp. or *Li* lesions. However, additional studies are required to identify other potential factors that promote coinfection of *Entamoeba* with other bacteria.

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