Molecular investigation on the occurrence of *Toxoplasma gondii* oocysts in cat feces using TOX-element and ITS-1 region targets

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**A R T I C L E  I N F O**

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**A B S T R A C T**

One of the most important routes of transmission for *Toxoplasma gondii* infection is the ingestion of foods contaminated with cat feces containing sporulated oocysts. The diagnosis of *T. gondii* infection by fecal microscopy is complicated, as other similar coccidian oocysts are often present in the same fecal specimen. This study aimed to identify *T. gondii* oocysts in cat feces using a novel PCR technique. Feline fecal specimens (*n* = 254) were screened for coccidian oocysts by light microscopy using the Sheather’s flotation method. PCR analysis performed on the same specimens targeted a 529 bp repeat element and internal transcribed spacer-1 (ITS-1) regions were used to confirm the presence of *Toxoplasma* oocysts. By light microscopy, 49/254 (19.3%) of specimens contained coccidian oocysts. PCR analysis demonstrated 2/254 (0.8%) and 17/254 (6.7%) positive results using *Tox* and ITS-1 primers, respectively. However, coccidian oocysts were not identified on microscopic examination of specimens that were PCR-positive by *Tox* primers. Coccidian oocysts were identified on microscopic examination of 6/17 (35.3%) of the PCR-positive fecal specimens using ITS-1 primers. The BLAST results of 16 ITS-1 sequences were identified as *T. gondii* (*n* = 12; 4.7%) and *Hammondia hammondi* (*n* = 4; 1.6%). There was slight agreement between the 529 bp and ITS-1 PCR results (*κ* = 0.148). This is the first report of the detection of *Toxoplasma* oocysts using PCR analysis on feline fecal specimens from Southern Thailand. The ITS-1 region has potential as an alternative marker to identify *T. gondii* oocysts in feline fecal specimens.

**Introduction**

*Toxoplasma gondii* is a protozoan pathogen known throughout the world. In particular, it is mostly prevalent in Europe and North America (Diza et al., 2005; Fromont et al., 2009; Jones and Holland, 2010). It can infect various intermediate hosts including nearly all warm-blooded animals and humans, but wild and domestic cats are the definitive hosts. While infection is often subclinical or produces only mild clinical signs, there can be more serious consequences for immunocompromised hosts due to the rapid multiplication of *Toxoplasma* tachyzoites in infected tissues (Ho-Yen, 1992). In addition, *T. gondii* was reported as one of the five most common food-borne pathogens in American people resulting in common food-borne pathogens in American people resulting in hospitalisation and death (CDC, 2011). There are two principal routes of disease transmission to humans: (1) accidental oral ingestion of food or water contaminated with infectious oocysts, or the consumption of raw or undercooked meat containing bradyzoite tissue cysts; and (2) transplacental transmission to the fetus during a primary infection in a pregnant woman (Wong and Remington, 1994). Of these modes of transmission, the oral route is considered more common (Zhou et al., 2011).

*T. gondii* is a heteroxenous and cyst-forming coccidian parasite of which there are seven genera: Besnoitia, Hammondia, Cystoisospora (Isospora), Frenkelia, Neospora, Sarcocystis and Toxoplasma (Frenkel and Smith, 2003). Infected wild and domestic felines serve as definitive hosts of *T. gondii* and can produce large quantities of oocysts in their feces. Mature oocysts can survive in soil and water, and remain infectious for long periods due to their high tolerance to harsh conditions (Frenkel et al., 1975; Dubey, 1998; Dumètre et al., 2008). Infective oocysts are also resistant to a variety of chemical reagents or disinfectants, such as 2% sulfuric acid and 100 mg/L sodium hypochlorite solutions (Wainwright et al., 2007a, 2007b). In previous studies, the incidence of *Toxoplasma* oocysts in cat feces by light microscopic observation was 0.9% in the USA (Dabritz et al., 2007), and 2% in Egypt (Amany and Merwad, 2012). However, other published surveys failed to detect any *Toxoplasma* oocysts in feces by light microscopy (Miró et al., 2004; Karatepe et al., 2008), perhaps because oocysts are shed in cat feces over relatively short periods after primary infection (median 8 days; Miró et al., 2004; Karatepe et al., 2008). Variations in feline diets could also potentially contribute to varying environmental prevalences of *Toxoplasma* oocysts (Lopes et al., 2008; Hong et al., 2013). Although *T. gondii* oocysts...
in cat feces are traditionally identified by their morphological characteristics on light microscopy (Foreyt, 2001), this technique is time consuming and requires specific technical skills. Additionally, other related coccidian oocysts are similar enough to make them indistinguishable from *T. gondii*. Consequently, the present study aimed to determine the prevalence of *Toxoplasma* oocysts in cat fecal specimens collected from Southern Thailand using a PCR amplification test for the TOX-element and ITS-1 region.

**Materials and methods**

**Fecal specimen collection and microscopic examination**

Fresh cat fecal specimens (*n = 254*) were collected by cat owners and veterinarians in Songkhla Province between January 2013 and August 2013. All fecal specimens were excoriated by household pet cats (age range, 1 month to 13 years old) and the majority of these cats were fed commercial pet foods. Screening for coccidian oocysts was performed using fecal flotation in Sheather’s sugar solution (Dubey and Beattie, 1988). Each specimen was examined in triplicate. A positive fecal specimen was reported based on the presence of coccidian oocysts with three morphological characteristics: (1) 6–48 μm in diameter; (2) oval or elliptical in shape; and (3) two distinct internal sporocysts in each oocyst.

**Disruption of oocyst walls and DNA extraction**

Supernatant specimens (4 mL) from Sheather’s flotation solutions were pipetted into fresh tubes. Approximately 36 mL of distilled water was added to each specimen, and then centrifuged at 2500 g for 10 min. The supernatant was discarded and pellets were collected. Oocyst walls were disrupted and the DNA was extracted as described previously (Salant et al., 2007) with a minor modification for DNA elution step resulting in 100 μL of final elution volume. The concentration of the purified DNA was assessed by a spectrophotometer (MaestroNano Spectrophotometer, MAESTROGEN). The DNA was stored at −20°C for later use in the PCR assay.

**PCR amplification of the parasite DNA and DNA sequencing**

DNA amplification was conducted using a thermal cycler (T100 thermal cycler, Bio-Rad). The PCR working mixture for amplifying each of target genes contained 200 μM of each primer, 200 μM for each dNTP, 1.5 mM MgCl₂, 1 × PCR buffer (35 mM Tris-HCl, 25 mM KCl, pH 9.0), 0.5 units of i-Taq Plus DNA Polymerase (Intron Biologicals); 5 μL of each DNA specimen was used as a template for a total reaction volume of 25 μL. To detect *T. gondii* DNA, specimens were amplified using primers Tox4/Tox5 targeting a non-coding 529 bp repetitive DNA fragment (Homan et al., 2000). The PCR conditions started with an initial denaturation step at 94°C for 5 min, followed by an amplification profile for 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The final elongation step was conducted at 72°C for 5 min. For the amplification of the ITS-1 of the rRNA gene, three different primer sets were selected to amplify an amplicon of approximately 600 bp from the DNA specimens. The first primer pair was Tim3/Tim11, which was used to amplify the target sequence by a conventional PCR. The second primer set was a nested PCR using primers GS1/GS2 as external primers and J5/JS4 as internal primers, and the last primer set was a nested PCR with GS1/Tim11 as external primers and JS4/GS2 as internal primers. The PCR cycling conditions used for amplifying ITS-1 region were performed using the methods described by Schaeres et al. (2008b). Additionally, the specimens were screened for *Hammondia hammondi* using primer pairs Hham34F/Hham3R (Schaeres et al., 2008a). In each set of PCR reactions, a negative control (deionised water) and a positive control (DNA extracted from tachyzoites of *T. gondii*) were run simultaneously with DNA specimens. The amplified products were electrophoresed on a 1.3% agarose gel (Promega) in 0.5 × TAE buffer along with a 100-bp DNA ladder (New England Biolabs), stained with ethidium bromide, and visualised by UV light. The DNA fragments obtained from the PCR using ITS-1 primers were excised and purified from the gels using a GeneHelp Gel/PCR kit (Geneaid). The purified ITS-1 amplicons were sequenced in both forward and reverse directions with an ABI3730xl automated DNA sequencer (Applied Biosystems) at Macrogen. The nucleotide sequences were assembled with BioEdit Software¹ and compared with the sequence data available from GenBank database using a BLAST search. The oligonucleotide primers used in this study are shown in Table 1.

**Statistical analysis**

Cohen’s kappa (κ) coefficient was used to evaluate the agreement between the positive and negative results for each specimen detected by PCR amplification of TOX-element and ITS-1 region with confidence intervals of 95%. The χ² values were defined using five levels of agreement: slight, 0.00–0.20; fair, 0.21–0.40; moderate, 0.41–0.60; substantial, 0.61–0.80; and almost perfect, 0.81–1.00 (Landis and Koch, 1977). Statistical analysis was performed using SPSS version 17.0 (SPSS). P values <0.05 were considered statistically significant.

**Results**

Using light microscopy, coccidian oocysts (approximately 8–42 μm in diameter) were detected in 49/254 specimens (19.3%). For PCR using Tox primers, only 2254 specimens (0.8%) were positive for the DNA fragment of 529 bp (Fig. 1; Table 2). These two specimens were negative for coccidian oocysts by light microscopy. For the amplification of the ITS-1 region, 17/254 specimens (6.7%) were positive by either a conventional PCR or nested PCRs. Of the 17 ITS-1 positive specimens, one was positive using Tim3/Tim 11 primers, and 12 and four specimens were positive using the second and third primer sets, respectively (data not shown). Of these 17 specimens, six specimens were positive for coccidian oocysts by microscopic examination. A total of 11/205 (5.4%) of the coccidial oocyst-negative specimens were successfully amplified with the ITS-1 primers (Table 2). All 16 PCR products amplified from the ITS-1 region were sequenced to identify the species of parasite, except for one specimen (number 74), in which the DNA template was insufficient for sequencing. The BLAST results indicated that the tested sequences were *T. gondii* (12/16) and *H. hammondi* (4/16). The ITS-1 sequences obtained were submitted to GenBank (accession numbers KF895857 to KF895872; Table 2). A slight agreement (κ = 0.148, 95% confidence interval, −0.109 to 0.405; P < 0.05) was observed between the results obtained by PCR amplification of the two targets tested.

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![Fig. 1. PCR amplification products of TOX-element identified on 1.5% agarose gel electrophoresis. Lane 1, positive control; Lane 2, negative control; Lanes 3–4, fecal specimen numbers 45 and 74, respectively; Lane M, 100 bp molecular weight marker.](image-url)
**Discussion**

The identification of feline *Toxoplasma* infections is often based on measuring the level of specific antibodies against the parasite or the identification of *Toxoplasma* oocysts in fecal specimens (Al-Kappany et al., 2010; Mosallanejad et al., 2011; Amany and Merwad, 2012). It has been reported that most *Toxoplasma*-seronegative cats have oocyst-positive feces, whereas *Toxoplasma*-seropositive cats no longer excrete oocysts in their feces (Sumner and Ackland, 1999), thus providing challenges for determining the precise time of infection. Furthermore, several coccidian oocysts reported in cat feces have morphological similarity, such as *Cystoisospora* spp., *Sarcocystis* spp., *H. hammondi* and *T. gondii* (Barutzki and Schaper, 2003). The differentiation of these oocysts is mainly based on their size on light microscopy, with average sizes varying from 11 μm to 40 μm in length and 9 μm to 30 μm in width; *Cystoisospora* has the largest oocysts (Dubey et al., 2009). Two studies investigating the sensitivity of microscopic techniques for the detection of *Toxoplasma* oocysts clearly showed disparities in detection limits in experimentally infected cats, with 250 oocysts/g of feces (*Cystoisospora* felis, *Cystoisospora rivolta*, *Besnoitia darling*, *Besnoitia wallacei*) reported. Thus, the expertise of individual microscopists is potentially an important factor, especially if low numbers of oocysts are shed by naturally infected cats. Our results demonstrated that 49/254 (19.3%) of coccidian oocysts with sizes ranging from 8 μm to 42 μm in diameter were observed in feline fecal specimens. However, morphological examination is not always reliable for species identification. Considering our PCR results, only 2/254 specimens were positive by PCR targeting TOX-elements, yet these were microscopy-negative. Our findings were similar to others who demonstrated that no cat fecal specimens that were positive by PCR based on the 529 bp repeat element (11/122 specimens) were positive by microscopic examination (Salant et al., 2007). It is possible that the oocyst load was insufficient for microscopic detection, and that PCR was more sensitive and specific. A non-coding 529 bp DNA fragment and the ITS-1 of the rRNA gene were the highly conserved repetitive DNA sequences used for detection of *T. gondii* DNA by PCR. The *T. gondii* genome consisted of 200–300 copies of the 529 bp repeat element (Homan et al., 2000), resulting in a higher detection rate than the 35-copy *B1* gene (Calderaro et al., 2006; Fallahi et al., 2014). The 110-copy ITS-1 has been applied in some labora-

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Conclusions

Our findings indicate that the ITS-1 of the rRNA gene could be used as an alternative marker for the identification of T. gondii, especially in fecal specimens with trace amounts of target DNA. This is the first report of the detection of Toxoplasma DNA from naturally infected Thai cats using a PCR technique. Since T. gondii oocysts can contaminate soil and water, increasing the risk of mammalian Toxoplasma infection, accurate and rapid diagnostic methods for the detection of T. gondii are essential in epidemiological surveys, to locate the source of contamination and track its spread.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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