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Epidemiological Studies on Intestinal Protozoa in Pigs in Saitama, Japan

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Abstract

We surveyed the rates of internal infection of swine with gastrointestinal tract protozoa by checking the stools of 334 pigs (suckling pigs, growing pigs aged 1 to 6 months and sows or sow candidates) on 8 hog farms in Saitama, Japan from September to November 2009. Oocysts and cysts per gram of stools in all pigs were calculated. Parasite detection rates were statistically analyzed for each of the farms, age groups, and stool condition scores, and the relationship between parasitic infection and diarrhea was investigated. *Cryptosporidium* was detected in 79 pigs (23.7%) of all pigs checked, *Giardia* was found in 53 (15.9%), *Balantidium* in 155 (46.4%), and coccidium in 20 (6.0%). The rate of *Cryptosporidium* infection was higher in the 2- and 3-month-old groups (55.6 to 60.0%) than in the others (2.5 to 27.6%) ($P < 0.05$): oocysts per gram of stools of 10^4 to 10^5 were detected in a total of 33 suckling pigs and 2- and 3-month-old pigs, including 10 of an outdoor farm. There was a correlation between the infection rate and diarrhea in five piglets aged 1 month or younger. In addition, the number of 6-month-old pigs infected with *Giardia* was 40.0%, as opposed to just 5.1% ($P < 0.05$) of suckling pigs. Detection rates differed among farms. The *Balantidium* infection rate was high in pigs older than 4 months (45.0 to 78.9%), as opposed to 15.4 to 17.2% ($P < 0.05$) in pigs younger than 1 month.

Discipline: Animal health
Additional key words: *Balantidium*, coccidium, *Cryptosporidium*, *Giardia*, pig

Introduction

Parasitic protozoa that inhabit the gastrointestinal tracts of pigs include *Cryptosporidium*, *Giardia*, *Balantidium*, coccidium, *Chilomastix*, *Entamoeba*, *Iodamoeba*, and *Tritrichomonas*[16]. Of coccidium species, *Eimeria scabra*[6], *Isospora suis*[23], and *Balantidium coli*[25] are known to cause diarrhea in pigs. *Cryptosporidium* species (*C. suis*, *C.* pig genotype II) has been reported as having weakly pathogenic results in pigs[4,20,21,27]. However, the pathogenicity of *Cryptosporidium* species remains unclear, and there have been only a few pathological studies on the induction of diarrhea by protozoa[13,26]. In this study, we investigated swine parasitism by *Cryptosporidium*, *Giardia*, *Balantidium*, and coccidium by calculating the number of oocysts and cysts per gram of stools in 334 pigs and analyzing the relationships between the detection rate and age group. The stool condition score was also analyzed to investigate the relationship between infection and diarrhea. Using the results of this, the first systematically classified study of swine protozoan infection, we clarified the status of *Cryptosporidium* and other protozoan infections and investigated their relationships with diseases.

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Materials and methods

1. Stool samples

Stools was sampled from 334 pigs in various age groups (39 suckling pigs, 29 pigs aged 1 month, 45 aged 2 months, 45 aged 3 months, 40 aged 4 months, 40 aged 5 months and 20 aged 6 months, as well as 76 sows or sow candidates) on 8 hog farms (A to H) in Saitama prefecture, Japan. For each specimen, the number (1 to 334) and score (formed stool, +; partly unformed stool, ++; and unformed stool, +++) were recorded.

2. Protozoa detection

Cryptosporidium, *Giardia*, *Balantidium*, and coccidium were detected by storing the specimens at 4 °C, sampling 1 gram from each, and condensing and purifying oocysts by a medical general laboratory method using ethyl acetate sedimentation (MGL method). Oocysts of *Cryptosporidium* and *Giardia* in the condensed specimens were detected using fluorescent antibodies (FA). Fifty micrograms of condensed and purified specimens were dried on a 12-holls slide glass (5 mmφ) (Matsunami Glass Industries Ltd., Osaka, Japan) and then reacted with *Cryptosporidium*/*Giardia* FITC-labeled antibody (A400FLR-1X Crypt-a-Glo, Waterborne, USA). Oocysts of *Cryptosporidium* and *Giardia* were counted under an incident-light fluorescence microscope (Olympus BX51, Olympus, Tokyo), and the counts were multiplied by 200. A new set of specimens was prepared and double-stained with *Cryptosporidium*/*Giardia* FITC-labeled antibodies and 4', 6 diamidino-2-phenyl indole (DAPI) to count *Cryptosporidium* sporozoites and *Giardia* under excitation wavelengths of 470 to 490 nm and 360 to 370 nm, respectively. For *Balantidium* detection, 50 µl of specimen was stained with methylgreen formalin saline (MFS) solution[14]; the presence of cysts and trophozoites was confirmed and the numbers counted on a ruled slide glass. In the case of coccidium, numbers were counted in 50 µl of the specimen on a ruled slide glass.

Using examples from reports, we detected based on the protozoa in 1 gram of samples[10,18,19]. The cysts per gram of stools of *Balantidium* and the oocysts per gram of stools of coccidium were determined by multiplying the counts by 200. The values for the oocysts per gram of stools for *Cryptosporidium* and *Giardia* were classified into low (1–10^3), medium (10^4), and high ($\geq 10^5$). The *Balantidium* cysts per gram of stools was classified into low (1–499), medium (500–999), and high (≥1,000). The oocysts per gram of stools of Coccidium were classified into low (1–999), medium (1,000–4,999), and high (≥5,000) (Table 1). These classifications of OPG or CPG counts of each protozoa followed the previous reports[3,7,11,12].

3. Statistical analysis

Detection scores of *Cryptosporidium*, *Giardia*, *Balantidium*, and coccidium were analyzed for each of the hog farms, hog age groups, and stools conditions. A test to highlight significant differences based on these analytical results was conducted.

Results

Cryptosporidium, *Giardia*, and *Balantidium* were detected on all hog farms. Of the 334 pigs tested, *Cryptosporidium* was found in 79 (23.7%), *Giardia* in 53 (15.9%), *Balantidium* in 155 (46.4%), and Coccidium in 20 (6.0%).

1. Differences in detection rates among age groups

Cryptosporidium, *Giardia*, and *Balantidium* were detected in all age groups (Table 1). The detection rates of *Cryptosporidium* were high in 2- and 3-month-old pigs, at 55.6% (25/45) and 60% (27/45), respectively. They were low in suckling pigs at 17.9% (7/39), 1-month-old piglets at 27.6% (8/29), 4-month-old pigs at 20.0% (8/40), 5-month-old pigs at 2.5% (1/40), 6-month-old pigs at 5.0% (1/20), and sows and sow candidates at 2.6% (2/76) (Table 1). The $\chi 2$ test showed a significant difference between 2- or 3-month-old pigs, in which there were high *Cryptosporidium* detection rates, and pigs of other ages; suckling, 1-month-old and 4- to 6-month-old pigs, and sows and sow candidates ($P < 0.05$) (Fig. 1).

Giardia was detected at low rates of 5.1% in suckling pigs (2/39), 8.9% (4/45) in 2-month-old pigs, 13.3% (6/45) in 3-month-old pigs, and 10.0% (4/40) in 4-month-old pigs, but at high rates of 24.1% (7/29) in 1-month-old pigs and 40.0% (8/20) in 6-month-old pigs (Table 1).

Balantidium was detected at low rates of 15.4% (6/39) in suckling pigs, 17.2% (5/29) in 1-month-old pigs, 28.9% (13/45) in 2-month-old pigs, and 31.1% (14/45) in 3-month-old pigs, but at high percentages of 62.5% (25/40) in 4-month-old pigs, 45.0% (18/40) in 5-month-old pigs, 70.0% (14/20) in 6-month-old pigs, and 78.9% (60/76) in sows and sow candidates (Table 1) respectively.

2. Differences in detection rates among farms

The $\chi 2$ test showed no significant differences in *Cryptosporidium* detection rates between 8 hog farms ($P < 0.05$). *Cryptosporidium* in terms of oocysts per gram of stools of 10^4 to 10^5 was detected in ten 2- and 3-month-old pigs on an outdoor hog farm (Farm C) (specimen nos. 98, 100, 101, 102, 103, 104, 105, 109, 114 and 115) (Table 2).

Table 1. Distribution (%) of (oo)cysts and cysts were counted per gram in stools

Age	Sample number	Protozoa	Low	Medium	High	Positive sample
Suckling piglets	39	*Cryptosporidium*	7.7 (3)	7.7 (3)	2.6 (1)	17.9 (7)
	39	*Giardia*	5.1 (2)	0 (0)	0 (0)	5.1 (2)
	39	*Balantidium*	12.8 (5)	2.6 (1)	0 (0)	15.4 (6)
	39	Coccidium	2.6 (1)	2.6 (1)	17.9 (7)	23.1 (9)
1 month	29	*Cryptosporidium*	13.8 (4)	10.3 (3)	3.4 (1)	27.6 (8)
	29	*Giardia*	10.3 (3)	6.9 (2)	6.9 (2)	24.1 (7)
	29	*Balantidium*	6.9 (2)	6.9 (2)	3.4 (1)	17.2 (5)
	29	Coccidium	0 (0)	0 (0)	3.4 (1)	3.4 (1)
2 months	45	*Cryptosporidium*	24.4 (11)	26.7 (12)	4.4 (2)	55.6 (25)
	45	*Giardia*	2.2 (1)	4.4 (2)	2.2 (1)	8.9 (4)
	45	*Balantidium*	20.0 (9)	6.7 (3)	2.2 (1)	28.9 (13)
	45	Coccidium	0 (0)	0 (0)	0 (0)	0 (0)
3 months	45	*Cryptosporidium*	37.8 (17)	22.2 (10)	0 (0)	60.0 (27)
	45	*Giardia*	4.4 (2)	6.7 (3)	2.2 (1)	13.3 (6)
	45	*Balantidium*	17.8 (8)	8.9 (4)	4.4 (2)	31.1 (14)
	45	Coccidium	4.4 (2)	0 (0)	0 (0)	4.4 (2)
4 months	40	*Cryptosporidium*	17.5 (7)	2.5 (1)	0 (0)	20.0 (8)
	40	*Giardia*	7.5 (3)	2.5 (1)	0 (0)	10.0 (4)
	40	*Balantidium*	30.0 (12)	20.0 (8)	12.5 (5)	62.5 (25)
	40	Coccidium	2.5 (1)	2.5 (1)	7.5 (3)	12.5 (5)
5 months	40	*Cryptosporidium*	2.5 (1)	0 (0)	0 (0)	2.5 (1)
	40	*Giardia*	2.5 (1)	12.5 (5)	2.5 (1)	17.5 (7)
	40	*Balantidium*	17.5 (7)	15.0 (6)	12.5 (5)	45.0 (18)
	40	Coccidium	2.5 (1)	5.0 (2)	0 (0)	7.5 (3)
6 months	20	*Cryptosporidium*	5.0 (1)	0 (0)	0 (0)	5.0 (1)
	20	*Giardia*	25.0 (5)	15.0 (3)	0 (0)	40.0 (8)
	20	*Balantidium*	20.0 (4)	20.0 (4)	30 (6)	70.0 (14)
	20	Coccidium	0 (0)	0 (0)	0 (0)	0 (0)
Sows, Sow candidates	76	*Cryptosporidium*	1.3 (1)	1.3 (1)	0 (0)	2.6 (2)
	76	*Giardia*	17.1 (13)	2.6 (2)	0 (0)	19.7 (15)
	76	*Balantidium*	32.9 (25)	17.1 (13)	28.9 (22)	78.9 (60)
	76	Coccidium	0 (0)	0 (0)	0 (0)	0 (0)
Total	334	*Cryptosporidium*	13.5(45)	9.0(30)	1.2(4)	23.7(79)
	334	*Giardia*	9.0(30)	5.4(18)	1.5(5)	15.9(53)
	334	*Balantidium*	9.0(72)	12.3(41)	12.6(42)	46.4(155)
	334	Coccidium	1.5(5)	1.2(4)	3.3(11)	6.0(20)

Low, medium and high; the numbers of (oo)cysts per gram of stools is defined respectively for *Cryptosporidium* and *Giardia* as: $1\sim10^3$, 10^4, $\geq10^5$.
Low, medium and high; the number of cysts per gram of stools is defined respectively for *Balantidium*: 1~499, 500~999, ≥1,000.
Low, medium and high; the number of oocysts per gram of stools is defined respectively for Coccidium: 1~999, 1,000~4,999, ≥5,000.
( ) : Positive sample number.

3. Differences in detection rates by feces condition score

For each stool condition score, the pigs were classified into three groups (low, medium, and high) based on the number of parasites. The relationship with diarrhea was analyzed by conducting a χ2 test, which showed no significant differences among the groups (Table 3). *Cryptosporidium* in terms of oocysts per gram of stools of 10^4

Table 2. Result of FA performed on fecal specimens collected from pigs known to be positive (>10^4) for *Cryptosporidium* oocysts per gram of stools

Farm	Sample number	Group	*Cryptosporidium*	*Giardia*	*Balantidium*	Coccidium	Properties of stools[1)]
A	13	<1 month	12,500	5,000	0	0	+
	16	2 months	255,000	0	0	0	+
B	51	2 months	17,500	0	0	0	++
	53	2 months	17,500	0	500	0	++
	54	2 months	12,500	0	0	0	++
	57	3 months	15,000	32,500	0	0	++
	58	3 months	15,000	25,000	0	0	++
	60	3 months	15,000	0	0	0	++
C	82	<1 month	162,500	0	0	0	+
	98	10 weeks	12,500	0	0	0	++
	100	10 weeks	40,000	0	0	0	++
	101	10 weeks	87,500	0	0	0	+
	102	10 weeks	37,500	0	1,000	0	+
	103	10 weeks	37,500	5,000	500	0	+
	104	10 weeks	87,500	0	1,000	0	++
	105	10 weeks	150,000	0	0	0	++
	109	12 weeks	17,500	0	0	0	++
	114	15 weeks	12,500	0	0	1,000	++
	115	15 weeks	10,000	0	1,000	0	++
D	156	3 months	57,500	0	5,000	0	+
	157	3 months	20,000	0	0	0	+
	159	3 months	25,000	0	500	0	+
	160	3 months	27,500	0	1,000	0	+
E	174	Sows, Sow candidates	20,000	0	1,000	0	++
	188	1 month	12,500	0	2,500	0	++
	200	4 months	17,500	0	1,000	0	+
F	224	<1 month	85,000	0	0	0	++
	228	<1 month	20,000	0	0	21,500	++
	233	2 months	12,500	0	500	0	++
G	276	2 months	70,000	>125,000	500	0	+
	278	3 months	10,000	>125,000	0	0	+
H	306	1 month	35,000	75,000	0	0	+++
	308	1 month	125,000	>125,000	0	0	+++
	309	1 month	45,000	12,500	0	0	+
	314	2 months	25,000	30,000	500	0	+

1) : +++ : Unformed stool, ++ : Partly unformed stool, + : Formed stool

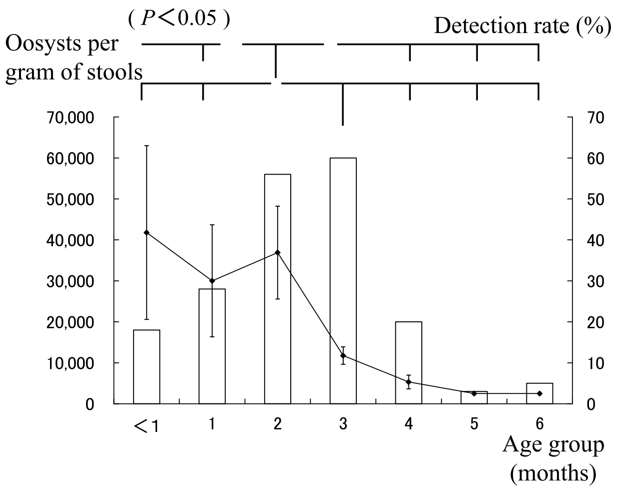

Fig. 1. ***Cryptosporidium*** **detection rates (bars) and oocysts per gram of stools (line) for each age group**
There was a 5% significant difference in detection rates between the 2- and 3-month-old groups and the other age groups. High number of oocysts per gram of stools were detected in suckling pigs and in 1- month-old or 2-month-old pigs (mean ± standard error).

to 10^5 was detected in five piglets aged 1 month or younger; these piglets had partly unformed or unformed stools (specimen nos. 188, 224, 228, 306 and 308) (Table 2).

Discussion

Comparison of the *Cryptosporidium* detection rates in each age group with those reported previously showed differences in detection rates[2,5,8,11,15,17,20,22,24,31]. Possible factors behind these differences included differences in the methods used to condense, purify, and detect *Cryptosporidium*, or in rearing conditions and environmental factors (temperature and humidity). In previous reports, detection rates of *Cryptosporidium* were high in 1- to 3-month-old pigs[8,17,20,22] and low in sows and sow candidates[31]. As with previous reports, in this study, detection rates of *Cryptosporidium* were significantly higher in 1-month-old pigs (27.6%, 8/29), 2-month-old pigs (55.6%, 25/45) and 3-month-old pigs (60.0%, 27/45) than in the other age groups (P < 0.05), and lower in sows and sow candidates (2.6%, 2/76) than in the other age groups. These results coincide with previous reports[8,17,20,22,31].

As proved by our study, pigpen structure and rearing control methods can affect the detection rates of *Cryptosporidium*, which were also reportedly higher in the stools of pigs grown outdoors than those of pigs reared indoors[1,20]. In our study, the detection rate in 2- to 3-month-old pigs grown in a simple pigpen installed outdoors (70%, 14/20, Farm C) exceeded that in pigs of the same age reared in indoor facilities (54.3%, 38/70, Farms

Table 3. Infection intensity of *Cryptosporidium*, *Giardia*, *Balantidium* and coccidium in the pigs with the diarrhoeal and non-diarrhoeal

Level of infection	Unformed stool (n=7)				Partly unformed stool (n=103)				Formed stool (n=224)			
intensity	*Cryptosporidium*	*Giardia*	*Balantidium*	Coccidium	*Cryptosporidium*	*Giardia*	*Balantidium*	Coccidium	*Cryptosporidium*	*Giardia*	*Balantidium*	Coccidium
Positive[1)]												
Low	1	0	1	0	17	6	19	1	26	23	52	5
Medium	1	1	0	0	17	5	10	3	13	12	31	9
High	1	1	0	0	1	2	6	6	2	3	36	10
Total	3[a)]	2[b)]	1[c)]	0	35	13	35	10	41[d)]	38[e)]	119[f)]	24
Negative	4[a)]	5[b)]	6[c)]	7	68	90	68	93	183[d)]	186[e)]	105[f)]	200

1) Low, medium and high (oo)cyst per gram of stools is defined respectively for *Cryptosporidium* and *Giardia* as: 1~10^3, 10^4, ≥10^5.
Low, medium and high cyst per gram of stools is defined respectively for *Balantidium*: 1~499, 500~999, ≥1,000.
Low, medium and high oocyst per gram of stools is defined respectively for Coccidium: 1~999, 1,000~4,999, ≥5,000.
a)-d), b)-e), c)-f): Two samples were compared by a χ^2 (P<0.05).
n: Total sample number of unformed stool samples, partly unformed stool samples, and formed stool samples, respectively.

A, B, and D to H). Furthermore, 10 of the 2- to 3-month old pigs grown outdoors (Farm C) showed high *Cryptosporidium* oocysts per gram of stools of 10^4 to 10^5 (Table 2).

We determined oocysts or cysts per gram of stools of protozoa[10,18,19]. After determining the number of oocysts or cysts per gram from stools, we investigated the relationship between infection and diarrhea of the specimens. No significant relationship was found between parasitic infection and diarrhea, i.e. the stool condition score. However, *Cryptosporidium* oocysts per gram of stools of 10^4 to 10^5 was detected in five pigs aged 1 month or younger with partly unformed or unformed stools (specimen nos. 188, 224, 228, 306 and 308). In humans and cattle, unformed stools in *Cryptosporidium* infection reportedly contained as many as 10^5 to 10^6 oocysts per gram of stools[28,29]. Experimental *Cryptosporidium* infection can cause serious diarrhea, loss of appetite and vomiting in suckling pigs[4]. Diarrhea caused by *C. suis* in outdoor-grown suckling pigs has also been reported[21], suggesting *C. suis* infection as a potential cause of diarrhea in the five pigs in our study that produced partly unformed or unformed stools with oocysts per gram of stools of 10^4 to 10^5. Conversely, it has been reported that diarrhea in 1- to 6-week-old pigs is unrelated to infection with *C.* pig genotype II or *C. suis*[20]. Therefore, further investigations are needed to clarify the relationship between diarrhea in suckling pigs and dense infection with *Cryptosporidium* species, and also ascertain the factors allowing *Cryptosporidium* growth in the intestines of 2- to 3-month-old pigs.

Diarrhea caused in pigs by *Cryptosporidium* allows increased superinfection with other pathogens such as rotavirus (Rota V), porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, and *Escherichia coli*[4,13,26]. Rota V infection facilitates the growth of *Cryptosporidium* in the digestive tract of the pig and increases mortality rates[4]. Pigs showing declines in immune function under compromised conditions can develop diarrhea due to exposure to numerous *Cryptosporidium* oocysts under stressed conditions[13,26]. This report was become evident that young pigs with diarrhea were infected with large numbers of *Cryptosporidium* oocysts in their stools. But it was considered to necessary that more detail examine the relationship between *Cryptosporidium* and other pathogen infections.

Giardia duodenalis, which infects pigs, is classified into assemblage A, which causes zoonosis, and assemblage E which infects ungulates[1,24]. *Giardia* infection can cause diarrhea in calves[30], lambs[15], and horses[9], but not in pigs[1,31], this agrees with the results of our study. We detected *Giardia* in 5.1 to 40.0% of pigs, and this finding agreed with reported detection ratios of 3.0 to 40.0%[11,15,31]. Detection of *Giardia* has been reported to be higher in pigpens on concrete floors than on drainboard floors[11,31]. We considered that more needs to be further explored the relationships between feeding environment and Giardia detection.

Balantidium, which infects the ileum and colon in pigs, is widely believed not to produce clinical signs, although it has been reported to cause diarrhea[25]. We found no relationship between *Balantidium* infection and diarrhea. Detection rates of *Balantidium* can increase as pigs grow[7], and our findings supported this trend (Table 2).

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