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Cryptosporidium rubeyi n. sp. (Apicomplexa: Cryptosporidiidae) in multiple *Spermophilus* ground squirrel species

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Previously we reported the unique *Cryptosporidium* sp. “c” genotype (e.g., Sbey03c, Sbey05c, Sbld05c, Sltl05c) from three species of *Spermophilus* ground squirrel (*Spermophilus beecheyi*, *Spermophilus beldingi*, *Spermophilus lateralis*) located throughout California, USA. This follow-up work characterizes the morphology and animal infectivity of this novel genotype as the final step in proposing it as a new species of *Cryptosporidium*. Analysis of sequences of 18S rRNA, actin, and HSP70 genes of additional *Cryptosporidium* isolates from recently sampled California ground squirrels (*S. beecheyi*) confirms the presence of the unique Sbey-c genotype in *S. beecheyi*. Phylogenetic and BLAST analysis indicates that the c-genotype in *Spermophilus* ground squirrels is distinct from *Cryptosporidium* species/genotypes from other host species currently available in GenBank. We propose to name this c-genotype found in *Spermophilus* ground squirrels as *Cryptosporidium rubeyi* n. sp. The mean size of *C. rubeyi* n. sp. oocysts is 4.67 (4.4–5.0) μm × 4.34 (4.0–5.0) μm, with a length/width index of 1.08 (n = 220). Oocysts of *C. rubeyi* n. sp. are not infectious to neonatal BALB/c mice and Holstein calves. GenBank accession numbers for *C. rubeyi* n. sp. are DQ295012, AY462233, and KM010224 for the 18S rRNA gene, KM010227 for the actin gene, and KM010229 for the HSP70 gene.

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1. Introduction

Cryptosporidium spp. are a group of protozoan parasites that infect a wide range of vertebrate hosts including companion animals, livestock, wildlife, and humans. Approximately 30 species of *Cryptosporidium* have been described in vertebrate hosts that include fish, amphibians, reptiles, birds and mammals (Šlapeta, 2013). Host specificity, when documented, is highly variable between *Cryptosporidium* species, with some species or genotypes, for example *Cryptosporidium parvum*, capable of infecting multiple vertebrate hosts, while other species, for example, *Cryptosporidium andersoni*, appear restricted to a much smaller number of hosts. Systematic challenge studies for many recently described species of *Cryptosporidium* in taxonomically-related or unrelated vertebrate hosts are often lacking. Although humans and livestock are considered major biological reservoirs of a number of *Cryptosporidium* species (MacKenzie et al., 1994; Xiao and Ryan, 2004; Atwill et al., 2006; Feltus et al., 2006; Brook et al., 2009), wildlife are increasingly recognized as significant sources of environmental dissemination (Jiang et al., 2005; Feng et al., 2007; Ruecker et al., 2007; Chalmers et al., 2010) which can help foster inter-species transmission between livestock, wildlife, and humans (Hill et al., 2008; Putignani and Menichella, 2010; Raskova et al., 2013).

Ground-dwelling squirrels of the genus of *Spermophilus* are ubiquitous across California, USA. Each *Spermophilus* species inhabits a different set of ecosystems, including coastal plains and lower agricultural valleys, foothills dominated by annual grassland or oak woodlands, meadow complexes surrounded by coniferous forests, and isolated groves of pinyon pines in the remote mountains of eastern California. Colonies of ground squirrels can reach relatively high densities in suitable habitats, resulting in high rates of environmental loading of *Cryptosporidium* oocysts (Atwill et al.,

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2001). For example, California ground squirrels (*Spermophilus beecheyi*) can reach densities as high as 92 adults hectare^{-1} (Owings et al., 1977; Boellstorff and Owings, 1995), which when combined with shedding of up to 2 × 10^5 oocysts animal^{-1} day^{-1} results in rates of environmental loading equivalent to 1 × 10^7 oocysts hectare^{-1} day^{-1} (Atwill et al., 2004).

Previously we have reported a unique *Cryptosporidium* sp. c-genotype in California ground squirrels (*S. beecheyi*) (Sbey03c, 05c), Belding's ground squirrels (*Spermophilus beldingi*) (Sbld05c), and Golden mantled ground squirrels (*Spermophilus lateralis*) (Sltl05c) from throughout California, USA (Pereira et al., 2010). Based on DNA sequences of multiple genes of *Cryptosporidium*, this c-genotype is consistently different from other *Cryptosporidium* isolated from a wide range of hosts, supporting its designation as a new species of *Cryptosporidium* in *Spermophilus* ground squirrels from throughout California (Atwill et al., 2004; Pereira et al., 2010). Oocysts of Sbey03c were not infectious to neonatal BALB/c mice (Atwill et al., 2004). In the present work, we describe oocyst morphology of the c-genotype, and assess its infectivity for BALB/c mice and calves. We further characterize this genotype using 18S rRNA, actin, and HSP70 genes. The objective of the present work is to provide data on phenotypic and genotypic characteristics of c-genotype oocysts to support our assertion that this novel *Cryptosporidium* species in *Spermophilus* ground squirrels of California, USA is a new species.

2. Materials and methods

2.1. Sample collection

In 2011, 100 *S. beecheyi* squirrels from the Central Coastal region of California were sampled for additional genetic analysis of *Cryptosporidium* isolates. Squirrels were collected according to the American Veterinary Medical Association's guidelines for harvesting wildlife and feces were obtained from the large intestine and colon. Fecal samples were placed into 15 ml tubes with 5 ml of antibiotic storage solution (0.1 ml 10% Tween 20, 0.006 g Penicillin G, 0.01 g Streptomycin Sulfate, 1.0 ml amphotericin B solution, and reagent grade water for a total of 100 ml). Fecal samples were placed on ice during transportation and stored at 4 °C in the laboratory and processed within one week of collection.

2.2. Detection of Cryptosporidium oocysts

Detection of *Cryptosporidium* oocysts in previous studies were conducted by direct immunofluorescent microscopy (IFA) as described previously (Atwill et al., 2004; Pereira et al., 2010). Similar methods were used for the feces collected in 2011. Briefly, fecal samples were processed within one week after collection. Feces and antibiotic solution were mixed in deionized water with 0.2% Tween 20 to a final volume of 40 ml. The fecal suspension was strained through 4 layers of cotton gauze into a 50 ml centrifuge tube, which was filled with deionized water to a final volume of 50 ml. Tubes were centrifuged at 1500 g for 15 min and supernatant discarded, leaving a 1:1 ratio of pellet to solution volume. This final suspension was homogenized and 10 μl was used for making slides using the Aqua-Glo G/C Direct kit (Waterborne Inc., New Orleans, LA, USA). Slides were examined using a fluorescent microscope (Olympus BX 60) at ×400 magnification.

2.3. Oocyst morphology

A subset of positive fecal samples were resuspended in 40 ml of deionized water with 0.2% Tween 20 and filtered through 4-fold gauze. Filtrates were centrifuged at 1500 *g* for 10 min, supernatants discarded by aspiration, and the pellet resuspended with an equal volume of deionized water. Oocysts were purified using a discontinuous sucrose gradient method (Arrowood and Sterling, 1987) and washed 3× in deionized water with centrifuging. Oocysts were counted using a phase contrast hemacytometer and concentrations were adjusted to 10^5 oocysts/ml deionized water and stored at 4 °C for up to 14 days before morphology was examined. Wet mount slides were prepared by pipetting 20 μl of each oocyst stock solution on to a glass slide, applying a coverslip and sealing with nail enamel. The length and width of each oocyst were measured using Nomarski Differential Interference Contrast (DIC) microscopy (Olympus BX 60) at ×1000 magnification, with an eyepiece micrometer etched with 0.2 μm divisions (reticule KR-230, Scientific Instrument Company, Napa, CA, USA). The mean length and width and the shape index (the ratio of length to width) of each isolate were calculated based on measurements of 20 intact oocysts of each isolate. These measurements were compared to the mean shape indices of 20 oocysts of *C. parvum* from a naturally infected dairy calf from central California (GenBank accession no. FJ752165).

2.4. Infectivity of Cryptosporidium sp. c-genotype oocysts

An *in vivo* neonatal BALB/c mouse assay (Li et al., 2010) was used to determine if *Cryptosporidium* oocysts from *Spermophilus* squirrels were infectious for this well-studied host species. Fresh oocysts were purified as described in Section 2.3 (above) and were stored in deionized water at 4 °C for approximately 3 weeks before inoculation to animals. Prior to inoculating to mice, oocysts were examined with DIC microscopy and confirmed to be intact. Female BALB/c mice with neonatal pups were purchased from Harlan Laboratories (San Diego, CA, USA), housed in cages fitted with air filters and given food and water *ad libitum*. Oocysts were administered to neonatal mice at 5 days of age by intragastric inoculation using a 24-gauge ball–point feeding needle. One hour prior to infection, the pups were removed from the dam to empty their stomachs for easier inoculation and the dam was returned to the pups after inoculation. Each litter of mice was given oocysts from only one isolate as shown in Table 2, using doses ranging from 10^2 to10^4 oocysts per mouse. *C. parvum* oocysts (GenBank accession no. FJ752165) purified from naturally infected California dairy calves were similarly administered to mice as a positive control, as was deionized water as a negative control. Heat inactivated (incubation at 70 °C for 2 h) *C. parvum* oocysts were also inoculated into mice to monitor pass-through of oocysts resulting from inoculation (Li et al., 2010).

Cryptosporidium infection in mice was assessed by staining intestinal homogenates with a FITC-labeled anti-*Cryptosporidium* immunoglobulin M antibody (Waterborne Inc., New Orleans, LA, USA) which has been shown to be a sensitive method for detecting *Cryptosporidium* oocysts from intestinal homogenates of infected mice (Hou et al., 2004). Seven days post-inoculation (PI) mice were euthanized by CO_2 asphyxiation and the entire intestine from duodenum to rectum was collected. Intestinal samples were suspended in 5 ml of deionized water in 50 ml tubes and homogenized with an IKA® Ultra-Turrax T8 tissue homogenizer (GmbH & Co. KG, Staufen, Germany). The homogenates were washed 1× in deionized water by centrifuging at 1500 *g* for 10 min and the supernatant removed. The pellets were resuspended in 10 ml of deionized water and filtered through a 20 μm pore nylon net filter (Millipore, Bedford, MA, USA) fixed on a Swinnex holder (Millipore, Bedford, MA, USA). The filtrates were concentrated to 1 ml by centrifuging at 1500 *g* for 10 min and mixed by vortexing. Fifty μl of the final homogenates were mixed with 50 μl of anti-*Cryptosporidium* monoclonal antibodies (Waterborne Inc., New Orleans, LA, USA) and 2 μl of 0.5% evans blue, then incubated at room temperature for 45 min in a dark box. Three wet mount slides were prepared from each sample using 20 μl of reaction mixture per slide. Slides were

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