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The development of a molecular approach for coprodiagnosis of *Toxoplasma gondii*

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Abstract

Copro-diagnostic methods for *Toxoplasma gondii* infected cats have been traditionally based on the identification of oocysts by light microscopy or by bioassays. The first method is not sensitive and also unable to differentiate between *Toxoplasma* oocysts from other coccidian parasites in cats, and the second is cumbersome, time consuming and expensive. We have adapted a polymerase chain reaction (PCR) method to detect *T. gondii* oocyst DNA in fecal samples. Oocysts were successfully disrupted by freeze thawing coupled with mechanical means, and DNA extraction was subsequently accomplished. The test, based on amplifying a 529 bp repeated sequence, proved sensitive for detecting 1–2 oocysts in 200 μg of stool sample. The test specificity was established by showing that DNA from other cat coccidia tested negative. Specificity was reconfirmed by Southern hybridization of the PCR products with a specific probe. Of 122 stool samples from Jerusalem cats surveyed for the presence of *Toxoplasma* oocysts, 11 were found positive by PCR while none was detected by microscopy.

Keywords: *Toxoplasma gondii*; Oocysts; Feces; PCR

1. Introduction

Cats, definitive hosts for *Toxoplasma gondii*, have been shown to excrete oocysts of this coccidian parasite for a limited and relatively short period of time when a primary infection takes place (Dubey, 1976; Davis and Dubey, 1995) During this period of excretion that lasts about 2 weeks, millions of oocysts are passed in the stool daily. Within 2–5 days, oocysts sporulate and become infective to mammals and birds (Dubey et al., 1970b). Excretion of fewer oocysts occasionally occurs in reinfected cats (Dubey, 1976; Dubey, 1995).

Historically, detection of infective cats has been based on the detection of the excreted oocysts, approximately 10–13 μm in diameter, under light microscopy after their prior concentration by flotation. The sensitivity of this method is low and thus detection is problematic when smaller oocyst numbers are excreted, as occurs, for example, after infection with some strains or after cats are infected by the tachyzoite or oocyst stages of this parasite (Dubey and Frenkel, 1976; Dubey, 1996). Furthermore, oocysts of other coccidian parasites appearing in cat feces, such as those of *Hammondia hammondi*, may not be differentiated microscopically from *T. gondii*. Bioassay in mice, although specific and relatively sensitive for the detection of *T. gondii* oocysts, relies on the presence of sporulated and thus infective oocysts in the sample. Not only does this method introduce a

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possible biohazard element, it is also expensive, requires animal facilities, and is time consuming.

Since molecular coprodiagnostic methods can be expected to prove superior for parasite detection (Singh, 1997; Abassi et al., 2003; Orlandi and Lampel, 2000), we have adapted a PCR method, amplifying a 529 bp repeated sequence (Homan et al., 2000) to detect *T. gondii* in feces, in search of an alternative method for detecting parasites in infective feces with high sensitivity, specificity and reproducibility.

2. Materials and Methods

2.1. Feces samples and parasites

Negative fecal samples used in spiking experiments, were kindly provided by Prof. V. Svobodova, Brno, Czech Republic. They were taken from cats that were shown to be seronegative for *T. gondii* on two serial indirect immunofluorescence tests and for which the feces were free of *Toxoplasma*-like oocysts by light microscopy. These feces samples were stored in 70% ethanol at 4 °C until used. Fecal samples from stray cats captured for the survey and examined for *Toxoplasma* infection were kept at 4 °C until used (see below). Oocysts of the VEG strain were kindly provided by Dr. J.P. Dubey (USDA, Beltsville, USA), and were stored in 2% sulphuric acid at 4 °C. DNA from coccidian parasites, *H. hammondi*, *Hammondia heydorni* and *Neospora caninum* (donated by Dr.Schares, Vienna, Austria), *Cryptosporidium parvum* (donated by Dr.Naser, Ministry of Health, Israel), *Besnoitia besnoiti* (donated by Dr. Shkap, Veterinary Institute, Israel), *Isospora suis* and *Cystoisospora* sp. (donated by Dr. Anja Joachim, Vienna, Austria) were used to assess the specificity of the test.

2.2. Examining the efficacy of mechanical disruption of oocysts

Approximately 10,000 sporulated oocysts were mixed in 200 μL double distilled water (ddw) with an equal volume of glass beads (400–600 nm, Sigma–Aldrich, Missouri, USA), then vortexed for 30 min with intermittent freeze–thawing every 10 min using 1 min dips in liquid nitrogen for freezing and 1 min at 60 °C for thawing. For assessing the disruption of oocysts, samples were taken from the suspension every 10 min and examined by light microscopy. Absence of sporulated oocysts and the presence of microscopically detectable naked sporocysts were the proof of complete oocyst disruption.

2.3. Preparation of fecal samples and extraction of DNA

2.3.1. Negative feces with oocysts added (spiking experiments)

Spiking experiments were carried out to determine conditions of oocyst disruption and for determining detection sensitivity.

Toxoplasma negative feces (samples of 200 μL) were spiked with 1–2, 5, 10, 25, 50, 100, 250, and 1000 sporulated oocysts, then mixed and washed twice with ddw. An equal volume of 2× Sheather's solution (106 g sugar, 100 mL ddw, 0.8 mL phenol; with specific gravity (SG) = 1.26) was added to each residue, then mixed and spun for 15 min at 900 × *g*. The supernatants were collected, mixed with nine volumes of ddw, and the samples were spun at 2500 × *g* for 15 min. The pelleted oocysts underwent disruption and DNA extraction as follows. Two hundred microliters of ASL, the lysis buffer provided in the DNA extraction kit (QIAamp DNA Stool Mini Kit, Qiagen, CA, USA), was added to the pelleted oocysts, and then an equal volume of glass beads (400–600 nm, Sigma Aldrich, Missouri, USA) was added, and disruption was carried out for 30 min (as described in Section 2.2, above). Following disruption, the samples were spun, supernatant collected and mixed with 1.2 mL ASL. Thereafter, extraction was according to the manufacturer's instructions with the following modifications: Proteinase K incubation was carried out at 60 °C for 1 h instead of 10 min, and DNA elution from spin columns was performed twice. The combined eluate (200 μL per sample) was stored at −20 °C until examined.

2.3.2. Preparation of fecal samples from stray cats for PCR

One gram of fecal material was mixed with 10 mL water, in 15 mL capped plastic tubes, and the mixture centrifuged at 2000 × *g* for 10 min in a swingout rotor. The supernatant was discarded and the residue (about 1 mL) mixed with an equal volume of Sheather's solution (SG = 1.26; see Section 2.3.1, above), then spun for 10 min at 1000 × *g*. To recover oocysts, nine volumes of water were added to the supernatant of the previous step, and the sample was spun at 2500 × *g* for 10 min. The pelleted material was transferred to 2 mL microcentrifuge tubes, spun at full speed for 10 min, and the pelleted oocysts underwent disruption and DNA extraction as described above (Section 2.3), and the product stored at −20 °C until used.