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MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF FIRST ISOLATES OF *HAMMONDIA HAMMONDI* FROM CATS FROM ETHIOPIA

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ABSTRACT: *Toxoplasma gondii* oocysts are morphologically and antigenically similar to oocysts of another feline coccidian, *Hammondia hammondi*. The distinction between *H. hammondi* and *T. gondii* is important from an epidemiological perspective because all isolates of *T. gondii* are potentially pathogenic for humans and animals, whereas *H. hammondi* is not known to cause clinical disease in any naturally infected intermediate or definitive hosts. In the present report, *H. hammondi* (designated HhCatEt1 and HhCatEt2) oocysts were found microscopically in the feces of 2 of 36 feral domestic cats (*Felis catus*) from Addis Ababa, Ethiopia. Oocysts were orally infective to Swiss Webster and gamma interferon gene knockout mice; the inoculated mice developed tissue cysts in their muscles. Laboratory-raised cats fed mouse tissues of infected mice shed *H. hammondi* oocysts with a prepatent period of 5 days. The DNA extracted from sporulated oocysts reacted with *H. hammondi*–specific primers, and sequences were deposited in GenBank (accession nos. JX477424, and KC223619). This is the first report of isolation of *H. hammondi* from cats from the African continent.

Domestic cats (*Felis catus*) and other felids are important in the epidemiology of *Toxoplasma gondii* infection because they are the only hosts known to excrete the environmentally resistant oocysts (Dubey, 2010). *Toxoplasma gondii* oocysts are morphologically similar to oocysts of another feline coccidian, *Hammondia hammondi* (Frenkel and Dubey, 1975). The distinction between *H. hammondi* and *T. gondii* is important from an epidemiological perspective because all isolates of *T. gondii* are potentially pathogenic for humans and animals, whereas *H. hammondi* is not known to cause clinical disease in any naturally infected intermediate or definitive host.

Hammondia hammondi was first described from the feces of a naturally infected cat from Iowa (Frenkel and Dubey, 1975). Since then, it has been isolated from feces of naturally infected domestic cats in the United States, Brazil, Germany, Italy, and Austria (Rommel and von Seyerl, 1976; Christie et al., 1977; Ogassawara et al., 1985; Schares, Vrhovec et al., 2008; Herrmann et al., 2010). Unlike, *T. gondii*, *H. hammondi* has a limited host range in nature (Dubey and Sreekumar, 2003). In Australia, Mason (1978) reported that laboratory-raised cats fed tissues of naturally exposed rats and mice shed *H. hammondi* oocysts. Shimura and Ito (1986, 1987) made similar observations in Japan by feeding naturally infected goat muscles to a cat. A cat fed naturally infected roe deer muscles in Germany also shed *H. hammondi* oocysts (Entzeroth and Scholtyseck, 1978). Thus, goats, rats, mice, and roe deer are potential natural intermediate hosts for *H. hammondi*. Experimentally, several other mammals, including primates, but not birds, have been infected with *H. hammondi* (Dubey and Sreekumar, 2003).

Although there are minor differences in structures of *H. hammondi* and *T. gondii* (Dubey and Sreekumar, 2003; Schares, Vrhovec et al., 2008), specific diagnosis of *H. hammondi* versus *T. gondii* is technically difficult because these parasites can cross react serologically (Frenkel and Dubey, 1975; Christie and Dubey, 1977) and molecularly, depending upon primers used (Schares, Herrmann et al., 2008). Bioassay in mice is required for a definitive diagnosis of *H. hammondi*. Unlike *T. gondii*, *H. hammondi* has an obligatory 2-host life cycle; the definitive host (cat) is infected by eating infected tissue of intermediate hosts (usually rodents), and intermediate hosts become infected by ingesting sporulated oocysts from the environment; there is no congenital transmission of this infection (Dubey and Sreekumar, 2003). Little is known of biological or molecular differences among *H. hammondi* isolates, and the parasite has not been reported from Africa.

MATERIALS AND METHODS

Naturally infected cats

As part of an epidemiological investigation of *T. gondii* infection in Ethiopia, 48 feral cats from the Addis Ababa area, Ethiopia, were examined for antibodies to *T. gondii* and other infections, and these serological results were reported by Tiao et al. (2013). Of these 48 cats, 36 cats were available for further studies. These 36 cats were killed at the Aklilu Lema Institute of Pathobiology, Addis Ababa, and their samples were brought to the United States for attempted isolation of *T. gondii* by bioassays in mice. Viable *T. gondii* was isolated from the hearts of 26 cats and feces of 7 cats as described by Dubey et al. (2013). Here, we report the isolation of 2 *H. hammondi* isolates (designated HhCatEt1from the feces of cat #9, and HhCatEt2 from the feces of cat #20) of these 36 cats.

Cat #9 was 1-yr-old female from Kirkos (suburb of Addis Ababa) caught on 26 August 2011. The cat was killed on 17 September 2011. This cat had a 1,600 *T. gondii* antibody titer in the modified agglutination test (MAT), but *T. gondii* was not isolated from its heart or feces by bioassay in mice (Dubey et al., 2013). Cat #20 was a 10-mo-old cat, was obtained on 5 September 2011 (12 days before euthanasia), and had a MAT titer of 400; *T. gondii* was not isolated from its heart by bioassay in mice (Dubey et al., 2013).

Examination of cat feces for oocysts

Feces (~10 g) collected at necropsy from the rectum of cats #9 and #20 were moistened with water, emulsified in 33% sucrose solution, filtered through gauze, and centrifuged for 10 min at 1,400 *g* in a 50-ml tube with a cap. A drop from the very top of the float was examined microscopically for oocysts. A 5–ml sample of float from the top of the meniscus was mixed with 45 ml of water and centrifuged for 10 min. After discarding the supernatant, the sediment was mixed with 10 ml of 2% sulfuric acid and aerated in a shaker at room temperature for 1 wk to induce oocyst sporulation. Sporulated oocysts had been stored at 4 C for 5 mo (cat #9) and 12 mo (cat #20) before use in the experiments.

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Bioassay of cat feces for *H. hammondi* infection

To distinguish *H. hammondi* oocysts from *T. gondii*, several bioassays were performed in mice and cats. For oocyst bioassay, sporulated oocysts from cat #9 (<100,000) and cat #20 (approximately 1,000,000) were neutralized with 3.3% NaOH and orally inoculated into Swiss Webster (SW) outbred albino mice (day 0 PI) and interferon gamma gene knockout (KO) mice (Dubey et al., 2013). The mice were bled 33–50 days postinoculation (PI), and 1:25 dilution of serum was tested for *T. gondii* antibodies using the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). The mice were killed 43 days PI, and squash preparations of their brains and leg muscles were examined microscopically for tissue cysts. To further characterize this parasite, muscles from the infected mice were fed to 3 *T. gondii*–free laboratory-raised cats (Dubey, 1995). Cats A and B were fed tissues from mice infected with HhCatEt1from the feces of cat #9, and cat C was fed tissues from mice infected with HhCatEt2 from the feces of cat #20. Feces of cats were examined for *T. gondii*– or *H. hammondi*–like oocysts, as described previously (Dubey, 2010).

To distinguish *H. hammondi* from *T. gondii*, tissues of mice that were found positive for tachyzoites or tissue cysts after consuming oocysts were bioassayed in mice with or without digestion (Dubey, 2010). Tachyzoites or tissue cysts of *H. hammondi* are not infectious for mice (Dubey and Sreekumar, 2003). For this, murine tissues were homogenized in aqueous NaCl solution (saline), and 1 ml of the homogenate was inoculated subcutaneously into mice. For pepsin digestion, the procedures described for *T. gondii* digestion were followed (Dubey, 2010). Briefly, tissue homogenate was incubated with pepsin for 30 min, centrifuged, suspended in saline, and neutralized with sodium bicarbonate, and 1 ml of the homogenate was inoculated subcutaneously into mice as described previously (Dubey, 2010). Tissues of the recipient mice were examined for *T. gondii* and *H. hammondi*. Pepsin digestion releases encysted bradyzoites from tissue cysts as the host tissue is digested.

To further distinguish *T. gondii* from *H. hammondi*, extra-intestinal organs (mesenteric lymph nodes, spleen, liver) of cat C killed 11 days after infection with *H. hammondi* tissue cysts were pooled and homogenized in saline, and an aliquot was inoculated into 2 SW and 2 KO mice to exclude *T. gondii* infection; *H. hammondi* does not invade extra-intestinal tissues of cats (Dubey and Sreekumar, 2003).

Pathogenicity of *H. hammondi* oocysts to mice

To determine the pathogenicity and infectivity of the Ethiopian isolate (HhCatEt1) of *H. hammondi* isolated from the feces of 2 experimentally infected cats (A, B), samples were pooled (>200 million), sporulated, and stored in 2% sulfuric acid at 4 C. Oocysts were neutralized and diluted in aqueous NaCl solution (saline), and aliquots were inoculated orally into KO mice (see results). The recipient mice were examined for tissue stages of *H. hammondi*.

A similar assay was performed with oocysts of the second *H. hammondi* isolate (HhCatEt2). Oocysts from cat C were counted and diluted 10-fold, so that inocula contained 1,000,000, or 100,000, or 10,000. Ten mice (5 SW, 5 KO) were inoculated orally with each of these 3 inocula. The experiment was terminated at day 40.

Tissue stages of *H. hammondi* in cat intestine

To study enteroepithelial stages, cat A was killed 10 days PI, sections of cat A ileum were fixed in 10% buffered neutral formalin, and paraffin-embedded histological sections were examined after staining with hematoxylin and eosin.

DNA characterization of *H. hammondi* isolates from Ethiopian cats

DNA was isolated from 3 preparations of sporulated oocysts from cats experimentally infected with HhCatEt1 and HhCatEt2 as follows: Oocysts were washed 3 times in 10 ml of Hank's balanced salt solution (HBSS; Life Technologies, Grand Island, New York) using centrifugation at 800 *g*, incubated in PBS containing 10% bleach for 15 min, and then washed again in HBSS until all traces of bleach were removed (~3 times). The pellets were resuspended in 500 µl of DNAzol (Life Technologies), passed 3 times through a 25 gauge needle, and then boiled for 10 min. DNA was precipitated with 0.5 volumes of 100% ethanol and washed in 75% ethanol, and dried pellets were resuspended in water. One microliter of the suspension was used in a PCR reaction with forward primers Hham34F (5′-ATCCCATTCCGGCTTCAGTCTTTC-3′) and Hham3R (5′-ACAGCGGAGCCGAAGTTGGTTT-3′), which are known to distinguish *H. hammondi* from *T. gondii* (Schares, Herrmann et al., 2008). Reactions were allowed to proceed for 30 cycles of 95 C for 30 sec, 58 C for 30 sec, and 72 C for 40 sec. A similarly obtained DNA sample from HhCatGer041 (Walzer et al., 2013) served as a positive control, and DNA from *T. gondii* strain VEG (Dubey et al., 1996) was used as a negative control. PCR products were gel-purified and directly sequenced using Hham34F and Hham3R primers, and assembled using SeqMan (DNASTAR, Madison, Wisconsin).

To further distinguish *H. hammondi* from *T. gondii*, tissues of infected mice were subjected to DNA isolation and PCR amplification by using appropriate primers indicated by Schares, Herrmann et al. (2008).

RESULTS

HhCatEt1 (Cat #9)

The 2 SW mice inoculated orally with oocysts from feces of cat #9 remained asymptomatic, and protozoal tissue cysts were not found when mice were killed 43 days PI. Therefore, the bioassay was repeated in 2 SW and 1 KO mouse; antibodies to *T. gondii* were not detected in 1:25 serum dilution tested 48 days PI. However, *H. hammondi*–like tissue cysts were seen in leg muscles of the KO mouse and 1 of the 2 SW mice, while protozoal tissue cysts were not seen in the brain squashes; cats A and B fed with tissues from these infected mice shed *H. hammondi*–like oocysts 5 days PI.

Oocyst, tissue cyst, and enteroepithelial stages of the Ethiopian isolate of *H. hammondi* are shown in Figure 1. Oocysts (Fig. 1A) were shed unsporulated in feces of cats A and B and were approximately 12 µm in diameter. Tissue cysts (Fig. 1B) found in muscles of infected mice followed the shape of infected myocytes. Schizonts, gamonts, and oocysts were found in histological sections of entrocytes of ileum of cat A fed *H. hammondi* tissue cysts and killed 10 days PI (Fig. 1C); these stages were identical to those of *H. hammondi* (Dubey and Sreekumar, 2003).

Tissue stages of *H. hammondi* were not infective to mice as evidenced by bioassays. The KO and SW mice inoculated with muscle homogenate of infected mice containing microscopically verified bradyzoites remained asymptomatic and did not have antibodies to *T. gondii*, and protozoal tissue cysts were not found in their tissues when killed 52 days PI.

The Ethiopian isolate of *H. hammondi* (HhCatEt1) from cat #9 was relatively avirulent for outbred SW mice. The SW mice each fed as many as 10,000,000 oocysts became infected but usually remained asymptomatic. Of the 40 KO mice, each fed 10,000,000 oocysts, 2 died 7 days PI; tachyzoites were found in their mesenteric lymph nodes. Five mice died 75, 121, 167, 170, and 180 days PI; tissue cysts were seen in sections of their muscles but not in brain material. The KO and SW mice each fed 5,000,000 became infected but remained asymptomatic.

HhCatEt2 (cat #20)

Many (70 million) *Hammondia*/*Toxoplasma*-like oocysts were present in the rectal contents of this cat #20. The 2 SW and 2 KO mice fed oocysts remained asymptomatic; 3 of these 4 mice were seronegative to *T. gondii* at 1:25 dilution, while 1 SW was seropositive at the 1:25 serum dilution. *Hammondia hammondi*–like tissue cysts were found in the muscles of all 4 mice killed 38 days PI, but no cysts were seen in the brain. *Toxoplasma gondii*

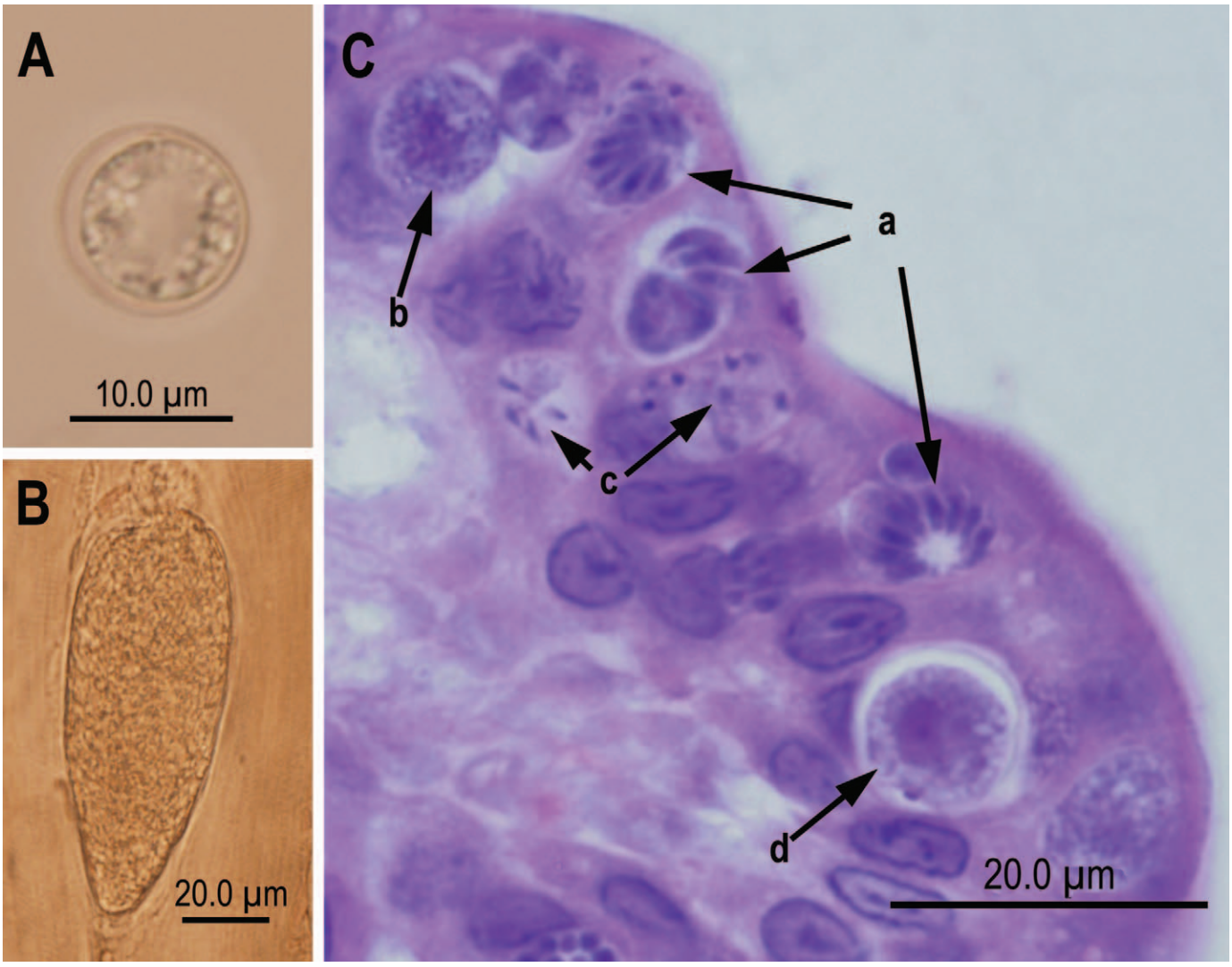

FIGURE 1. Stages of Ethiopian isolate of *Hammondia hammondi* . (**A**) Unsporulated oocyst in feces of an experimentally infected cat. Unstained. (**B**) Tissue cyst in quadriceps muscle of an experimentally infected KO mouse. Unstained. (**C**) Section of small intestine of cat A, 10 days after feeding on *H. hammondi*–infected muscles. Note schizonts (a), female gamont (b), male gamonts (c), and an oocyst (d) in surface entrocytes. Hematoxylin and eosin stain.

DNA was not detected by PCR from the DNA from infected mouse muscles. Cat C fed infected muscles shed *H. hammondi* oocysts with a prepatent period of 5 days. The 1 KO and 2 SW mice inoculated with lymph node, spleen, and liver homogenate of cat C did not become infected with *H. hammondi* or *T. gondii*.

The HhCatEt2 strain oocysts from cat C were more pathogenic to SW than KO mice. Of the mice fed 1,000,000 oocysts, all 5 SW mice died (day of death 6, 6, 10, 13, 13), but only 2 of 5 KO mice died (day of death 9). Tachyzoites were found in intestines and mesenteric lymph nodes of mice that died 6–13 days PI. Of the 10 mice fed 100,000 oocysts, 1 of 5 SW mice died (day of death 9), and none of the 5 KO mice died. All 10 SW or KO mice fed 10,000 oocysts remained asymptomatic. Numerous tissue cysts were seen in sections of skeletal muscle, including tongue, but not in other tissues in mice.

PCR and sequences

PCR products were amplified from oocysts from the Ethiopian isolates HhCatEt1, HhCatEt2, and HhCatGer041, but not *T. gondii* VEG strain (Fig. 2). The HhCatEt1 and HhCatEt2 PCR products were ethanol precipitated and directly sequenced with Hham34F and Hham3R. The HhCatEt1 sequence matched *H. hammondi* isolate P7 sequence (GenBank succession no. EU493281.1) at 278 of 282 residues. Two of the mismatches were due to ambiguous nucleotides in the PCR sequencing reactions at positions 94 (C or T) and 101 (C or G) that were present in each of the 2 sequence PCR products. These ambiguities (revealed by the similar-sized peaks for both nucleotides at each of these positions) are likely due to amplifications of different copies of this repetitive DNA region (Schares, Herrmann et al., 2008). This confirms that HhCatEt1 is indeed *H. hammondi* and not *T. gondii*. The HhCatEt2 sequence matched HhCatEt1 at 225 of 229

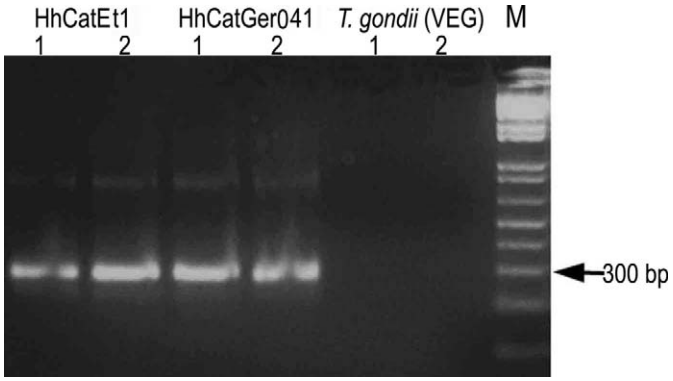

FIGURE 2. PCR amplification of a repetitive diagnostic *Hammondia hammondi* locus from HhCatEt1 and HhCatGer041 but not *Toxoplasma gondii* VEG. Primers were as described in materials and methods, and PCR reactions were carried out in duplicate on 2 distinct DNA preparations of both HhCatEt1 and HhCatGer041. Products in the first 2 lanes were directly sequenced, confirming that HhCatEt1 is *H. hammondi*.

residues and otherwise was most similar to *H. hammondi* P9 sequence (Gen Bank accession no. EU493282.1). Two of the polymorphisms between HhCatEt2 and HhCatEt1 were at ambiguous sites, while 2 others were true polymorphisms. HhCatEt1 and HhCatEt2 sequences have been deposited in GenBank (accession nos. JX477424 and KC223619, respectively).

Hammondia hammondi DNA was detected in the liver, lung, spleen, kidneys, and intestines of 2 KO mice killed day 46 after consuming HhCatEt2 oocysts, but tissue cysts were detected only in muscles of these mice.

DISCUSSION

In the present study, the HhCatEt1 isolate of *H. hammondi* was relatively nonpathogenic for mice. There is limited information on the pathogenicity of *H. hammondi* in outbred mice (Dubey and Sreekumar, 2003), but it is generally thought to be nonpathogenic for outbred SW mice (Frenkel and Dubey, 1975). *Hammondia hammondi* has an obligatory 2-host life cycle; oocysts are not infective to the definitive host, and tissue stages (bradyzoites and tachyzoites) are not infective to intermediate hosts, mice, and it is also nonpathogenic to cats, irrespective of the dose of tissue cysts (Dubey and Sreekumar, 2003). In mice, *H. hammondi* oocysts are not pathogenic by the parenteral route, but mice become ill after feeding on oocysts, depending on the dose and the strain of the parasite (Frenkel and Dubey, 1975; Dubey, 1993). Most studies on *H. hammondi* have been performed using the CR4 and the H.H.34 strains from United States (Frenkel and Dubey, 1975; Dubey and Sreekumar, 2003). With the CR4 strain, SW mice fed 10,000 or more oocysts generally became ill, and approximately 30% died after feeding on 100,000 and 1,000,000 oocysts (Frenkel and Dubey, 1975). With 7 other American strains of *H. hammondi*, SW mice survived oral inoculation with 100,000 oocysts (Christie and Dubey, 1977). There are no published data on pathogenicity of other isolates of *H. hammondi* in outbred mice.

The multiplication of *H. hammondi* in SW mice is limited. In histological sections of SW mice, tissue cysts are mainly in skeletal muscles, and cyst size varies, depending on the tissue; cysts in skeletal muscles are up to 350 μm long, but only 30 μm in cardiac mycocytes and in the brain (Frenkel and Dubey, 1975). Before the discovery of KO mice, mice were administered cortisone to increase the number of *H. hammondi* in SW mice (Frenkel and Dubey, 1975). Currently, KO mice are used to propagate *H. hammondi* (Dubey and Sreekumar, 2003). The KO mice fed 10,000 oocysts of the H.H.34 strain generally become sick and few die, but a dose titration has not been conducted because KO mice are expensive.

Schares, Hermann et al. (2008) bioassayed 2 *H. hammondi* isolates (HHA-dgGER1 and HHA-dgGER2) in 2 KO mice each. The 2 KO mice fed 15,000 HHA-dgGER1 oocysts were killed 155 days PI, and the 2 KO mice fed 10,000 HHA-dgGER2 oocysts were killed 181days PI. All 4 KO mice remained asymptomatic until necropsy, and portions of their brain, heart, lungs, kidneys, spleen, liver, and skeletal muscle were tested for *H. hammondi* DNA (Schares, Herrmann et al., 2008); *H. hammondi* DNA was detected in skeletal muscle, heart, and lungs of all 4 mice, in the brains of 3, and in spleens, kidneys, and liver of 2 mice. These observations are confirmed here. These data indicate that *H. hammondi* is not localized exclusively to the muscles of KO mice. However, tissue cysts have been detected only in muscles and rarely in brains of mice, including KO mice. This is the first study that characterizes *H. hammondi* isolates biologically and molecularly on their first isolation of the strain.

The genome sequencing of the H.H.34 strain is in progress, and there is great scientific interest in comparing the genomic structures of the pathogenic *T. gondii* with the nonpathogenic *H. hammondi*. In addition to the sequence from this isolate, we have also recently sequenced another *H. hammondi* isolate from Germany (HhCatGer041; Walzer et al., 2013), which should also be useful for these comparative studies and to begin identifying genes that are more, or less, prone to mutation and diversification in this species. At high inoculation doses, there seems to be variation in the virulence of *H. hammondi* in mice, especially in KO mice, and these comparative studies may shed new light on the genetic underpinnings of these distinct phenotypes. In this respect, the HhCatEt1 isolate from Ethiopia is nonpathogenic, even to KO mice, and future studies to sequence its genome may provide further insights into the evolution of mouse virulence in this species and the development of new hypotheses about key loci involved in virulence in both *H. hammondi* and *T. gondii*.

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