

Experimental infection of cats with *Cystoisospora felis*

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Abstract

Background: *Cystoisospora felis* is a common parasite of cats and is diagnosed by fecal flotation, but false-negative results can be common.

Hypothesis/Objectives: To experimentally inoculate cats with *C. felis* oocysts, to compare fecal flotation and polymerase chain reaction (PCR) results, and to describe any clinical signs consistent with infection.

Animals: Six cats.

Methods: *Cystoisospora felis* oocysts were identified morphologically from feces of a naturally infected kitten with diarrhea, sporulated oocysts (5000) were inoculated to 6 cats that were negative for fecal parasites by fecal flotation and by a fluorescent antibody assay (FA) for *Giardia* spp. and *Cryptosporidium* spp. Cats were observed daily for the presence of clinical signs consistent with infection. Fecal samples were evaluated by fecal flotation and FA up to 3 times per week post inoculation (PI) to Day 27. Thirty-six samples collected before inoculation and from Days 8, 10, 13, 15, and 20 PI were assayed using an internal transcribed spacer 1 (ITS1) PCR that amplifies DNA of *C. felis*.

Results: All cats were negative for *C. felis* by both assays before inoculation. All cats shed *C. felis* oocysts by Day 10 PI, oocysts were not detected by fecal flotation after Day 15 PI. *Cystoisospora felis* DNA was amplified from 24/36 (66.6%) fecal samples from 6/6 (100%) of the cats. Oocysts were not detected by fecal flotation in 4 of the samples that were positive for *C. felis* DNA by PCR. Clinical signs were not recognized in any of the study cats.

Conclusions and Clinical Importance: Fecal flotation is a convenient assay for detection of *C. felis* but could occasionally give false-negative results when compared to this ITS1 PCR.

KEYWORDS

cats, clinical signs, *Cystoisospora felis*, fecal flotation, ITS-1 PCR

Abbreviations: DNA, deoxyribonucleic acid; FA, fluorescent antibody assay; ITS1, internal transcribed spacer 1; PCR, polymerase chain reaction; PI, post inoculation.

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1 | INTRODUCTION

Cystoisospora spp. (previously *Isoospora* spp.) are protozoan parasites that belong to the coccidian group, are strictly host specific and have worldwide distribution.^{1,2} Cats are definitive hosts for *Cystoisospora felis* and *Cystoisospora rivolta*. Cats acquire *Cystoisospora* spp. by ingestion of sporulated oocysts from the environment or by ingestion of tissues of another infected paratenic vertebrate host.² The infection can also occur after ingestion of paratenic hosts such as flies, cockroaches, or dung beetles that have ingested sporulated oocysts.^{2,3} After ingestion, the enteroepithelial phase occurs in the small intestine of infected cats and results in the passage of unsporulated oocysts in feces. The prepatent periods for *C. felis* and *C. rivolta* are 7 to 11 days and 4 to 7 days, respectively.¹ The patent periods for *C. felis* and *C. rivolta* are 10 to 11 days and 14 or more days, respectively.¹ The numbers of oocysts shed can vary considerably by individual animal.

Clinical disease associated with *Cystoisospora* spp. infections occurs most commonly in young, debilitated and immunosuppressed animals. Diagnosis of coccidiosis is performed by demonstration of oocysts in fecal samples by microscopic examination after fecal flotation. However, cats with or without clinical signs of disease can pass *Cystoisospora* spp. oocysts, so the finding of oocysts does not prove a disease association. *Cystoisospora felis* is commonly believed to induce diarrhea in kittens but few experimental data are available from young adult cats. The aims of this study were to experimentally inoculate cats with *C. felis* oocysts isolated from a naturally infected kitten, to compare the results of fecal flotation and a conventional polymerase chain reaction (PCR) assay for their ability to detect *C. felis*, and to describe any clinical findings consistent with infection.

2 | MATERIALS AND METHODS

2.1 | Source of oocysts

Oocysts consistent with the structure of *C. felis* were identified morphologically and then concentrated from the feces of a naturally infected kitten with diarrhea.⁴ No other parasites were detected after sugar centrifugal flotation and by assessment of a commercially available fluorescent antibody assay (FA) for *Giardia* spp. and *Cryptosporidium* spp. (Merifluor *Crypto/Giardia* kit, Meridian Diagnostic Corporation, Cincinnati, Ohio).

2.2 | Experimental inoculation

Six domestic short-hair cats (8 months old) were purchased from a commercial breeder, housed individually, and shown to be negative for fecal parasites on 3 different samples collected a week before inoculation. An inoculum containing a total of 5000 *C. felis* sporulated oocysts was then administered to each cat by stomach tube while sedated. Food was withheld for 24 hours and then the cats were fed a commercial feline diet ad libitum and observed daily for the

presence of gastrointestinal signs such as inappetance, vomiting, or diarrhea.

The protocol for animal use in this project was approved by the Colorado State University Institutional Animal Care and Use Committee. The title of the protocol was: "Establishment of *Cryptosporidium felis* and *Isoospora felis* infection in experimentally inoculated cats," IACUC Protocol 12-3323A and it was originally approved on April 20, 2012.

2.3 | Assays performed

Fecal samples were evaluated by fecal flotation (1 g) and by FA (1 g) up to 3 times per week post inoculation (PI) to Day 27. An oocyst score was assigned after fecal flotation: Score 0 = 0 oocysts (per slide); Score 1 = 1 to 250 oocysts; Score 2 = 250 to 500 oocysts; Score 3 = greater than 500. A total of 36 samples (6 from each cat) collected before inoculation and from Days 8, 10, 13, 15, and 20 PI were assayed using an internal transcribed spacer (ITS1) PCR assay designed by IDEXX laboratories.

DNA was isolated from fecal samples using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Polymerase chain reaction primers *C. felis* ITS1 fp (5' CTAAGTGAATCCCATAATCAGGAC 3') and *C. felis* ITS1 rp (5' CCAAATCTCAAGGAGATAGGAG 3') amplified a 224 base pair fragment of the *C. felis* ITS1 sequence (EU124689). The reactions contained 25 pmol of each primer (Integrated DNA Technologies, Coralville, Iowa), 2.2 U of ActiQ[®] DNA polymerase (Roche Diagnostics, Mannheim, Germany), 200 μ M of each dNTP (Roche Diagnostics, Mannheim, Germany) and 2 mM of MgCl₂ (Roche Diagnostics, Mannheim, Germany) in a final volume of 50 μ L. Cycling conditions for the assay consisted of a pre-incubation step (95°C for 10 minutes) and 50 amplification cycles (95°C for 20 seconds, 55°C for 20 seconds, 72°C for 20 seconds).

Analytical specificity was determined by the specificity of the primers and by testing a panel of DNA from common feline parasites. Deoxyribonucleic acid from various *Giardia duodenalis* isolates (Assemblages F and D), *Cryptosporidium* spp. (*Cryptosporidium parvum*, *Cryptosporidium ubiquitum*, and other *Cryptosporidium* species isolated from mouse, and wild felids) and *Toxoplasma gondii* were tested by the ITS1 PCR.

A cat fecal sample that tested positive for *C. felis* by fecal flotation and by PCR was used to calculate the analytical sensitivity. Five fold serial dilutions were prepared from this sample, from 30 oocysts/g of feces to 0.93 oocysts/g of feces, in PCR water. Multiple titrations were performed to detect analytical sensitivity.

Diagnostic sensitivity and specificity were calculated using Fecal flotation (FF) as a gold standard.

Sequencing analysis was performed in the samples with discordant fecal float and PCR results. Deoxyribonucleic acid sequences were analyzed in forward and reverse directions using an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, California). The sequence data from *C. felis* isolates by ITS1 gene was compared by

BLAST analysis with sequences from the nucleotide database from the GenBank.

3 | RESULTS

All cats were negative for *C. felis* by both assays before inoculation. After *C. felis* inoculation, 20 of 30 fecal samples tested were positive for *C. felis* oocysts by FF. Oocysts were seen in feces by FF on 4 of 6 cats on Day 8 PI (Score 1), on all 6 cats on Days 10 PI (Score 3) and 13 PI (Scores 1-3), and on 4 of 6 cats on Day 15 PI (Score 1). For all other time points assessed (Days 17, 20, 22, 23, and 27), all cats were negative after fecal flotation.

After *C. felis* inoculation, 24 of 30 fecal samples tested were positive for *C. felis* DNA in feces. *Cystoisospora felis* DNA was amplified from feces from 5 of 6 cats on Day 8 PI, from all 6 cats on Days 10 and 13 PI, and on 5 of 6 cats by Day 15 PI. On Day 20 PI, *C. felis* DNA was amplified from 2 out of the 6 cats. Oocysts were not detected in 4 of the selected samples that were positive for *C. felis* DNA (Days 8, 15, and 20).

The specificity of the primers was determined by BLAST analysis and they matched *C. felis* (EU124689 and HK 430064.1) sequences. Deoxyribonucleic acid from various *G. duodenalis* isolates (Assemblages F and D), *Cryptosporidium* spp. (*C. parvum*, *C. ubiquitum* and other *Cryptosporidium* species isolated from mouse, and wild felids), and *T. gondii* was not amplified by the ITS1 PCR.

The ITS1 PCR assay consistently detected up to the 1.78 oocysts/g of feces. Considering that only 300 μ L of sample are used in the DNA extraction, the detection limit of the assay is below 1 oocyst.

The 4 PCR positive FF negative samples tested showed 100% homology to 2 *C. felis* isolates (MK430064 and EU124689) by BLAST analysis.

The diagnostic sensitivity and specificity of FF (defined at the gold standard) in comparison to ITS1 PCR assay were 100% and 75%, respectively.

Clinical signs were not recognized in any of the cats.

4 | DISCUSSION

Fecal flotation is the most commonly used technique for detection of *Cystoisospora* spp. oocysts in cat feces. To the best of our knowledge, the minimum detection limit of *Cystoisospora* spp. oocysts by fecal flotation has not been reported. Polymerase chain reaction has been used to amplify DNA of a variety of fecal parasites in cats and some assays could be more sensitive than microscopy.^{5,6} In this study, oocysts were not detected in 4 of the selected samples that were positive for *C. felis* DNA. These 4 samples could be considered false PCR positives or true PCR positives. Each PCR was run with DNA extraction negative controls in addition to positive (*Cystoisospora* spp. DNA) and negative PCR (water) controls, since the results of the controls worked as expected, we believe that these discordant results were

not false-positive PCR results. The DNA sequencing of the *C. felis* ITS1 amplicons for the discordant samples confirmed the presence of *C. felis* DNA. *Cystoisospora* shedding is variable between animals; maybe the number of oocysts shed was below the threshold of detection of the FF. The detection limit of this PCR assay is below 1 *C. felis* oocyst, and *C. felis* DNA can be detected in feces in the absence of intact cysts, so we believe that these results are true PCR positives.

The primers used in the PCR in the study were specific for *C. felis*. The specificity of the primers was determined by BLAST analysis, by assessment of DNA of other feline parasites and DNA sequencing of the amplicons. Polymerase chain reaction for detection of *Cystoisospora* spp. in cats is not routinely used but in certain situations can be useful. The ITS1 is commonly used as a biomarker of *Cystoisospora* spp. due to its high copy number element and represents a promising target for diagnosis. Amplification of the 18S rRNA and ITS1 genes for detection of *Cystoisospora* spp. infections was previously used for research purposes in dogs and cats.⁵⁻⁷ False-negative fecal flotation results in cats with diarrhea are infrequent.⁸ Previous studies showed that several PCR assays were more sensitive than microscopy, so we assumed that false-negative PCR results will also be uncommon.^{5,9} However, in some cases clinical signs precede oocysts shedding and so a second sample might be examined to prove infection. Conversely, false-negative fecal float results could also occur, if the sample was not collected during the shedding period or if the shedding was below the detection limit of the fecal flotation. *C. felis* induces shedding of *T. gondii* oocysts in cats chronically infected with *T. gondii* in the absence of clinical signs.² The number of *C. felis* oocysts shedding varied among individuals, so this assay could be useful for monitoring *T. gondii* chronically infected cats for detection of *C. felis* infections.

The ITS1 target can also be included as part of a multiplex PCR for simultaneous detection of various fecal parasites. Diagnostic sensitivity and specificity and predictive values need to now be evaluated in samples from naturally infected cats.

In regard to clinical signs, young adult cats orally inoculated with *C. felis* sporulated oocysts in this model developed self-limiting, sub-clinical infections. This finding is consistent with previous research that showed that *C. felis* is not a significant primary pathogen in cats over 1 month of age.¹

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Colorado State University IACUC (IACUC Protocol 12-3323A).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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