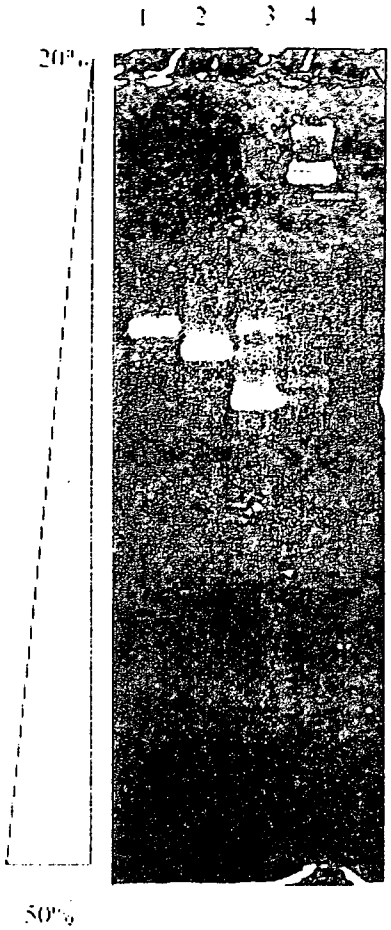

Fig. 2 Ethidium bromide staining of acrylamide gel applied DGGE. *Lane 1*, *C. muris* RN66 strain; *Lane 2*, *C. muris* Japanese field mouse genotype; *Lane 3*, *C. andersoni* Kawatabi strain; and *Lane 4*, *C. parvum* HNJ-1 strain, respectively. *Numbers of percentage* and *triangle on the left* of figure indicate the gradient of concentration of the chemical denaturing solution

PCR-RFLP is based on the presence or absence of restriction enzyme cleavage sites on genes. In contrast, as DGGE relies on the base composition of a DNA fragment, it was developed to avoid failure of detection due to the absence of alternative restriction enzyme cleavage sites (Myers et al.). We confirmed that PCR amplicons of the *C. muris* RN66 strain and *C. andersoni* Kawatabi strain could not be differentiated using PCR-RFLP, as reported previously (Awad-el-Kariem et al.). Furthermore, because *C. andersoni* and the two genotypes of *C. muris* showed different banding patterns, DGGE is useful for differentiating species that cannot be differentiated by PCR-RFLP. In particular, despite the absence of restriction enzyme cleavage sites, this method can be used for the discrimination of the genotypes of *C. muris*.

Although it is necessary to carry out further studies by using a number of species and genotypes of *Cryptosporidium*, this DGGE method will be available for the discrimination of *Cryptosporidium* species and genotypes because the minor difference of the nucleotide sequence of the PCR fragments, such as 1 bp, can be differentiated as mentioned for bacteria (Muyzer et al.).

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Short communication

Isolation of *Cryptosporidium andersoni* Kawatabi type in a slaughterhouse in the northern island of Japan

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Abstract

Fecal samples were collected from 325 adult cattle and 108 pigs in a slaughterhouse in Hokkaido, the northern island of Japan. Five adult cattle were found to be positive for oocysts of *Cryptopsoridium* (1.5%). The oocysts were morphologically similar to those of *Cryptosporidium andersoni*. The partial sequence of the 18S rRNA gene of the isolate was 100% identical with that of the *C. andersoni* Kawatabi strain. SCID mice were infected after oral administration. Based on the morphology of the oocysts, the sequence of the 18S rRNA gene and the infectivity to SCID mice, the isolate was concluded to be of the same type as the *C. andersoni* Kawatabi strain that has been isolated in Honshu, the main island of Japan.

Keywords: Cattle; *Cryptosporidium andersoni*; Slaughterhouse

1. Introduction

Cryptosporidium in the stomach of mice was first described by Tyzzer in 1907. This parasite has been reported to be present in a wide range of hosts including mammals, birds, reptiles and fishes (Fayer, 1997). It causes chronic diarrhea, especially in calves and immunocompromized human beings. Outbreaks of cryptosporidiosis have been reported worldwide, including in Japan. In 1996, approximately 9000 patients were reported (Yamamoto et al., 2000) in the Saitama prefecture in Honshu, which is the main island of Japan. In Hokkaido, which is the northern island of Japan and the main area for milk and beef production, Akamatsu et al. (1987) first reported cryptosporidial infection in cattle. Later, many cases of bovine infection were reported in various areas of Hokkaido (Saga et al., 1987; Sakai et al., 2003; Shimizu et al., 1990). However, the slaughterhouses in this area have not been extensively surveyed. There-

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fore, we investigated *Cryptosporidium* oocysts in livestock in a slaughterhouse in Hokkaido and characterized the isolated oocysts biologically and genetically.

2. Materials and methods

Rectal feces were collected from 325 adult cattle and 108 adult pigs in a slaughterhouse in Hokkaido in September 2001. All the fecal samples were maintained at 4 °C. The ages of the cattle were between 2 and 10 years and all of them were Holsteins. The age of the pigs was approximately 6 months. Oocysts were detected using the sucrose centrifugal flotation method. The DNA from the detected oocysts was extracted using the MagExtractor-Genome (Toyobo, Osaka, Japan), following the manufacturer's instructions. We designed a primer set specific to the partial sequence of 18S ribosomal RNA (rRNA) gene; it was based on the sequences of a bovine and a hylax strain of *Cryptosporidium muris* (GenBank accession numbers: AF093496 and AF093498), using Oligo 5.0 (National BioScience Inc., Plymouth, MN). The specific primer set was 5′-AACTTTACGGATCGCATCTCTGA-3′ and 5′-CCCATCACCATGCATACTCATAA-3′. The partial sequence of 18S rRNA gene was amplified by PCR using the primer set. PCR amplification was performed in 1× PCR buffer, 1 mM $MgSO_4$, 0.2 mM dNTPS (each), 0.3 μM of each primer and 1.0 unit KOD polymerase (Toyobo, Osaka, Japan) (final concentrations). The reactions were pre-heated at 94 °C for 2 min and cycled 40 times at 94 °C for 15 s, 58 °C for 30 s, 68 °C for 2 min and then 68 °C for 10 min. The PCR fragment of 18S rRNA was cloned using the pT7 Blue Perfectly Blunt Cloning Kit (Novagen, Darmstadt, Germany). The PCR products were purified using the MagExtractor-PCR and Gel Clean up Kit (Toyobo, Osaka, Japan). The purified PCR products were sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Japan Ltd., Tokyo, Japan), according to the manufacturer's instructions, in an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.). Sequence accuracy was confirmed by bi-directional sequencing. In order to assess the infectivity of the isolate, we orally inoculated 1×10^5 purified oocysts into three 4-week-old female SCID mice. The feces of each mouse were collected and the discharge of oocysts was monitored for 204 days. All the mice were maintained in accordance with the Tohoku University's guidelines for animal experiments.

Table 1
Characteristics of cattle discharging *Cryptosporidium* oocysts

Cattle no.	Age (year)	Sex[a]	Fecal condition[b]
1	7	F	N
2	6	F	N
3	2	C	D
4	4	F	D
5	8	F	D

[a] F: female, C: cast.
[b] N: normal, D: diarrhea.

3. Results

Cryptosporidium oocysts were detected in 5 of 325 adult cattle (Table 1). The oocysts were not detected in any of the pigs. These oocysts were ovoid in shape and (7.9 ± 0.9) × (5.2 ± 1.1) μm in size (Table 2). Since many oocysts were obtained only from No. 1 cattle, sequences and experimental infection were performed with the oocysts from No. 1 cattle. Partial sequences for the 18S rRNA gene of the oocysts discharged from No. 1 cattle (shown in Table 1) and from mice infected with oocysts from No. 1 cattle were sequenced (1253 bp). The DNA sequences of both oocysts showed a 100% similarity with that of the *Cryptosporidium andersoni* Kawatabi strain, a 100% similarity with that of *C. muris* calf genotype and a 98% similarity with that of *C. muris* mouse genotype (IDRH-13) (GenBank accession numbers: AB089285, AF093496 and AF093498, respectively). All the SCID mice infected with the oocysts showed fecal oocysts

Table 2
The measurements of oocysts (n = 50)

Cattle no.	Size (μm)[a]
1	(7.62 ± 0.44) × (5.48 ± 1.05)
2	(7.34 ± 1.11) × (4.76 ± 1.09)
3	n.d.
4	(7.06 ± 0.99) × (5.22 ± 1.10)
5	n.d.
1-p[b]	(7.75 ± 1.14) × (5.58 ± 1.33)

[a] Mean ± S.D.
[b] Oocysts from mice infected with oocysts discharged from No. 1 cattle.

Table 3
Oocyst discharge[a] from mice inoculated with 1×10^5 *Cryptosporidium* oocysts

Mouse no.	Days after inoculation																			
	−1	1	2	3	4	5	6	7	8	9	10	17	24	38	66	87	108	129	150	171
1	–	–	–	–	–	–	–	–	+	+	+	+	++	+++	+++	+++	+++	+++	+++	+++
2	–	–	–	–	–	–	–	–	+	+	+	+	++	+++	+++	+++	+++	+++	+++	+++
3	–	–	–	–	–	–	–	–	+	+	+	+	++	+++	+++	+++	+++	+++	+++	+++

Mouse No. 1 died on day 198 PI, No. 2 died on day 204 PI.
[a] The number of oocysts in a microscopic field: (+) 1–2, (++) 10–30, (+++) >30.

from day 8 post-inoculation until day 204, which was the last day of the experiment (Table 3).

4. Discussion

C. andersoni Kawatabi strain is first described by Satoh et al. (2003) and subsequently reported by Matsubayashi et al. (2004), which isolate for the first time in Hokkaido, and Nakai et al. (2004). Matsubayashi et al. (2004) isolated oocysts from a 1-month-old calf and identified them to be of the Kawatabi type of *C. andersoni*, based on the genetic analysis and transmission experiment in SCID mice. In the present study, we detected in 5 of 325 fecal samples from cattle aged between 2 and 7 years on the same island, Hokkaido. Nakai et al. (2004) detected the *C. andersoni* Kawatabi strain from neonatal calves and adult cattle on the main island of Japan and observed that the positive cattle discharged the oocysts for several years. Therefore, the Kawatabi type of *C. andersoni* may widely spread in young and adult cattle in Japan.

C. muris or *C. muris*-like oocysts were reported to be present in cattle in many slaughterhouses in Japan, and the frequency of its occurrence was 4.7% in Miyagi (Okazaki, 1996), 1.2% in Fukushima (Miyano et al., 1999), 1.2% in Saitama (Nakamura et al., 1998), 3.9% in Kanagawa (Kojima, 1997), 2.8% in Shizuoka (Suzuki et al., 1997) and 3.4% in Tokushima prefecture (Tani et al., 1999). These areas are on the main or the southern island of Japan. This is the first report on the survey of *Cryptosporidium* large-type oocysts from a slaughterhouse in the northern island (Hokkaido). In this investigation, the positive rate of detection was found to be 1.5%. This rate is considered to be similar to the positive rates in other areas in Japan.

No *Cryptosporidium* oocysts were detected in pigs in this survey; this finding is in agreement with the previous reports from Fukushima (Miyano et al., 1999), Miyagi (Nakai et al., 2004; Okazaki, 1996) and Saitama (Nakamura et al., 1998). Although the rate of detection was low, *Cryptosporidium* oocysts were detected from pigs in Aichi (1.9%) (Tsubouchi et al., 1999). These results suggest that *Cryptosporidium* invariably existed (1.2–4.7% positivity) in cattle and was negligible in pigs in the slaughterhouses in Japan.

Oocysts isolated from cattle in the U.S., previously identified as *C. muris*-like oocysts, did not infect mice or even cattle (Lindsay et al., 2000). Oocysts similar to those from the cattle in the U.S. were not transmissible to neonatal or adult BALB/c mice and SCID mice (Koudela et al., 1998). However, Pavlasek (1994), Kaneta and Nakai (1998), Satoh et al. (2003) and Matsubayashi et al. (2004) reported the successful transmission of bovine-derived large oocysts to SCID mice. Satoh et al. (2003) observed that the isolate in the Miyagi prefecture differed from the published strains of *C. andersoni* in terms of its ability to infect SCID mice and concluded that it was a novel type of *C. andersoni* and referred to it as the *C. andersoni* Kawatabi strain.

Satoh et al. (2003) sequenced the partial DNA sequence of the 18S rRNA gene (1255 bp) of the *C. andersoni* Kawatabi strain. Sréter et al. (2000) registered only a sequence of 265 bp of *C. andersoni* in GenBank (accession number: AJ275963), but the sequence was homologous to the *C. andersoni* Kawatabi strain. The detailed sequencing of the original U.S. strain of *C. andersoni* should be performed for comparison with isolates from other countries. However, the original U.S. isolate has not been maintained (personal communication with Dr. Lindsey).

In this study, the size, form and structure of the isolates were found to be closely similar to those of the

C. andersoni Kawatabi strain ((7.4 ± 7.6) × (5.1 ± 5.9) μm) (Satoh et al., 2003) and the original U.S. strain of *C. andersoni* ((6.0 ± 8.1) × (5.0 ± 6.5) μm) (Lindsay et al., 2000). The DNA sequence of the 18S rRNA gene of the isolate was found to be 100% identical with the *C. andersoni* Kawatabi strain and the original U.S. strain. The isolate in this study is *C. andersoni*, and it is one of the members of the type of *C. andersoni* Kawatabi strain because of its infectivity to SCID mice. Therefore, we may refer to this type as *C. andersoni* Kawatabi type.

Further studies are required to clarify the distribution of the Kawatabi type of *C. andersoni* in Japan and in other countries and to define the genetic and biological differences among types of *C. andersoni*.

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Morphological and Immunohistochemical Features of *Cryptosporidium andersoni* in Cattle

K. Masuno, T. Yanai, A. Hirata, K. Yonemaru, H. Sakai, M. Satoh, T. Masegi, and Y. Nakai

Abstract. Light and electron microscopic features and immunohistochemical features of *Cryptosporidium andersoni* (*C. andersoni*) and host reaction in the mucosa were studied. Although the affected cattle demonstrated no apparent clinical signs, a severe infection of *C. andersoni* was observed in the abomasum. *C. andersoni* were round in shape, measured 6–8 μm in size and were mainly observed to be freely located in the gastric pits, being attached in occasional cases to the surface of the abomasum epithelium. Frequent inflammatory cells had infiltrated the lamina propria of the affected mucosa, and frequent mitotic figures were observed in epithelial cells at the dilated isthmus. To access the cell kinetics, the number of epithelial cells infected with *C. andersoni* were counted and compared with noninfected cattle. The number of gastric pit cells in infected cattle was significantly higher than that in the controls. The number of proliferative cells determined by the Ki-67 antigen in *C. andersoni* infected cattle was also significantly higher than that in the controls. Transmission electron microscopy and scanning electron microscopy revealed that the morphology of the *C. andersoni* organism was common to those of other *Cryptosporidium* spp. Immunohistochemically, several commercial antibodies against *Cryptosporidium* spp. showed positive reactions at the wall of these oocysts or parasitophorous vacuoles. This report is possibly the first to discuss the prominent hyperplasia of the abomasum mucosa, as well as morphologic features of *C. andersoni* in cattle.

Key words: Abomasum; cattle; cryptosporidium; hyperplasia; immunohistochemistry; ultrastructure.

Genus *Cryptosporidium* is an intracellular protozoan parasite and causes gastrointestinal disease in a wide variety of mammals and other vertebrate species worldwide.[1,9,11] *Cryptosporidium* spp. are transmitted by the ingestion of oocysts that are excreted in the feces of humans and animals.[9,14,17,22] In cattle, three subspecies of *Cryptosporidium* have been reported: *Cryptosporidium parvum*,[9,10,20] *Cryptosporidium felis*,[3] and *Cryptosporidium andersoni*, recently renamed from *Cryptosporidium muris*.[2,4,8,10,12,13,18,20]

Cryptosporidium resides on the apical surface of intestinal epithelial cells. It is viewed as a minimally invasive, mucosal pathogen, which causes infection at the microvillus border of the gastrointestinal epithelium. However, infection elicits a cell-mediated response following both primary and secondary infections.[9] Some *Cryptosporidium* spp. cause acute diarrhea in immunocompetent animals and chronic life-threatening disease in immunocompromised animals.[15,23]

Many studies have been conducted on morphology, immunohistochemistry and epidemiology for some species of *Cryptosporidium*, particularly *C. parvum*.[5,9] *C. andersoni* is a newly established member of *Cryptosporidium* spp., and there have been few reports on morphologic studies in cattle, including histopathological and ultrastructural features, as well as immunohistochemical reactivity. Furthermore, there has been no study on the histopathological alterations in the mucosa of host animals.

In the present study, we attempted to clarify the histopathological characteristics of host reactions in the mucosa, as well as morphological characteristics of *C. andersoni*, by using light and electron microscopy.

Eight Japanese black hair cattle with or without fecal excretion of *Cryptosporidium* spp. were examined. Four cattle (Nos. 1 and 3–5) were obtained from the farm of Tohoku University in Kawatabi, Miyagi, Japan. The other cattle (Nos. 2 and 6–8) were kept at dairy farms in Gifu Prefecture and were sent to Gifu University. In four cattle maintained at the farm of Tohoku University, oocysts of *Cryptosporidium*. spp. were collected from fecal samples using the sucrose flotation method and were identified as *C. andersoni* by sequencing the 18S ribosomal DNA, heat-shock protein 70 and oocyst wall protein genes.[18] Oocysts of *C. andersoni* were defecated for at least 14 months in cattle No. 1; 31 months in cattle Nos. 3 and 4; and 35 months in cattle No. 5 before euthanization, respectively (Table 1). The cattle were euthanatized, and complete necropsy was done. Organs and tissues, including the liver, spleen, kidneys, heart, lungs, rumen, reticulum, omasum, abomasum, ileum, cecum, colon, and adrenal glands, were collected and fixed in 10% buffered formalin and embedded in paraffin. For the abomasum, three samples were collected from the anterior, middle, and posterior parts. Specimens of abomasum from three Japanese black hair cattle with no lesions were used as a control (Nos. 6–8). Each of the sections was cut at

Table 1. Summary of the clinical data of cattle.

Cow No.	Age	Sex*	Origin	Period of Shedding Oocyst	Species of *Cryptosporidium*
1	4y2m	F	Miyagi	<14m	*Cryptosporidium andersoni*
2	6y	F	Gifu	ND	*Cryptosporidium* spp.
3	3y	F	Miyagi	31m	*Cryptosporidium andersoni*
4	3y	F	Miyagi	31m	*Cryptosporidium andersoni*
5	3y1m	F	Miyagi	35m	*Cryptosporidium andersoni*
6	9m	M	Gifu	ND	ND
7	5m	M	Gifu	ND	ND
8	10m	M	Gifu	ND	ND

* M = male; F = female; ND = not done.

a thickness of 4 μm, stained with hematoxylin and eosin (HE), and examined by light microscopy. In the three infected cattle (Nos. 3–5) and the three control cattle (Nos. 6–8), the number of cells was counted at 10 gastric pits per cow.

Samples of the abomasums from three cattle (Nos. 3–5) were prepared for transmission electron microscopy (TEM). The specimens were rinsed in phosphate buffer, postfixed with 1% buffered osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in epoxy resin. Individual samples were sectioned at 1 μm and stained with toluidine blue. Areas where *C. andersoni* were present were selected by light microscopy, and thin-cut sections, mounted on copper grids, were stained with lead citrate/uranyl acetate and examined with TEM (Hitachi, Tokyo, Japan, H-8100). Samples of formalin-fixed abomasum from one cow (No. 3) were washed in phosphate buffer and directly examined with scanning electron microscopy (SEM) (Hitachi, Tokyo, Japan, S-3000N) under low-vacuum conditions (150 Pa).

Immunohistochemical stain was determined in four of the infected cattle (Nos. 1, 3–5) and three of the control cattle at the anterior part of the abomasum. The stain was performed on prepared slides using the avidin-biotin-horseradish peroxidase (ABC) method. The primary antibodies used were mice monoclonal antibodies against *Cryptosporidium parvum* oocyst (Chemicon, Temecula, CA), *Cryptosporidium parvum* (Argene, Varilhes, France), and *Cryptosporidium* (VMRD, Pullman, WA). For analysis of cell kinetics at the affected sites, immunohistochemistry for Ki-67 antigen was conducted, and examination was made by light microscopy. In the three infected cattle (Nos. 3–5) and three control cattle (Nos. 6–8), the total number of epithelial cells and the number of Ki-67-positive epithelial cells were counted at 10 gastric pits per cow, and the percentage of Ki-67-positive epithelial cells in the three infected cattle was compared with the three noninfected controls. The number of gastric pit cells and the incidence of Ki-67-positive cells in the affected cattle and noninfected control cattle were expressed as averages ± SD and compared using the Wilcoxon signed rank test. Differences between groups were considered significant if the probability values evinced under 0.01.

Grossly, no abnormalities were observed in any organs or tissues, including the gastrointestinal tract, in any case examined.

Histopathologically, no abnormalities were detected except in the abomasum. In the abomasum of all five positive cases, numerous weakly basophilic, round-to-oval organisms measuring 6–8 μm in size were observed on the apical surface of the epithelium in the gastric pits at the anterior and middle parts (Fig. 1). The length of the gastric pits was significantly extended, and the number of epithelial lining cells had increased in comparison with the noninfected control cattle (Fig. 2a, 3a). The average number of gastric pits cells for three *C. andersoni* infected cattle (291.9 ± 55.3 SD) was found to be significantly greater than that of the three control cattle (146.7 ± 29.2 SD). The level of significance was less than 0.01 in nonparametric testing. Many fragment nuclear epithelial cells were observed at the isthmus of the gastric pits (Fig. 1). In the lamina propria of the affected mucosa, mild-to-moderate diffuse infiltration of lymphoid cells,

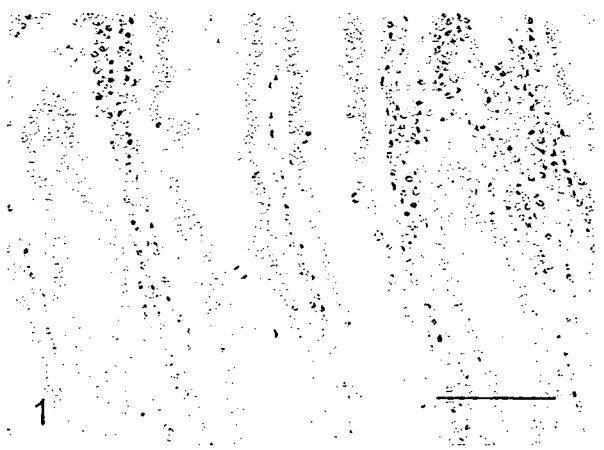


Fig. 1. Abomasum; cow No. 3. Numerous *C. andersoni* in gastric pits with diffuse infiltration of inflammatory cells in the lamina propria, and mitotic figures of epithelial cells. HE, paraffin section. Bar = 100 μm.

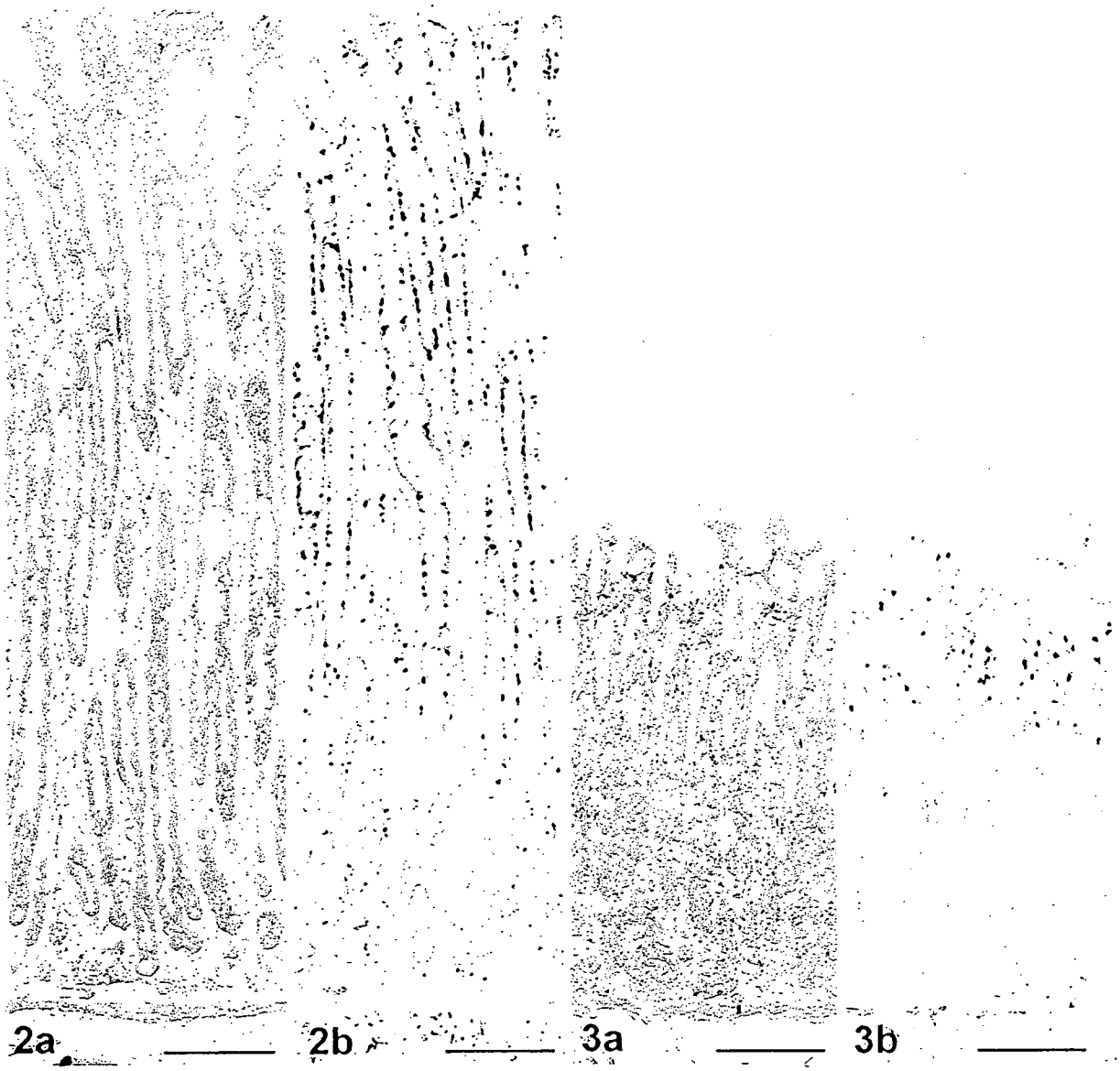


Fig. 2a. Abomasum; cow No. 5. The length of the gastric pits is greatly extended because of increase in the number of epithelial cells. HE, paraffin section. Bar = 200 µm. **Fig. 2b.** Abomasum; cow No. 5. Cells with brown nuclei in all active phases of the cell cycle. The length of the gastric pit is greatly extended due to increased numbers of gastric pit cells. Immunohistochemical stain for Ki-67 antigen, paraffin section. Bar = 200 µm.

Fig. 3a. Abomasum; cow No. 6. No lesions. HE, paraffin section. Bar = 200 µm. **Fig. 3b.** Abomasum; cow No. 6. No lesion. Immunohistochemical stain for Ki-67 antigen, paraffin section. Bar = 200 µm.

plasma cells, and eosinophils and mild edema were observed (Fig. 1). There were no lesions in the mucosal epithelium of the posterior part of the abomasum.

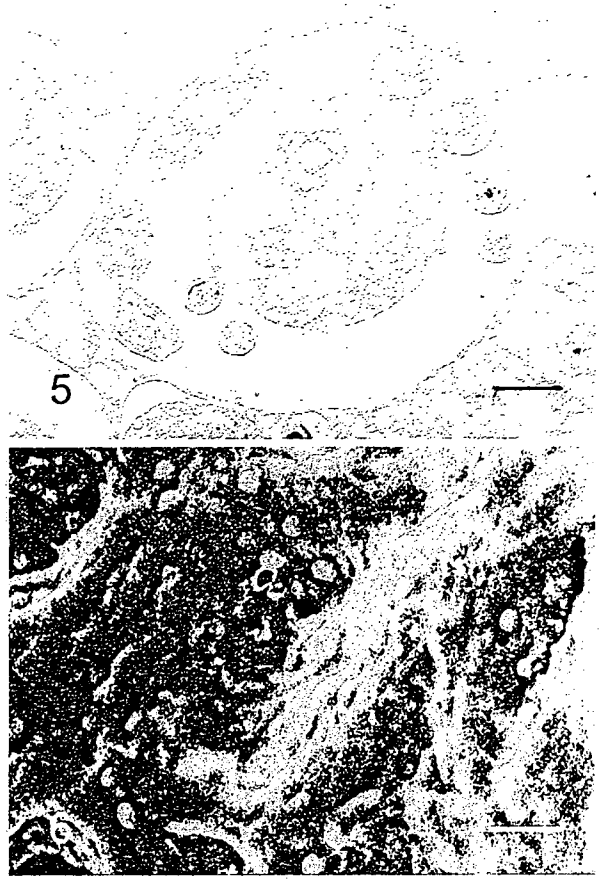
TEM revealed various life-cycle stages of *C. andersoni* on the epithelial surface of the gastric pits. Trophozoites enveloped the microvilli lining on the surface of the mucosal epithelium in the gastric pits, and were located within a parasitophorous vacuole. They exhibited well-developed feeder organelle and a rough endoplasmic reticulum in the cytoplasm (Fig. 4). Generally, schizonts contained eight merozoites within a parasitophorous vacuole (Fig. 5).

Under SEM, numerous round-to-spherical organisms consistent with *C. andersoni* were observed on the

Fig. 4. *C. andersoni*; cow No. 3. Trophozoite attaching to the epithelial cells of gastric pits with large parasitophorous vacuole. TEM. Bar = 1 μm.

surface of the abomasum mucosal epithelium (Fig. 6). Occasionally, hatching organisms of *C. andersoni* and the remaining shells were observed.

Immunohistochemically, in the infected cattle that were examined (Nos. 1 and 3–5), most of the weakly basophilic, oval organisms in the gastric pits showed a positive reaction for primary antibodies against *Cryptosporidium parvum* oocyst (Chemicon, Temecula, CA), *Cryptosporidium parvum* (Argene, Varilhes, France) and *Cryptosporidium* (VMRD, Pullman, WA) (Fig. 7). Those organisms that were reactive to antibodies were larger in size than those observed with HE stain. The Ki-67 antigen positive proliferation cells were clearly identifiable by their brown nuclei. A large number of epithelial cells showed positive reaction in the cervix portion of the gastric pits (Fig. 2b). The length of the cervix portion of the gastric pits was largely extended in comparison with the noninfected control cattle (Figs. 2b, 3b). The average number of Ki-67-positive index in the gastric pits in the three cattle with *C. andersoni* infection was 56.2% ± 11.1 SD, and the average number in the corresponding control cattle was 47.2% ± 16.9 SD. The average frequency of the proliferative cells in the gastric pits of infected cattle was significantly higher than that of



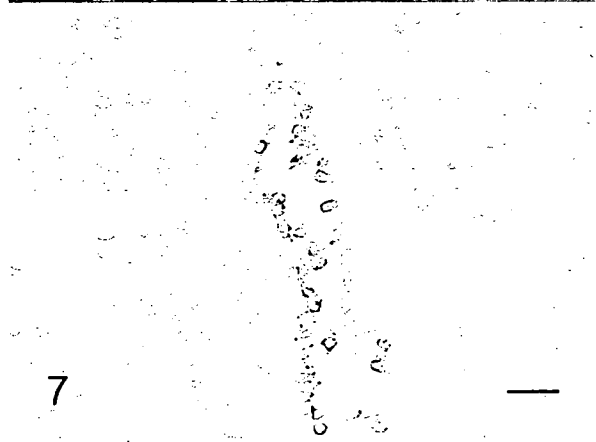


Fig. 5. *C. andersoni*; cow No. 3. A mature shizont with eight merozoites in the parasitophorous vacuole. TEM. Bar = 1 μm.

Fig. 6. Abomasum; cow No. 3. Large numbers of *C. andersoni* on the epithelial cells. SEM. Bar = 20 μm.

Fig. 7. Abomasum; cow No. 3. Only the wall of parasitophorous vacuoles and oocysts of *C. andersoni* positive for primary antibody. Immunohistochemical stain for *Cryptosporidium parvum* oocyst (Chemicon, Temecula, CA), paraffin section. Bar = 20 μm.

the controls. The level of significance was less than 0.01 in nonparametric testing.

HE sections revealed that *C. andersoni* infection was specifically limited to the mucosa of the abomasum in the present study. On the other hand, infection caused by other *Cryptosporidium* spp., such as *C. parvum*, showed up in the small intestine, particularly in the ileum. Unlike *C. parvum*, *C. andersoni* and *C. muris* were thought to have different infection sites. This is a characteristic feature of these species. Furthermore, on the same abomasum, there was a clear predisposition to infection of *C. andersoni*, which was located in the anterior and middle parts of the abomasum. These predisposed infection sites in the abomasum may be dependent on environmental factors, such as favored pH, ion balance, or the characteristics of the host cells, but detailed factors were not fully clarified.[4,6]

As with the in vitro examination of *C. parvum*, *C. andersoni*[4,6,9] showed increased nuclear fragmentation and enhanced apoptosis in the epithelial cells of the affected area in the present study. It was hypothesized that the cell division served as defense mechanisms or host response against attachment and invasion by *C. andersoni* organisms.

The length of the gastric pits was extended as a result of the accumulation of increased epithelial cells in the cervix portion, and the proliferation portion was not the principalis of the gastric pits, as it is in monkeys with *Cryptosporidium muris*-like infections.[7] The number of the gastric pit cells in cattle with *C. andersoni* had significantly increased ($P < 0.01$). This increase in the number of cells could conceivably correlate strongly with the larger frequency of Ki-67-positive cells. This result indicated that the extension of the isthmus of the gastric pits might be caused by the increasing number of cells as a consequence of the high frequency of cell divisions.

A moderate degree of infiltration of lymphocytes, plasmacytes, and eosinophils was found in the lamina propria in the affected abomasum. The severity of the inflammatory cell reaction in the lamina propria was more intense in infections caused by other *Cryptosporidium* spp., especially *Cryptosporidium parvum*, than in those caused by *C. andersoni*.[6,8,9,16,21] This relatively mild host reaction might relate to the long term colonic infection.

The ultrastructural features of *C. andersoni* were similar to those in other *Cryptosporidium* spp. for the most part,[7,9] except for the size of the parasitophorous vacuole. The size of the *C. andersoni* parasitophorous vacuole was larger than that in other *Cryptosporidium* spp. This may be one of the characteristic features of *C. andersoni* on TEM observation. SEM revealed numerous *C. andersoni* organisms with a spherical shape attached to the surface of the mucosal epithelium of the abomasum. We concluded that *C. andersoni* mainly infected the apical surface of the pits of the abomasum and then grew and propagated in the abomasum.[5]

Immunohistochemically, most *C. andersoni* organisms were reactive to all primary antibodies examined. However, *C. andersoni* with positive reactivity by immunohistochemistry were larger than those examined with HE stain. This discrepancy might be due to the presence of parasitophorous vacuole around the organisms. TEM observation showed that the size of the parasitophorous vacuole of *C. andersoni* was much larger than the size of the nucleus and cytoplasm, which might account for the discrepancy in size between the HE stain and immunohistochemical stain.

In conclusion, most histological and ultrastructural features of *C. andersoni* organisms and its mechanisms of attachment to epithelial cells might be common on the whole to those in other *Cryptosporidium* spp. However, the host response was different. Only in *C. andersoni* mucosal hyperplasia was observed.[9a] It is unknown whether the same phenomenon occurs in other animals. Further studies are needed to clarify the pathogenesis of *C. andersoni* in animals including humans.

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Short communication

Quantification of the infectivity of *Cryptosporidium parvum* by monitoring the oocyst discharge from SCID mice

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Abstract

Doses of 1–10^5 oocysts of *Cryptosporidium parvum* HNJ-1 were inoculated into severe combined immunodeficient (SCID) mice, and the discharge of oocysts was monitored for 30 days post inoculation. None of the mice discharged any oocysts after oral inoculation of one oocyst. Only one of five SCID mice discharged oocysts after oral inoculation of 10 oocysts, and the prepatent period was 17 days. The other four mice did not discharge any oocysts. All the SCID mice discharged oocysts after oral inoculation of 10^2–10^5 oocysts. The prepatent periods were 13–17, 8–10, 8, and 10 days in SCID mice inoculated with 10^2, 10^3, 10^4, and 10^5 oocysts, respectively. A proportional correlation was observed between inoculation doses of oocysts ranging from 10 to 10^4 oocysts and the corresponding prepatent periods. The prepatent period can be used to evaluate the infectivity of *C. parvum* oocysts.

Keywords: *Cryptosporidium parvum*; Infectivity; SCID mouse; Experimental infection

1. Introduction

Cryptosporidiosis in humans is reported all over the world. It occurs as a sporadic infection or as outbreaks followed by zoonotic transmission from farm animals, person-to-person spread, or the contamination of water supplies (O'Donoghue, 1995). *Cryptosporidium parvum* causes self-limiting diarrhea in immunocompetent individuals; however, immunocompromised individuals experience chronic, long-term infection, often lasting several months (Fayer, 2004). Many new species and new genotypes of *Cryptosporidium* have been reported based on recent molecular studies (Hajdušek et al., 2004), and the determination of the infectivity of these strains is important. In particular, *C. parvum* has been reported to be resistant to many common disinfectants including chlorine (Korich et al., 1990). In order to develop and improve the methods for disinfection of oocysts, it is essential to evaluate the viability of the oocysts after their exposure to disinfectants. Therefore, tests that adequately determine the viability of *Cryptosporidium*

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oocysts should be semiquantitative, reproducible, and easy to perform. In the present study, we inoculated severe combined immunodeficient (SCID) mice with different doses of *C. parvum* oocysts, and observed the oocyst discharge and prepatent periods.

2. Materials and methods

C. parvum HNJ-1 genotype 2 oocysts that were originally isolated from a Japanese patient and maintained at Osaka City University and Tohoku University (Satoh et al., 2003) were used for the study. These oocysts were purified from feces by the sugar centrifugal floatation method. They were then preserved at 4 °C in sterile phosphate-saline (PBS), and used for the experiments within a week after collection (Nakai et al., 2004; Koyama et al., 2005). The concentration of oocysts in the stock suspension was generally 1.0×10^8 to 3.0×10^8 per ml.

A dose of one oocyst was prepared under an inverted microscope using a capillary tube. The other oocyst doses ranging from 10 to 10^5 oocysts per mouse were prepared from the stock suspension of oocysts by serial dilution.

Four-week-old female SCID mice were purchased from Charles River Co. Ltd., Japan, and were inoculated orally with 100 μl *C. parvum* oocyst suspensions 1 week after purchase. The mice were housed individually in cages with wire mesh floor, and were fed on standard laboratory feed pellets for experimental mice and water ad libitum. Feces were collected from the pan under the mesh floor, and the absence of *C. parvum* oocysts in them was confirmed by the sugar floatation method prior to the start of the experiments. This method was also used to monitor the discharge of oocysts for 30 days post inoculation. All the mice were maintained in accordance with the Tohoku University's guidelines for animal experiments.

3. Results

None of the SCID mice discharged *C. parvum* oocyst after oral inoculation of one oocyst (Table 1). Only one of five mice discharged oocysts after oral inoculation of 10 oocysts, and the prepatent period was 17 days. The other four mice did not discharge any oocysts. All the mice discharged oocysts after oral inoculation of 10^2–10^5 oocysts (Table 2). The prepatent periods were 13–17, 8–10, 8, and 10 days in the SCID mice inoculated with 10^2, 10^3, 10^4, and 10^5 oocysts, respectively. All the mice survived during the experiment, but only mice inoculated with 10^5 oocysts were debilitated on and after day 25 post inoculation.

4. Discussion

The present study indicated that there was good correlation between the inoculation doses of oocysts ranging from 10 to 10^4 oocysts and the corresponding prepatent periods. Similar results were also observed in preliminary experiments (data not shown).

Table 1
Oocyst discharge[a] from SCID mice inoculated with 1 or 10 *Cryptosporidium* oocysts

Mouse no.	Number of inoculated oocysts/mouse	Days after inoculation												
		−1	6	7	8	9	10	11	12	13	14	15	16	17–30
1	1	−	−	−	−	−	−	−	−	−	−	−	−	−
2	1	−	−	−	−	−	−	−	−	−	−	−	−	−
3	1	−	−	−	−	−	−	−	−	−	−	−	−	−
4	1	−	−	−	−	−	−	−	−	−	−	−	−	−
5	1	−	−	−	−	−	−	−	−	−	−	−	−	−
6	10	−	−	−	−	−	−	−	−	−	−	−	−	−
7	10	−	−	−	−	−	−	−	−	−	−	−	−	+
8	10	−	−	−	−	−	−	−	−	−	−	−	−	−
9	10	−	−	−	−	−	−	−	−	−	−	−	−	−
10	10	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] Oocysts were detected (+) and not detected (−) using the sucrose floatation method.

Table 2
Oocyst discharge[a] from SCID mice inoculated with 10^2–10^5 *Cryptosporidium* oocysts

Mouse no.	Number of inoculated oocysts/mouse	Days after inoculation													
		−1	6	7	8	9	10	11	12	13	14	15	16	17	18–30
11	10^2	−	−	−	−	−	−	−	−	−	−	−	−	+	+
12	10^2	−	−	−	−	−	−	−	−	+	−	−	+	+	+
13	10^2	−	−	−	−	−	−	−	−	−	−	+	+	+	+
14	10^3	−	−	−	−	−	+	+	+	+	+	+	+	+	+
15	10^3	−	−	−	+	−	−	−	−	+	+	+	+	+	+
16	10^3	−	−	−	−	+	+	+	+	+	+	+	+	+	+
17	10^4	−	−	−	+	−	+	+	+	+	+	+	+	+	+
18	10^4	−	−	−	+	−	+	−	+	+	+	+	+	+	+
19	10^4	−	−	−	+	+	+	+	+	+	+	+	+	+	+
20	10^5	−	−	−	−	−	+	+	+	+	+	+	+	+	+
21	10^5	−	−	−	−	−	+	+	+	+	+	+	+	+	+
22	10^5	−	−	−	−	−	+	+	+	+	+	+	+	+	+

[a] Oocysts were detected (+) and not detected (−) using the sucrose floatation method.

The oocyst viability of the *Cryptosporidium* spp. has been determined by vital dyes, in vitro excystation (Black et al., 1996), cell culture (Upton et al., 1994), and PCR (Joachim et al., 2003); however, neonatal mouse infectivity assays are the reference methods. Although animal bioassays are costly and require tedious and lengthy procedures for handling animals and scoring for infection, the infection method is more reliable than any other laboratory method. Therefore, this bioassay is considered the gold standard for assessing the infectivity of *Cryptosporidium* oocysts (Korich et al., 2000). Most of the methods for evaluating *Cryptosporidium* infectivity in neonatal mice require the dissection and histological observation of the mice (Finch et al., 1993; Buraud et al., 1995; Delaunay et al., 2000). The sugar floatation method that we used for evaluating in SCID mice is inexpensive and can be easily performed. In the method using neonatal mice, 400 oocysts per mouse was the minimum dose required to establish the infection in all the mice (Finch et al., 1993); however, in the present method, the minimum dose was 100 oocysts per mouse. The former method could detect approximately 25 viable oocysts (Finch et al., 1993; Korich et al., 2000). However, our method detected 10 oocysts and could quantify the infectivity of 10–10^4 oocysts. Consequently, our method using SCID mice is easier, more cost effective, and more precise than the method using neonatal mice. Therefore, the present method might be useful for evaluating the infectivity of *Cryptosporidium* oocysts in field samples and laboratory samples in experiments for developing or improving medication and disinfectants against *C. parvum*. Since there are few reports on this evaluation method in SCID mice, further studies are required to standardize this method.

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ORIGINAL PAPER

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A novel genotype of *Cryptosporidium muris* from large Japanese field mice, *Apodemus speciosus*

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Abstract *Cryptosporidium muris*-like oocysts were isolated from large Japanese field mice, *Apodemus speciosus*. Morphologically, these oocysts resembled those obtained from a *C. andersoni* Kawatabi isolate but were smaller in size than those from a *C.muris* isolate. Following oral inoculation of the oocysts into large Japanese field mice and SCID mice, developing stages were found in the stomach epithelium. The infectivity of the isolate to wild and laboratory mice was slightly different from that of *C.muris*. DNA sequences of the 18S ribosomal RNA (rRNA) gene of the isolate were not identical to those of any known *Cryptosporidium* spp.; however, phylogenetic analysis indicated that the isolate was a member of the *C.muris* cluster. Differences between the isolate and *C. muris* are not significant at this point; therefore, we propose that this isolate is a novel genotype of *C.muris* and denote it as *C. muris* Japanese field mouse genotype.

Introduction

Cryptosporidium species are intracellular protozoan organisms that infect the gastrointestinal epithelial cells of a wide range of vertebrates, including humans (Dubey et al. 1990; Fayer et al. 2000). Cryptosporidiosis in humans has been reported globally and occurs sporadically or as outbreaks following zoonotic transmission from farm animals, person-to-person spread, or contamination of water supplies (O'Donoghue 1995). Since large numbers of oocysts are released from animals to surface waters, animal husbandry is a potential source of human infection (de Graaf et al. 1999). Wild mammals, particularly rodents, have been identified as reservoirs of *Cryptosporidium* (Chalmers et al. 1997; Morgan et al. 1999). In Japan, the presence of *Cryptosporidium* has been extensively studied in domestic animals raised on farms and slaughter houses (Kaneta and Nakai 1998; Koyama et al. 2005); however, only a few wild animals have been investigated (Iseki 1998). We studied the presence of *Cryptosporidium* oocysts in wild animals and zoo housed animals in Japan and detected oocysts in large Japanese field mice, *Apodemus speciosus*, which were captured on the beef farm where the *C. andersoni* Kawatabi strain was isolated (Satoh et al. 2003, Nakai et al. 2004). *Cryptosporidium* has been classified on the basis of oocyst size, host specificity, and parasitized organs. Following elucidation of the DNA sequences of the genes for 18S rRNA (Morgan et al. 1999), heat shock protein (HSP) (Sulaiman et al. 2000), and cryptosporidian oocyst wall protein (COWP) (Xiao et al. 2000) of various *Cryptosporidium* spp., differences in the DNA sequences have become a new and additional criterion for the classification of *Cryptosporidium*.

In this study, we used morphological, biological, and genetic analysis to characterize an isolate from the large Japanese field mice, *A. speciosus*.

Materials and methods

Animals

From April end to mid November 2000 and from mid April to early September 2001, Sherman traps were used to capture small animals on the Kawatabi farm of Tohoku University. This was the farm where *C. andersoni* had been constantly detected in several beef cattle (Nakai et al. 2004). Kawatabi farm is located in the northern part of the main island of Japan (38°45′N, 140°45′E), and the altitude ranges from 200 to 630 m. Live caught animals were housed individually in cages with a wire

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mesh floor for 1–4 weeks prior to the experiments. Animals were fed on standard laboratory feed pellets for experimental mice and water ad libitum. Feces were collected, and the sugar flotation method was used to detect *Cryptosporidium* oocysts in them. Captured large Japanese field mice were mated, and their infants were housed in separate cages. All mice, including the laboratory mice, were kept according to the guidelines for animal experiments of Tohoku University.

Oocyst size

Feces that tested positive for *Cryptosporidium* oocysts were collected for 3 weeks and were preserved in 2.5% (w/v) potassium dichromate solution at 4°C. Oocysts were purified from feces by the sugar centrifugal flotation method and were preserved at 4°C in PBS; they were used for experiments within 1 week of their collection. Using an optical microscope, at 1,000 × magnification, 50 oocysts were measured from each of the following strains: *C. muris* RN66, which was originally isolated from a house rat (Iseki 1986); *C. andersoni* Kawatabi strain, which was originally isolated from grazing cattle in the Kawatabi farm (Satoh et al. 2003); and *C. parvum* HNJ-1, which was genotype 2 and was originally isolated from a Japanese woman (Masuda et al. 1991). These strains had been serially passaged in SCID mice (Charles River Co., Ltd. Japan) in our laboratory. The lengths, widths, and shape indices of these oocysts were compared using non-parametric Kruskal–Wallis analysis of variance between groups because their variances were different. Individual differences were determined by multiple comparisons of average rank.

Experimental transmission

The transmission experiment was carried out using large Japanese field mice, 1–4 weeks after their capture from the field, 2-week-old infant large Japanese field mice, 4-week-old female SCID mice, and 4-week-old male Crj:CD-1 (ICR) mice (Charles River Co., Ltd.). The absence of *Cryptosporidium* oocysts in these mice was confirmed by the sugar flotation method prior to the start of experiments. This method was also used to monitor the discharge of oocysts for 30 days post-inoculation.

Histological analysis

Samples were obtained from the liver, heart, kidney, lung, stomach, duodenum, jejunum, intestinum ileum, cecum, colon, and pancreas of a large Japanese field mouse and a SCID mouse that were infected with a *Cryptosporidium* field isolate; these samples were fixed with 10% formalin in PBS and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE).

Genetic analysis

DNA from oocysts of a *Cryptosporidium* field isolate was extracted using the Mag Extractor Genome (Toyobo, Osaka, Japan). A primer set was used to amplify fragments of genes, namely, the 18S ribosomal RNA (rRNA) (5′-AACCTGGTTGATCC-3′ and 5′-GA-ATGATCCTTCCGCAGGTTC-3′). We designed the primer set using OLIGO 5.0 (National BioScience Inc., Plymouth, Minn.), and its sequence was based on the sequence of a rock hyrax strain of *C. muris* (GenBank accession no. AF093496). PCR amplification was performed in a mixture that had the following final composition: 1× PCR buffer, 1 mM $MgSO_4$, 0.2 mM each dNTP, 0.3 μM each primer, and 1.0 U KOD polymerase (Toyobo). The reactions were preheated at 94°C for 2 min and cycled 40 times at 94°C for 15 s, 58°C for 30 s, and 68°C for 2 min and then at 68°C for 10 min. The PCR products were electrophoresed on 1.0% agarose and visualized by ethidium bromide staining. The PCR fragment of 18S rRNA was cloned using the pT7Blue Perfectly Blunt Cloning Kit (Novagen, Darmstadt, Germany). The PCR products were purified using the Mag Extractor-PCR & Gel Clean up-kit (Toyobo). At least three clones of each purified PCR product were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instruction.

Phylogenetic analysis

Nucleotide sequences obtained from our isolate and its homologues were identified using the Blast database search programs (Altschul et al. 1997). Homologous sequences were obtained from GenBank. The following additional *Cryptosporidium* 18S rRNA sequences were obtained from GenBank: *C. muris* rock hyrax (AF093498), *C. muris* bactrian camel (AF093497), *C.muris* RN66 (AB089284), *C. andersoni* Kawatabi strain (AB089285), *C. serpentis* (AF093502), *C. baileyi* (AF093495), *C. felis* (AF108862), *C. canis* (AF112576), *C.* mursupial genotype (AF108860), *C. suis* (AF115377), *C. meleagridis* (AF112574), *C. wrairi* (AF115378), *C. parvum* mouse isolate (AF112571), *C. hominis* (AF093489), *C. parvum* deer isolate (AF093494), and *C. parvum* (AF093490). The sequence of our isolate was aligned against those of the others using the ClustalW multiple alignment program (Thompson et al. 1994). The neighbor joining tree was constructed using the MEGA version 2.1 program (Kumar et al. 2001), and the genetic distance was calculated using Kimura's 2-parameter model. The maximum parsimony analysis was also conducted with the same alignments using the DAMBE program (Xia and Xie 2001). The maximum likelihood tree was also constructed using the DAMBE program. The substitution model was set to an algorithm in which the transition to transversion ratio was 2.

Bootstrap proportions computed for the maximum likelihood using the resampling were estimated by the log likelihood (RELL) method. In the construction of both the neighbor joining and maximum likelihood trees, a sequence of *Eimeria tenella* (AF026388) was used as the outgroup for the 18S rRNA analysis as reported previously (Xiao et al. 1999, 2002).

Results

Survey of *Cryptosporidium* oocysts from wild animals

Twenty-five large Japanese field mice, *A. speciosus*; five Norway rats, *Rattus norvegicus*; two house mice, *Mus musculus*; and a Japanese field vole, *Microtus montebelli* were captured on the Kawatabi farm (Table 1). *Cryptosporidium* oocysts were detected only from two large Japanese field mice (Fig. 1).

Table 1 Prevalence of *Cryptosporidium* species in wild rodents caught in the Kawatabi Farm

Captured rodents	Fecal specimens	
	Total number	Number positive
Large Japanese field mice	25	2
Norway rats	5	0
House mice	2	0
Japanese field voles	1	0

Fig. 1 Nomarski interference contrast photomicrographs of oocysts from a large Japanese field mouse. Magnification is 1,000×. *Bar* = 5 μm

Oocyst size

Oocysts isolated from the large Japanese field mouse were 5.70 ± 0.59×7.66 ± 0.64 μm in size and had a length/width ratio of 1.36 ± 0.17 (Table 2). The sizes were significantly larger than those of *C. parvum* HNJ-1 ($P < 0.01$), while the lengths were significantly smaller than those of *C. muris* RN66 ($P < 0.05$).

Experimental infection

The *Cryptosporidium* field isolate was infective to adult and infant large Japanese field mice, adult ICR mice, and SCID mice (Table 3). The prepatent periods for the *Cryptosporidium* field isolate were 17, 19, 11, and 11 days in these mice, respectively (data were not shown). Although *C. muris* was infective to adult large Japanese field mice at a high dose of oocysts, it was not infective to adult and infant Japanese field mice and adult ICR mice at a medium dose of oocysts. *C. andersoni* was not infective to adult and infant large Japanese field mice and ICR mice, even at a high dose. Clinical signs were not observed in any animals infected with the *Cryptosporidium* field isolate.

Histological observation

On day 24 post-inoculation of oocysts of the *Cryptosporidium* field isolate, a large number of developing stages of *Cryptosporidium* were detected in the gastric gland of the stomach of the large Japanese field mouse. However, no stages were detected from the liver, heart, kidney, lung, stomach, duodenum, jejunum, ileum, cecum, colon, and pancreas. The majority of the gastric glands were dilated, hypertrophied, and filled with numerous parasites (Fig. 2). The result was similar to that observed in the infected SCID mouse.

Genomic analysis of the *Cryptosporidium* field isolate

The 18S rRNA gene (1,673 bp) was partially sequenced, and the sequence of the field isolate submitted to GenBank had the accession number AY642591. In the 18S rRNA gene, the *Cryptosporidium* field isolate had 6 bp substitutions and 2 bp deletions compared with *C. muris* RN66 and 13 bp substitutions and 2 bp deletions compared with the *C. andersoni* Kawatabi strain (data not shown).

Phylogenetic analysis of 18S rRNA

The neighbor joining phylogenic tree and the maximum likelihood tree were constructed for the 18S rRNA gene