Isolation of *Hammondia hammondi* in Japan

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ABSTRACT. A cat which was fed muscles of a goat shed *Toxoplasma*-like oocysts (G-8 isolate) 43 days after feeding. The oocysts were subspherical to ovoid, measuring $12.7 \times 11.5 \mu\text{m}$ (unsporulated oocysts) and $13.0 \times 11.4 \mu\text{m}$ (sporulated oocysts). When mice were inoculated orally with 10^5 oocysts, tachyzoites were observed in the kidney, lung, mesenteric lymph nodes, small intestine and spleen of the mice 1 to 15 days after infection. Cysts were seen in the brain and various muscles. Cysts measured $25\text{--}224 \times 20\text{--}80 \mu\text{m}$ in the skeletal muscle and $35 \times 35 \mu\text{m}$ in the brain 181 days after infection. Bradyzoites in mice were not infectious to other mice. Sometimes antibody to *Toxoplasma* was observed in the mouse sera but the titer was low. Cats fed bradyzoites began to discharge oocysts from 7 to 8 days after ingestion. But cats inoculated with 10^6 oocysts of G-8 isolate shed no oocysts within 60 days after inoculation, and cats fed the muscles of these oocyst-inoculated cats shed no oocysts. The life cycle of G-8 isolate was obligatorily heteroxenous, and cysts in the muscles of the infected mice morphologically resembled those of *Hammondia hammondi* (Frenkel and Dubey 1975). G-8 isolate was identified to be *H. hammondi*.—**KEY WORDS:** *Hammondia hammondi*.

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Recently, the life cycles of many coccidia in dogs and cats have been studied. The authors isolated *Toxoplasma*-like oocysts in feces of 8 out of 17 cats fed goat muscles [11]. It is, however, difficult to identify such micro-organisms only on the morphology. Because it is known that cats shed the oocysts of *Toxoplasma gondi* and *Hammondia hammondi* both of which are structurally similar each other [7]. Seven of 8 isolates were identified as *T. gondii* because their tachyzoites and bradyzoites were infectious to mice and the serial passages of them among mice were possible. However, one remaining isolate (G-8) was different from other 7 isolates. When the 10^4 oocysts were orally administered to mice, neither cyst in the brain nor anti-*Toxoplasma* antibody was found in the mice 44 days after infection [11]. This article reports on the characteristics and the identification of this isolate.

MATERIALS AND METHODS

G-8 isolate: The oocysts of G-8 isolate were originated from feces of a cat which had ingested goat muscle. The oocysts used were discharged from a cat which fed the mice inoculated with the isolated oocysts.

Animals: Cats used were unwanted kittens collected in the neighborhood of the National Institute of Animal Health. After fecal examination, coccidia-negative cats were raised with heated food and examined for oocysts more than two weeks before use.

Mice used were commercial ddY strain weighed 20 grams.

Examination of tachyzoites and cysts in mice: Each of mice was inoculated orally with 10^5 sporulated oocysts of G-8 isolate.

For the examination of tachyzoites, the mice were bled and killed under ether anesthesia from 1 to 17 days after inocula-

tion. Each saline suspension of the intestine (duodenum, jejunum, ileum and colon), kidney, liver, lung, mesenteric lymph nodes, spleen and thymus of each mouse was made and examined for tachyzoites in fresh preparation under the microscope. Smears of these organs were made, fixed in methanol and stained with Giemsa solution.

For the examination of cysts, the mice were bled and killed under ether anesthesia on each given day from 9 to 587 days after inoculation. The cardiac muscle, diaphragm, esophagus, skeletal muscle and tongue of these mice were individually minced, mixed with saline and examined for cysts under the microscope. The brains of these mice were examined for cysts in fresh squash preparation. In addition, the brains and muscles were fixed in 10% buffered formalin solution, embedded in paraffin, cut at 5 μm and stained with hematoxylin and eosin (H-E) or periodic acid Schiff and hematoxylin (PAS-H).

Inoculation of tachyzoites and cysts into mice: Three mice were inoculated intraperitoneally with the pooled saline suspension of the mesenteric lymph nodes and spleen of a mouse fed 10^5 oocysts 6 days previously. Six days after the inoculation, one of them was killed and the pooled suspension of the mesenteric lymph nodes and spleen was subinoculated intraperitoneally into two clean mice. Thirty days after the subinoculation, all the mice were killed and examined for the presence of cysts.

Four mice were inoculated intraperitoneally with the saline suspensions of the brain and skeletal muscle of a mouse fed 10^5 oocysts 60 days previously. The inoculated mice were killed for the examination of cysts 30 days after inoculation.

Infection in cats: Two cats were inoculated orally with 10^6 oocysts. Feces of these cats were examined for oocysts over a period of 60 days after oocyst-inoculation. Eighty-six days after inoculation, one cat

was killed and the brain, diaphragm, esophagus, heart, kidney, liver, lung, mesenteric lymph nodes, skeletal muscle and spleen were pooled and fed to the third cat. Another oocyst-inoculated cat was killed 120 days after inoculation and the muscles and other organs were fed to the fourth cat. Feces of the recipient cats were examined for oocysts over a period of 30 days after ingestion.

The adrenal gland, brain, kidney, liver, lung, skeletal muscle, small intestine, spleen, stomach, thymus and uterus of each mouse inoculated with 10^5 oocysts 9, 10, 11 and 12 days previously were given to each of four cats, respectively. Feces of the cats were examined every days for oocysts by sugar flotation method. The number of oocysts per gram of feces (OPG) was examined in oocyst-positive cases. The cat fed organs of a mouse inoculated with oocysts 12 days before was killed 7 days after feeding, and the intestine of the cat was examined for the parasites in fresh preparations, Giemsa stained smears and paraffin sections.

Serological examination: Sera of all mice and cats were examined for the presence of antibody to *Toxoplasma* antigen by the latex agglutination test (Toxo-Check, Eiken) [13, 14].

RESULTS

Oocysts: Unsporulated oocysts were subspherical to ovoid, measuring $12.7 \times 11.5 \mu\text{m}$ ($11.0\text{--}14.0 \times 9.5\text{--}13.0 \mu\text{m}$); their length-to-width ratio was 1.11 (1.04–1.18) ($N=100$). The oocyst wall was colorless and smooth (Fig. 1).

At 25°C, using 2% potassium dichromate, the sporulation was completed after 2–3 days. Sporulated oocysts were subspherical to ovoid, measuring $13.0 \times 11.4 \mu\text{m}$ ($11.5\text{--}14.0 \times 9.5\text{--}13.0 \mu\text{m}$); their length-to-width ratio was 1.14 (1.08–1.29) ($N=100$).

Fig. 1. An unsporulated oocyst of G-8 isolate. Bar=10 μ m, $\times$ 1,250.

Fig. 2. A sporulated oocyst of G-8 isolate. Compact sporocyst residuum were present. Bar=10 μ m, $\times$ 1,250.

Table 1. Detection of tachyzoites in mice inoculated with oocysts of G-8 isolate

Organs	Days after inoculation																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Intestine																	
Doudenum	—	+	+	—	—	—	—	—	—	—	—	+	—	—	—	—	—
Jejunum	+	—	—	+	+	+	—	+	—	—	—	—	—	—	—	—	—
Ileum	—	—	+	+	++	++	+	+	+	++	++	+	++	—	—	—	—
Colon						—	—	—	—	—				—			
Kidney	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—
Liver	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lung	—	—	—	—	—	—	—	—	+	+	—	+	—	+	+	—	—
MLN ^{a)}	+	—	+	+	++	++	++	+	+	+	+	—	—	—	—	—	—
Spleen	+	+	—	+	+	—	—	—	—	—	—	+	—	—	—	—	—
Thymus										—	—	—	—	—	—	—	—
Peritoneal exudate							—	—	—	—	—	—	—	—			

a) Mesenteric lymph nodes.

Micropile, polar granules and oocyst residuum were absent (Fig. 2). The sporocysts were ellipsoidal without Stieda body, measuring $8.6 \times 6.4 \mu\text{m}$ ($7.5\text{--}9.5 \times 5.5\text{--}7.2 \mu\text{m}$); their length-to-width ratio was 1.35 ($1.19\text{--}1.53$) ($N=100$). They contained 4 sporozoites and a sporocyst residuum each. The sporozoites were elongated and curved measuring $7.4 \times 2.1 \mu\text{m}$ ($5.5\text{--}10.3 \times 1.4\text{--}2.7 \mu\text{m}$) ($N=50$).

Tachyzoites in mice: After the oral inoculation of 10^5 sporulated oocysts, tachyzoites were observed in the fresh preparations of the kidney, lung, mesenteric lymph nodes, small intestine and spleen of mice from 1 to 15 days after inoculation. Especially, many

tachyzoites were seen in the mesenteric lymph nodes and ileum (Table 1). Tachyzoites measured $5.6 \times 2.2 \mu\text{m}$ ($4\text{--}7 \times 1\text{--}3 \mu\text{m}$) in Giemsa stained specimens (Fig. 3). No tachyzoites were observed in the peritoneal exudate whereas the exudate increased in quantity from 7 to 13 days after infection.

Cysts in mice: A cyst was observed in the fresh preparation of the femoral muscle 9 days after oocyst inoculation. The cysts increased in number from 13 days and were seen in all the examined muscles after 30 days. Only a few cysts were observed in the muscles 400 days after infection. Cysts in the muscle were ellipsoidal, ovoid and sometimes elongated, and stained positive with

PAS (Figs. 4–6). Metrocytes were not seen. In fresh squash preparations of the brain, cysts appeared from 60 days after infection (Table 2). Cysts in the brain resembled those of *Toxoplasma gondii* in shape (Fig. 7). Cysts in the muscles increased in size and measured $25\text{--}224 \times 20\text{--}80\ \mu\text{m}$ in fresh preparation 181 days after infection. The thick-

ness of cyst wall was $2\text{--}3\ \mu\text{m}$. The other hand, cysts in the brain did not increase in size, measuring $35 \times 35\ \mu\text{m}$ 181 days after infection (Table 3). Bradyzoites measured $7 \times 1.6\ \mu\text{m}$ ($6\text{--}8 \times 1.5\text{--}2\ \mu\text{m}$) in fresh preparation.

Infectivity of tachyzoites and bradyzoites: Tachyzoites were not observed in the tachyzoite-inoculated mouse 6 days after the first passage. A cyst was seen in the abdominal muscle of the tachyzoite-inoculated mouse and the low titer of antibody to *Toxoplasma* antigen was detected. But, in the subinoculated mice, no cysts and no antibody to *Toxoplasma* antigen were demonstrable 30 days after the second passage.

Thirty days after inoculation, no cysts and no antibody to *Toxoplasma* antigen were detected in the mice inoculated with the saline suspension of the brain or muscle containing cysts.

Infection in cats: Two cats inoculated with 10^6 oocysts shed no oocysts in their feces within 60 days after inoculation. Two recipient cats fed the muscles and other organs of oocyst-inoculated cats also shed no oocysts in their feces within 30 days after feeding.

Fig. 3. Tachyzoites of G-8 isolate in the mesenteric lymph nodes of a mouse 4 days after infection. Giemsa stained smear, $\times 750$.

Table 2. Detection of cysts in mice inoculated with oocysts of G-8 isolate

Organs	Days after inoculation														
	9	10	11	13	15	16	17	30	60	100	110	181	400	578	
Brachial m. ^{a)}	—	—	—	+	ND ^{b)}	+	+	+	+	+	+	+	+	+	
Cardiac m.	—	—	—	—	+	+	+	(+) ^{c)}	+	+	+	+	—	—	
Diaphragm	—	—	—	—	ND	—	+	+	+	+	+	+	—	+	
Dosal m.	—	—	—	+	+	+	+	+	+	+	+	+	+	+	
Esophagus	—	—	—	—	ND	+	—	+	+	+	+	+	—	—	
Femoral m.	+	—	—	—	ND	—	+	+	+	+	+	+	—	(+)	
Masseter	—	—	+	—	ND	+	—	+	+	+	+	+	(+)	+	
Pectrials	—	—	—	+	ND	ND	+	+	+	+	+	+	—	+	
Tongue	—	—	—	+	ND	—	—	+	+	+	+	+	(+)	+	
Ventral m.	—	—	—	—	ND	ND	+	+	+	+	+	+	+	+	
Brain	ND	ND	ND	—	ND	—	—	—	+	+	+	+	—	—	

a) Muscle.

b) Not done.

c) Only bradyzoites were seen.

Fig. 4. A cyst of G-8 isolate in the skeletal muscle of a mouse 10 days after infection. PAS-H, $\times 750$.

Fig. 6. An elongated cyst in the skeletal muscle of a mouse 60 days after infection. H-E, $\times 320$.

Fig. 5. A cyst of G-8 isolate in the skeletal muscle of a mouse 30 days after infection. PAS-H, $\times 500$.

Fig. 7. A cyst of G-8 isolate in the brain of a mouse 60 days after infection. Fresh squash preparation, $\times 750$.

Antibody to *Toxoplasma* antigen was not detected in sera of donor cats on 86 and 120 days after oocyst inoculation and of the recipient cats on 30 days after feeding.

Each of 4 cats was fed the mouse inoculated with 10^5 oocysts 9, 10, 11 and 12 days before examination, respectively. The cat fed the mouse inoculated 9 days before did not shed any oocysts within 30 days after feeding. Both of 2 cats fed the mouse inoculated 10 and 11 days before, respectively, shed unsporulated oocysts. The pre-

patent period was 7 to 8 days and the patent period was 6 to 21 days. The maximum OPG value was more than 10^6 in both cats. The cat fed the mouse inoculated 12 days before was killed 7 days after feeding. Schizonts, merozoites, gametes and oocysts were observed in the small intestine. Parasites were found in the epithelial cells in paraffin sections (Fig. 8). Seven days after feeding, schizonts were $5-10 \times 3-9 \mu\text{m}$ in size, and merozoites $4-6 \times 1-1.5 \mu\text{m}$ in Giemsa stained smears (Fig. 9). On the 30

Fig. 8. Schizonts of G-8 isolate (arrows) in the epithelial cells of small intestine of a cat 7 days after infection. H-E, $\times 500$.

Fig. 9. Merozoites of G-8 isolate in the small intestine of a cat 7 days after infection. Giemsa stained smear, $\times 750$.

Table 3. Antibody and cyst development in mice inoculated with oocysts of G-8 isolate

Days after inoculation	LA ^{a)}	Cyst dimensions in fresh preparations (μm)	
		Muscles	Brain
9	—	13 $\times$ 8	ND ^{b)}
10	—	(13–15 $\times$ 8) ^{c)}	ND
11	—	23–30 $\times$ 10–22	ND
13	—	15–40 $\times$ 8–25	—
15	—	40–45 $\times$ 20	ND
16	—	30–58 $\times$ 18–30	—
17	—	45–75 $\times$ 15–30	—
30	—	27–115 $\times$ 17–50	—
60	: 32	24–142 $\times$ 15–65	25 $\times$ 23–25
100	—	28–125 $\times$ 16–65	20–35 $\times$ 15–23
110	: 128	40–190 $\times$ 20–80	20–28 $\times$ 19–27
181	: 64	25–224 $\times$ 20–80	35 $\times$ 35
400	—	80–375 $\times$ 20–120	—
578	ND	80–330 $\times$ 32–95	—

a) Titer by the toxoplasma latex agglutination test.

b) Not done.

c) Measurement from PAS stained sections.

days after feeding, no antibody against *Toxoplasma* antigen was detected in each serum of all cats fed inoculated mice.

DISCUSSION

The authors isolated *Toxoplasma*-like oocysts from 8 out of 17 cats fed the muscles of goats [11]. One of these 8 isolates resembled *Hammondia hammondi*. *H. hammondi* was

first isolated from cat feces in Hawaii and reported as *Toxoplasma*-like oocysts [15, 16]. Frenkel and Dubey [6, 7] described a protozoon which was isolated in Iowa, USA and named it *H. hammondi* as a new genus and a new species. Since then, *H. hammondi* was recorded in Germany [10], Ohio, USA [1] and Australia [9]. The independence of genus *Hammondia* has not always been agreed among scientists. Levine [8]

referred to this species as *Toxoplasma hammondi*, and Tadros and Laarman [12] synonymized genus *Hammondia* with genus *Isospora*. The problem on the classification of this species seems to have been caused by the confusion on the classification of isosporan coccidia. Several proposals on their classification have been made, but the confusion has still remained. We use tentatively the name *Hammondia hammondi* designated by Frenkel and Dubey [6, 7] in this paper. *H. hammondi* morphologically resembles *T. gondii* and both species shed their oocysts with cats feces. The life cycle of *H. hammondi*, unlike *T. gondii*, is obligatorily heteroxenous. Bradyzoites of *H. hammondi* infect only the final host. Those of *T. gondii* are infectious not only to the final host but also to the intermediate host. Cats shed no oocysts after ingesting oocysts of *H. hammondi*, but in the case of *T. gondii* they sometimes shed the oocysts. Some hosts infected with *H. hammondi* develop low level of antibody to *Toxoplasma* antigen [3, 5, 6, 7, 10, 15, 16]. Consequently, it may be difficult to differentiate *H. hammondi* only morphologically and serologically from *T. gondii*.

Oocysts of G-8 isolate were similar in shape and in size to those of *T. gondii*. But the bradyzoites did not infect mice, and oocyst-inoculated cats shed no oocysts. Recipient cats fed the muscles of oocyst-inoculated cats shed no oocysts.

Tachyzoites of G-8 isolate were observed in the ileum of mouse until 13 days after oocyst inoculation and until 15 days in the lung. Frenkel and Dubey [7] reported that tachyzoites of *H. hammondi* multiplied at least for 11 days in mice, and tachyzoites taken from the mouse on the 9th day after oocyst inoculation were not infectious to other mice. On the other hand, tachyzoites of G-8 isolate taken from the mouse on the 6th day after oocyst inoculation could be transferred from mouse to mouse and pro-

duced a cyst in a recipient mouse whereas the subsequent serial passage of this isolate did not succeed.

The cyst development of G-8 isolate in mice was almost the same as that of *H. hammondi* reported by Frenkel and Dubey [7]. They observed cysts at least for 468 days after oocyst inoculation but no cysts on the 518th day in mice. The cysts of G-8 isolate were still seen in mice even 578 days after oocyst inoculation, though they were few in number. G-8 isolate in mice began to have the infectivity to cats from 10 days after oocyst inoculation. This result was the same as that described in report of Dubey and Streitel [4]. But, cysts of G-8 isolate in mice were not infectious to other mice.

As mentioned above, the life cycle of this isolate was obligatorily heteroxenous, and the shape of cysts in the mouse muscle was similar to that reported by Frenkel and Dubey [6, 7], so it was identified as *H. hammondi* (Frenkel and Dubey 1975). And we designate this isolate as *H. hammondi* the G-8 strain.

Many authors reported that *H. hammondi* was isolated from feces of naturally infected cats [1, 6, 7, 10, 15, 16]. Mason [9] found *H. hammondi* oocysts in the feces from a cat which had eaten rats. In the case of G-8 isolate, oocysts were first detected in the feces of a cat 43 days after the ingestion of goat muscle [11]. The prepatent period of this isolate was too long and the infectivity to goats has not been clear. So, it is not yet clear whether the cat had been infected with this isolate before use and re-shed the oocysts after the ingestion of goat muscle or the parasites were present in the goat muscle. Because it has been reported that cats spontaneously re-shed *H. hammondi* oocysts [1, 2] and that the prepatent period is sometimes prolonged when cats fed the oocyst-inoculated cats or dogs which were not so suitable intermediate hosts for this species [1, 3]. It must be necessary to carry

out an experimental infection to goats for learning the origin of G-8 isolate.

This article is the first report of *H. hammondi* in Japan. It must be emphasized that we need to pay attention to the identification of *T. gondii* from *H. hammondi* when *Toxoplasma*-like organisms will be isolated.

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要 約

日本における *Hammondia hammondi* の分離：志村亀夫・伊藤進午（農林水産省家畜衛生試験場）——ネコにヤギの筋肉を食べさせたところ、43日後からトキソプラズマ様オーシスト（G-8株）を排出した。オーシストは類円ないしは卵円形で、大きさは $12.7 \times 11.5 \mu\text{m}$ （孢子未形成）および $13.0 \times 11.4 \mu\text{m}$ （孢子形成後）であった。孢子形成オーシスト 10^5 個をマウスに経口投与したところ、タキゾイトが肺臓、脾臓、腎臓、小腸および腸間膜リンパ節に1日目から15日目まで観察され、シストが骨格筋に9日目から認められ、その後心筋、舌、食道、横隔膜および脳にも認められた。摂食後181日のシストの大きさは筋肉内で $25 \sim 224 \times 20 \sim 80 \mu\text{m}$ で、脳では $35 \times 35 \mu\text{m}$ であった。一部の感染マウス血清に抗トキソプラズマ抗体が認められたが、抗体価は低かった。シストは他のマウスへの感染性を示さなかったが、シストを食べたネコは7～8日後からオーシストを排出した。ネコにG-8株のオーシストを経口投与してもオーシストを排出せず、またそのネコの筋肉を食べたネコもオーシストを排出しなかった。G-8株は生活環が obligatorily heteroxenous であることと、筋肉内のシストの形態から *Hammondia hammondi* (Frenkel and Dubey 1975) と同定され、わが国にも本原虫が存在することが明らかとなった。