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Identification of *Giardia duodenalis* and *Enterocytozoon bieneusi* in an epizoological investigation of a laboratory colony of prairie dogs, *Cynomys ludovicianus*

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Abstract

Since 2005, black-tailed prairie dogs (*Cynomys ludovicianus*) have been collected for use as research animals from field sites in Kansas, Colorado, and Texas. In January of 2012, *Giardia* trophozoites were identified by histology, thin-section electron microscopy, and immunofluorescent staining in the lumen of the small intestine and colon of a prairie dog euthanized because of extreme weight loss. With giardiasis suspected as the cause of weight loss, a survey of *Giardia duodenalis* in the laboratory colony of prairie dogs was initiated. Direct immunofluorescent testing of feces revealed active shedding of *Giardia* cysts in 40% (n = 60) of animals held in the vivarium. All tested fecal samples (n = 29) from animals in another holding facility where the index case originated were PCR positive for *G. duodenalis* with assemblages A and B identified from sequencing *triosephosphate isomerase* (*tpi*), *glutamate dehydrogenase* (*gdh*), and *ß-giardin* (*bg*) genes. Both assemblages are considered zoonotic, thus the parasites in prairie dogs are potential human pathogens and indicate prairie dogs as a possible wildlife reservoir or the victims of pathogen spill-over. Molecular testing for other protozoan gastrointestinal parasites revealed no *Cryptosporidium* infections but identified a host-adapted *Enterocytozoon bieneusi* genotype group.

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Keywords

Giardia duodenalis; Prairie dog; *Enterocytozoon*; Microsporidia; *Cynomys ludovicianus*; Giardiasis

1. Introduction

The black-tailed prairie dog, *Cynomys ludovicianus*, is a common rodent of the family Sciuridae in the Great Plains region of North America and is often found in urban landscapes (Morse et al., 2012). Prairie dogs are susceptible to a number of diseases, including some potentially acquired from introduced and peri-domestic animals in their natural and domestic habitats (Landolfi et al., 2003; Levine and Ivens, 1988; Stapp et al., 2009). In 2005, a colony of black-tailed prairie dogs was housed at the Centers for Disease Control and Prevention (CDC) for use in monkey-pox studies. Previously, animals were collected from areas of Kansas, Colorado, and Texas. In 2012, morbidity and mortality was observed in the laboratory colony, with weight loss and loose stool being the major clinical signs. Several enteric parasites were suspected potential causes of disease post-necropsy of the index case, including *Giardia*, *Cryptosporidium*, and *Enterocytozoon*.

Giardia, *Cryptosporidium*, and *Enterocytozoon* are intestinal protozoan and fungal parasites that infect animals via direct and indirect transmission cycles. The infective stage of each is environmentally resistant, which allows for multiple possible transmission routes, including person-to-person, animal-to-person, water-borne, and food-borne. Since multiple species can infect a very broad host range that includes domestic animals, wildlife, and humans, advanced molecular genotyping tools must be used to distinguish species and genotypes (Appelbee et al., 2005; Feng and Xiao, 2011; Santin and Fayer, 2011; Xiao, 2010). The current study describes the index case and utilizes molecular tools to investigate these enteric pathogens within a captive prairie dog population.

2. Materials and methods

2.1. Collection sites, animal housing, and sampling

Prairie dogs were supplied by private vendors that collected individuals from four locations at various time points since 2005 (2005: Kansas; 2008: Colorado; 2009: Texas; 2011: previous location and a city farm irrigated with treated waste water, Texas). All animals received a medical examination with a standard fecal exam that does not routinely include *Giardia* and are treated with one dose of 0.2–0.4 mg/kg subcutaneous Ivermectin upon arrival to the CDC. In an off-site facility, animals are housed in large groups (~40–60 animals/group). When needed, animals were moved to the vivarium on-site and grouped in two rooms with no contact and staggered holding times (room A: <1 year hold; room B: >1 year hold). At the time of the index case, room A contained 35 individuals from the 2011 group and room B contained 29 individuals from the 2009 group. The 2009 group on-site (room B) had no history of exposure to the 2011 animals (room A) at either the on-site or off-site location. The 2011 animals (room A) had previously been housed with their cohort in the off-site location. All animals were housed and treated at the CDC facilities under an IACUC-approved protocol.

Following the index case, fresh fecal samples were taken from 29 animals from three housing groups at the off-site holding facility for PCR analysis. Additionally, fresh stool samples were taken from all but four animals housed in the vivarium (2011 (Room A) n = 33; 2009 (Room B) n = 27), immediately placed in 10% buffered formalin, and analyzed using an immunofluorescent assay. Stool from these vivarium animals were not tested by PCR analysis.

2.2. Pathology of index case

In January of 2012, one prairie dog in the off-site holding facility was euthanized due to extreme weight loss. Full necropsy was performed. Tissues were fixed in 10% neutral buffered formalin, processed by routine paraffin histology, sectioned, and stained with hematoxylin–eosin for histopathologic evaluation.

Thin-section electron microscopy (EM) was performed on formalin fixed paraffin embedded tissue. A punch biopsy was taken directly from the paraffin block and deparaffinized in xylene. The tissue was fixed in glutaraldehyde, post-fixed in osmium tetroxide, and en block stained with uranyl acetate. A graded alcohol series and acetone were used to dehydrate and infiltrate the tissue, respectively, prior to eponaraldite resin exchanges (Mollenhauer, 1964). Following the final resin exchange, resin infiltrated tissue was placed in a 60 °C oven to polymerize overnight. Ultrathin sections were cut and stained with uranyl acetate and lead citrate prior to examination at the transmission electron microscope (Tecnai Spirit, FEI, Hillsboro, OR).

2.3. Pathogen detection

IFA was performed on index case tissues using rabbit antibody raised against culture-grown trophozoites of *Giardia duodenalis* and commercially prepared goat anti rabbit Ig conjugated with fluorescene isethionate (FITC) (Visvesvara and Healey, 1984). Deparaffinized tissue sections were flooded with a 1:200 dilution of the rabbit anti-*Giardia duodenalis* antibody, incubated at 37 °C for 30 min, washed three times with PBS (pH 7.2), flooded with a 1:1000 dilution of the FITC-goat anti Ig, and incubated at 37 °C for 30 min as above. The sections were washed thrice as above, mounted with anti-fade reagent, coverslipped, examined and photographed using an Olympus BX60 fluorescence microscope. Appropriate positive and negative control tissue sections were tested in parallel.

DNA was isolated from stool specimens from off-site animals using the FastDNA spin kit for soil (MP Biomedicals) following the manufacturer's protocol and stored at −20 °C until further analysis. A multi-locus genotyping method was used to detect and identify *Giardia* spp. by amplifying the *triosephosphate isomerase* (*tpi*), *glutamate dehydrogenase* (*gdh*), and *β-giardin* (*bg*) gene targets from isolated DNA (Caccio et al., 2008; Ye et al., 2014). The internal transcribed spacer (ITS) region of *E. bieneusi* and 18S rRNA gene of *Cryptosporidium* were used for molecular analyses of these organisms as previously described (Ye et al., 2014). Stringent protocols and controls were used in all PCR assays to prevent and to detect contamination.

PCR products were purified using Montage PCR filters (Millipore, Billerica, MA) prior to direct sequencing. Amplicons were bi-directionally sequenced using the secondary reaction primers with Big Dye terminator chemistries (Applied Biosystems, Foster City, CA) and run on an ABI 3130xl/Genetic Analyzer (Applied Biosystems). Complementary sequences were assembled and verified in ChromasPro 1.5 (Technelysium Pty Ltd). Alignments with reference sequences were completed using MEGA 5.1 (Tamura et al., 2011) and Geneious (BioMatters). Consensus neighbor-joining phylogenetic trees were developed using the Kimura 2 parameter model with 100 bootstrap replicates in MEGA 5.1.

Formalin-preserved feces from vivarium animals were screened for *Giardia* cysts and *Cryptosporidium* oocysts using the MERIFLUOR immunoflourescent assay (Meridian BioScience, Inc., Cincinnati, Ohio, USA). Fecal samples were concentrated into fecal pellets using a formalin-ethyl acetate stool concentration technique (Weber et al., 1992) and resuspended in a concentration of 1 g of feces to 1 mL of PBS. Each fecal sample was evaluated by screening 10 μL of the 1 g/mL solution of concentrated feces which was evenly distributed across the slide to ensure appropriate visibility during florescent microscopy (Salzer et al., 2007). This method was validated by spiking negative fecal samples with known quantities of *Cryptosporidium* oocysts and *Giardia* cysts (Salzer et al., 2007). Statistical methods included Fisher's Exact tests to assess differences in shedding between weight, hair loss, and overall health classifications.

3. Results

Necropsy findings for the index case included alopecia and ulcerative pododermatitis. The gastrointestinal tract was grossly unremarkable. Histopathologic examination revealed lymphoplasmacytic inflammation in the small and large intestines, without overt mucosal epithelial damage. Lining the mucosal epithelial surface and mixed with luminal debris were multiple 10–15 μm, round to pyriform or crescent-shaped trophozoites containing a thin wall and one or two deeply basophilic nuclei, consistent with *Giardia* trophozoites (Fig. 1A and C).

Transmission electron microscopy revealed organisms with morphology consistent with *Giardia* spp. Multiple organisms were present within the prairie dog's gastrointestinal tract. Organisms measured 6–10 μm and contained two nuclei, flagella, and a ventral disk (Fig. 2).

In the IFA assay the *Giardia* trophozoites reacted intensely with the anti-*Giardia duodenalis* antibody and produced bright green fluorescence (Fig. 1B). At higher magnifications characteristic features of *G. duodenalis* trophozoites that included a ventral disk (VD), two nuclei (N), and ventral flagella (VFl) were visualized (Fig. 1D).

Individual stools from all 29 animals tested from the off-site facility were positive for *Giardia* by PCR. Using the *tpi* (GenBank accession numbers: KP780949–KP780976), *bg* (GenBank accession numbers: KP780895–KP780924), and *gdh* (GenBank accession numbers KP780925–KP780948) gene targets for *Giardia*, *G. duodenalis* assemblage A (n = 19), assemblage B (n = 6), and mixed infections with assemblages A and B (n = 4) were detected in animals (Table 1). There was no difference in assemblages between animals from different collection years or housing group at time of sampling. A multi-locus

genotyping approach could not resolve the subassemblage in several animals because of incongruent phylogenies (Fig. 3). Analysis of concatenated sequences from assemblage A animals revealed further characterization into subassemblage AI, with a large group of samples comprising a single clade (Fig. 3). Additionally, a novel subtype, A1/A5-like, based on the *gdh* gene was identified that contained a single nucleotide polymorphism (SNP; T substitution for C) at nucleotide 621; this SNP was identified in 16 animals. The six assemblage B sequences were identical at all loci. Following treatment with two rounds of 50 mg of Fenbendazole (on food once a day for 5 days with a 7-day rest between treatment rounds), two animals were still positive for *Giardia*, with one having a different infecting assemblage after the treatment, indicating a previous co-infection or a re-infection with a new genotype (Table 1).

Animals from the 2011 collection group (14 of 17) were positive for *E. bieneusi*, while no animals from other collection years were positive for the microsporidian species (Table 1; GenBank accession numbers KP780977–KP780990). Using the ITS sequence, a novel *E. bieneusi* genotype, named "Row," was identified in all positive animals. The Row genotype shared 97.5% sequence similarity with reference sequences AF101198 (B/human), AF101197 (A/human), and AY237220 (WL12/beaver otter) in Group 1 (Fig. 4). No animals were positive for *Cryptosporidium* by PCR.

Using fluorescent microscopy we identified *Giardia* cysts in 24 of the 60 (40%) fecal samples collected from animals housed in the on-site vivarium facility. In animals from the 2011 collection (room A) group, 67% (22 of 33) were positive compared to 7% (2 of 27) from the 2009 animals (room B). No significant associations between *Giardia* infection and weight loss, hair loss, or overall health were found (data not shown). All animals were negative for the presence of *Cryptosporidium* oocysts.

4. Discussion

Animals sampled from both 2009 and 2011 collection times were found to be positive for *G. duodenalis*. The zoonotic assemblages A and B were identified in both collection year and all housing groups, indicating prairie dogs are susceptible to multiple genotypes of *G. duodenalis* as well as the potential for multiple infection sources in the population. These findings are different from previous studies that identified assemblage A as the only subtype found in wild and captive prairie dogs (Appelbee et al., 2005; van Keulen et al., 2002). A new subtype at the *gdh* gene target of assemblage A-infected animals further indicated that *G. duodenalis* may be established in some prairie dogs as the subtype has not been detected previously (Feng and Xiao, 2011).

In addition to the detection of *G. duodenalis*, a novel genotype of *E. bieneusi* was identified in animals in the 2011 collection group only. *Enterocytozoon bieneusi* is an obligately intracellular parasite in a diverse fungal group identified as Microsporidia. While *E. bieneusi* has been found in a number of wildlife, livestock, and human surveillance studies, its zoonotic potential and epidemiology are not fully understood (Widmer and Akiyoshi, 2010). Studies of the ITS region have shown significant host-specificity, with over 200 genotypes being known (Fayer and Santin-Duran, 2014; Santin and Fayer, 2011; Widmer and

Akiyoshi, 2010). This information, taken with the novel *E. bieneusi* Row genotype identified in the CDC prairie dogs, suggests that it may be a prairie dog-specific genotype that was likely endemic in the 2011 animals from Texas.

The introduction and transmission of *G. duodenalis* into the prairie dog colony was likely influenced by several factors. Firstly, *G. duodenalis* and *E. bieneusi* were likely endemic in animals from one or both Texas collection sites. In addition to the novel subassemblage and genotype evidence, two *Giardia* positive 2009 animals did not have contact with animals from the 2011 collection year. These animals were likely naturally infected with *G. duodenalis* prior to arrival at the animal facilities. Secondly, wild-captured animals are known to become stressed when first introduced to a captive setting. While great care is taken to reduce stress at animal care facilities, decreases in immune responses have been noted that can lead to both increased susceptibility to pathogens and increased shedding of endemic pathogens (Morgan and Tromborg, 2007; Thompson et al., 2005). Lastly, transmission was likely occurring within the facility as evidenced by some animals that tested positive with different *G. duodenalis* assemblages post-treatment compared to the initial screening.

The novel *G. duodenalis* subtype and *E. bieneusi* genotype indicated as endemic pathogens highlight the gaps in prairie dog parasite population studies. Despite its broad host range, giardiasis has rarely been reported for wild or captive prairie dogs, and no reports of *E. bieneusi* infections exist in this species. In one case report from Korea, a captive prairie dog in a colony with high morbidity and mortality was found to have profuse *Giardia* trophozoites throughout the duodenum and jejunum (Cho et al., 2005). Because the prairie dog is considered a keystone species and often exists at the human-wildlife interface, further characterization of this species' endemic parasites would be beneficial to ecosystem conservation efforts and one health studies (Kotliar et al., 1999; Morse et al., 2012).

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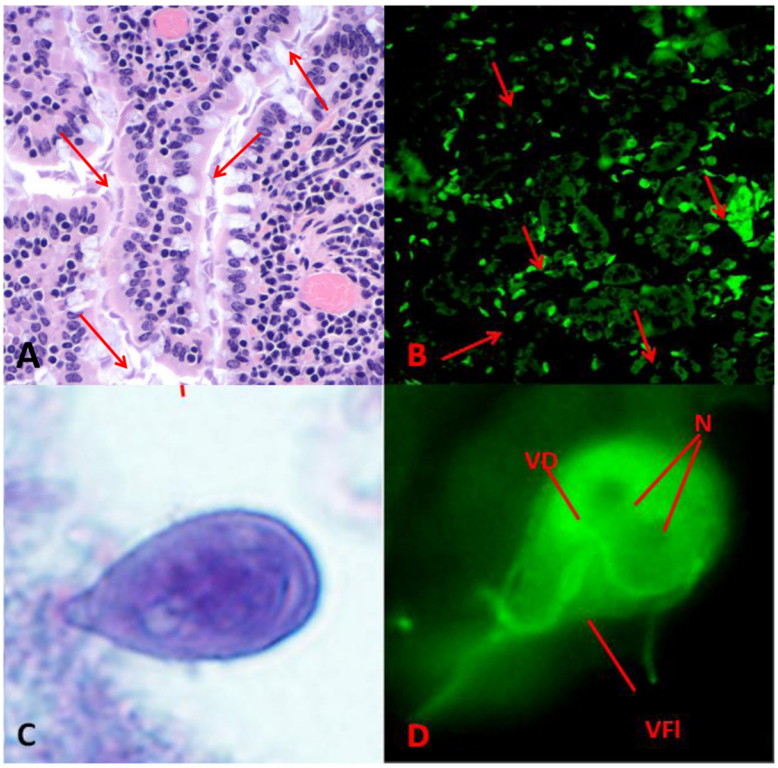

Fig. 1.

Microscopic evaluation of H&E and indirect immunofluorescent stained lumen section from index case. (A and C) Ten to fifteen micrometers, crescent-shaped trophozoites, consistent with *Giardia* spp. line the intestinal mucosal surface. (B) *Giardia* trophozoites reacted intensely with the anti-*Giardia duodenalis* antibody and produced bright green fluorescence (original magnification 25×). (D) Characteristic features of *G. duodenalis* trophozoites that included a ventral disk (VD), two nuclei (N) and ventral flagella (VFl) were visualized (original magnification 100×).

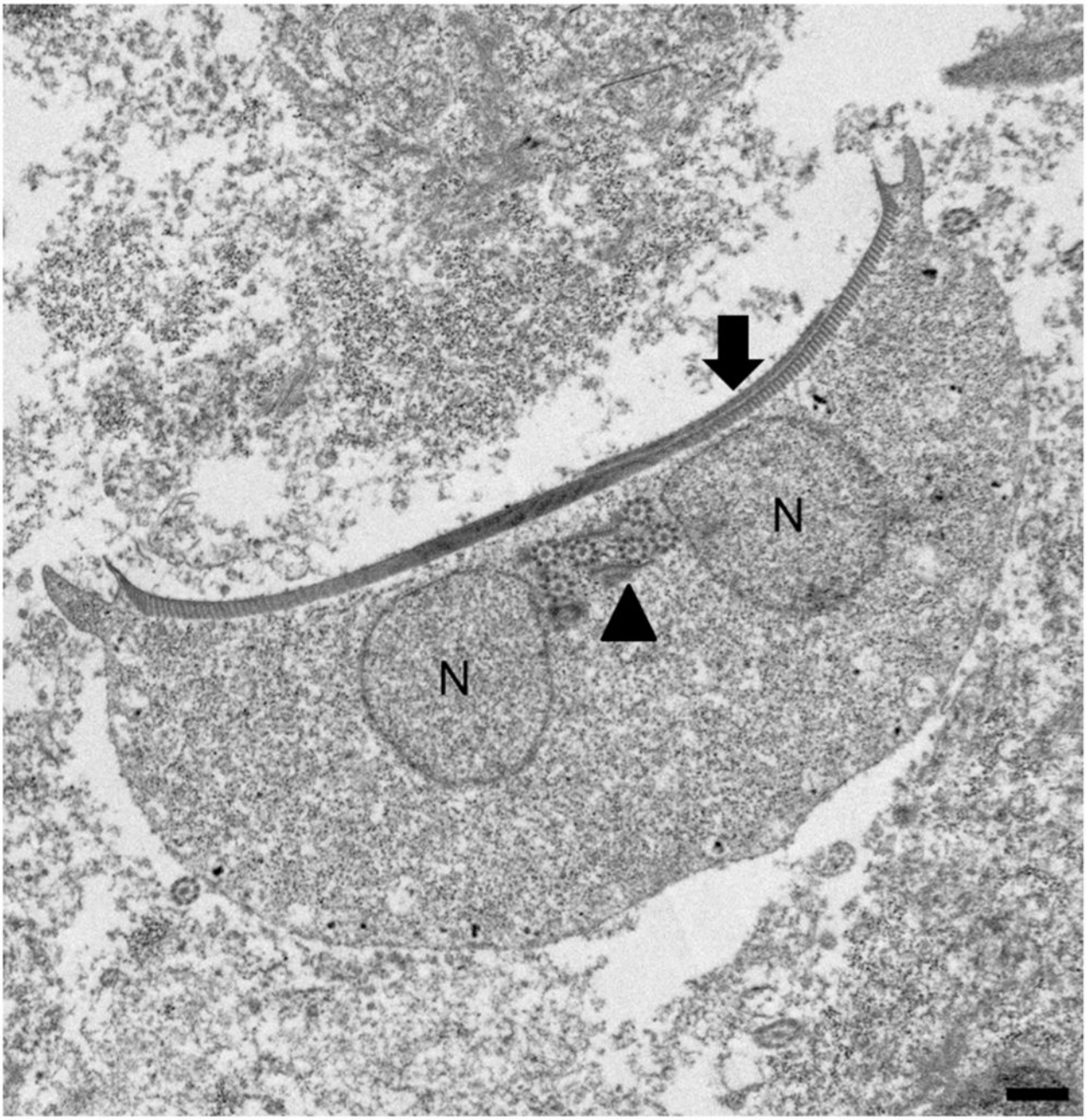

Fig. 2.

Thin-section electron microscopy of *Giardia* spp. A single *Giardia* organism containing two nuclei (N), basal bodies of flagella (arrowhead) and a ventral disk (arrow). Scale bar corresponds to 500 nm.

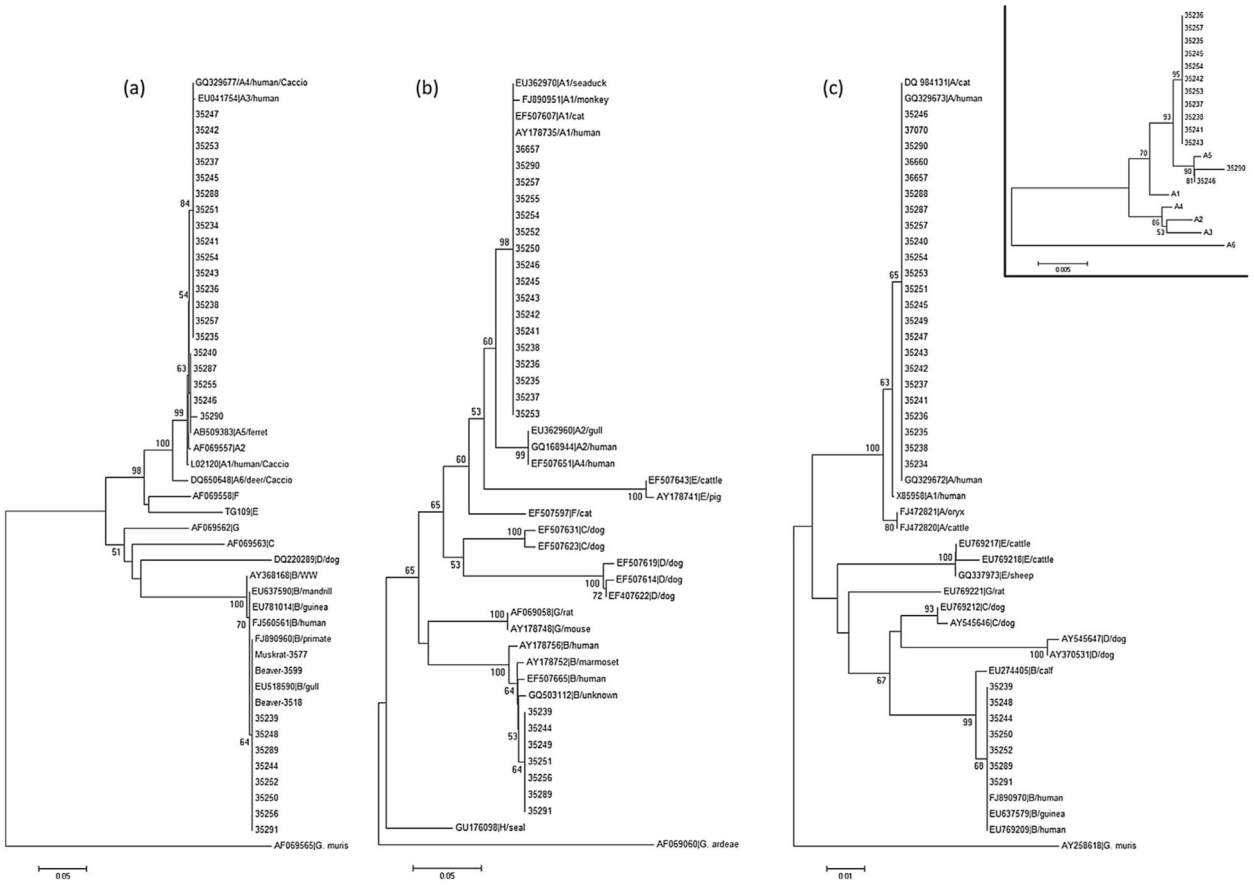

Fig. 3.

Consensus neighbor-joining phylogenetic tree of *Giardia duodenalis*. Phylogenetic tree of (a) *tpi* (b) *gdh* (c) *bg* gene targets and (inset) concatenated sequences at all targets for assemblage A samples in a laboratory colony of prairie dogs using the Kimura-2 parameter model with 100 bootstrap replicates in MEGA 5.1.

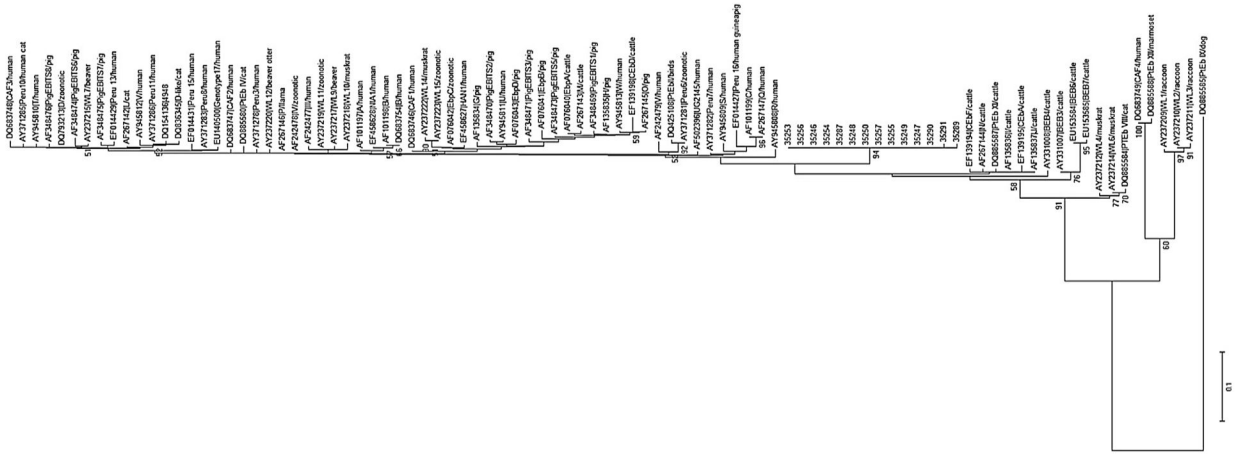

Fig. 4.

Consensus neighbor-joining phylogenetic tree of *Enterocytozoon bieneusi*. Phylogenetic tree of internal transcribed spacer (ITS) region in a laboratory colony of prairie dogs using the Kimura-2 parameter model with 100 bootstrap replicates in MEGA 5.1.

Table 1

Molecular detection and subtyping of *Giardia* and *Enterocytozoon bieneusi* in prairie dogs.[a,b]

Animal ID	Collection year	*Giardia* assemblage	*Giardia* subassemblage	*tpi*	*gdh*	*bg*	*E. bieneusi*
35234[c]	2009	A	AI/AII	A4	n.a.	A5	−
35235[c]	2009	A	AI	A4	A1/A5-like	A5	−
35236[c]	2009	A	AI	A4	A1/A5-like	A5	−
35237[c]	2009	A	AI	A4	A1/A5-like	A5	−
35238[c]	2009	A	AI	A4	A1/A5-like	A5	−
35240[c]	2009	A	AI	A5	n.a.	A5	−
35241[c]	2009	A	AI	A4	A1/A5-like	A5	−
35242[c]	2009	A	AI	A4	A1/A5-like	A5	−
35243[c]	2009	A	AI	A4	A1/A5-like	A5	−
35244[c]	2009	B	B	B	B	B	−
35245[c]	2009	A	AI	A4	A1/A5-like	A5	−
35251[c]	2011	A/B	Ind.	A4	B	A5	−
35255[c]	2011	A	AI	A5	A1/A5-like	n.a.	+
35256[c]	2011	B	B	B	B	n.a.	+
35287[c]	2011	A	AI	A5	n.a.	A5	+
35289[c]	2011	B	B	B	B	B	+
35290[c]	2011	A	AI	A5	A1/A5-like	A5	+
35288[d]	2011	A	AI/AII	A4	n.a.	A5	−
35239[d]	2009	B	B	B	B	B	−
35246[e]	2011	A	AI	A5	A1/A5-like	A5	+
35247[e]	2011	A	AI/AII	A4	n.a.	A5	+
35248[e]	2011	B	B	B	n.a.	B	+
35252[e]	2011	A/B	Ind.	B	A1/A5-like	B	−
35253[e]	2011	A	AI	A4	A1/A5-like	A5	+

Animal ID	Collection year	*Giardia* assemblage	*Giardia* subassemblage	*tpi*	*gdh*	*bg*	*E. bieneusi*
35291[e]	2011	B	B	B	B	B	+
35254[e]	2011	A	AI	A4	A1/A5-like	A5	+
35250[e]	2011	A/B	Ind.	B	A1/A5-like	B	+
35257	2011	A	AI	A4	A1/A5-like	A5	+
35249	2011	A/B	Ind.	n.a.	B	A5	+

[a] All animals tested negative for *Cryptosporidium* by PCR.

[b] n/a = sequencing could not be achieved; Ind = Indeterminable due to mixed infections or incongruences between loci.

[c–e] Indicate animals that shared rooms at the off-site holding facility.