

# Copro-PCR in the detection and confirmation of *Toxoplasma gondii* oocysts in feces of stray and domiciled cats

Copro-PCR na detecção e confirmação de oocistos de *Toxoplasma gondii* em fezes de gatos errantes e domiciliados

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## Abstract

Molecular methods such as Copro-PCR stand out in the diagnosis of *T. gondii* because they are highly sensitive and specific, and can distinguish *T. gondii* from other morphologically similar coccids. The purpose was the detection of *Toxoplasma gondii* copro-prevalence by polymerase chain reaction in 149 fecal samples from stray and domiciled cats, using three distinct markers (B5-B6, 18S and 529bp RE). Oocysts of *T. gondii*/*H. hammondi* were detected in 15.4% by parasitology fecal tests (PFT), and 4% of these oocysts were positively identified as *T. gondii* by Copro-PCR. The presence of *T. gondii* genetic material was detected in 16.1%, but 12% of the samples that tested positive by Copro-PCR were negative in PFT. Samples with discordant results were subjected to a new Copro-PCR with 18S marker and a 529, and of the 17 samples, 9 contained *T. gondii* genetic material. A comparison of the PFT and the molecular methods showed the latter was more sensitive, since it detected 22.1% while the PFT detected 15.4%. Demonstrating the high sensitivity and specificity of the Copro-PCR, particularly with the association of primers ( $k=0.809$ ), but also confirms the importance of using molecular techniques in laboratories, since Copro-PCR was able to detect samples considered negative by PFT.

**Keywords:** *Toxoplasma gondii*, Copro-PCR, cats.

## Resumo

Métodos moleculares como a Copro-PCR se destacam no diagnóstico de *T. gondii*, por serem altamente sensíveis e específicos, podendo distinguir *T. gondii* de outros coccídeos morfologicamente semelhantes. O objetivo foi a detecção da copro-prevalência de *Toxoplasma gondii* por reação em cadeia da polimerase, em 149 amostras fecais de gatos errantes e domiciliados, utilizando-se três marcadores distintos (B5-B6, 18S e 529pb RE). Oocistos de *T. gondii*/*H. hammondi* foram detectados em 15,4% pelos exames parasitológicos de fezes (EPF), e 4% desses oocistos foram identificados positivamente como *T. gondii* pela Copro-PCR. A presença de material genético de *T. gondii* foi detectada em 16,1%, mas 12% das amostras positivas pelo Copro-PCR foram negativas no EPF. As amostras com resultados discordantes foram submetidas a uma nova Copro-PCR com os marcadores 18S e 529 e, das 17 amostras, 9 continham material genético de *T. gondii*. A comparação do EPF com os métodos moleculares mostrou que esse último foi mais sensível, pois detectou 22,1%, enquanto o EPF detectou 15,4%. Isso demonstra a alta sensibilidade e especificidade da Copro-PCR, principalmente com a associação de marcadores ( $k = 0,809$ ); mas também confirma a importância do uso de técnicas moleculares em laboratórios, uma vez que a Copro-PCR foi capaz de detectar amostras consideradas negativas pelo EPF.

**Palavras-chave:** *Toxoplasma gondii*, Copro-PCR, gatos.

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## Introduction

*Toxoplasma gondii* is a protozoan of worldwide prevalence, found in every continent and every type of climate. The routes for *T. gondii* include vertical transmission from mother to fetus and horizontal transmission, which involves three life-cycle stages, i.e. ingesting infectious oocysts from the environment or ingesting tissue cysts or tachyzoites which are contained in meat or primary offal (viscera) of many different animals. Transmission may also occur via tachyzoites contained in blood products, tissue transplants, or unpasteurised milk (Tenter et al., 2000). This parasite is transmitted by oocysts present in the feces of stray and domiciled felines, the definitive hosts of this coccidian, which shed millions of immature oocysts in their feces after the primary infection, thereby contaminating the environment. The domestic cat (*Felis catus*) is able to shed about 10 million oocysts in a single defecation, thus representing an important source of environmental contamination and consequently one of the main routes of transmission of toxoplasmosis (Dubey & Beattie, 1988; Lindsay et al., 2002; Mancianti et al., 2010). Hence, the presence of stray cats may be a factor that contributes to the high urban prevalence of this parasite (Lima et al., 2018).

Domestic cats also play a major role in the worldwide (human and veterinary) prevalence of *T. gondii*, and their numbers have been increasing considerably in Brazilian households. However, most people are unaware of the zoonotic potential of this species. A contributing factor is that many owners allow their cats to roam freely in the streets, where the animals can ingest prey contaminated with *T. gondii*. Not only domiciled cats but also stray cats, which are considered more susceptible to contact with this parasite, can shed oocysts in the environment after they become infected (Dabritz & Conrad, 2010; Costa, 2015).

*T. gondii* oocysts can be detected in cat feces using microscopy through parasitology fecal tests (PFT), bioassays of experimental animals, and via molecular methods (Dubey, 2010; Elmore et al., 2010; Salant et al., 2010). However, the sensitivity of microscopy is too low to distinguish *T. gondii* oocysts from other coccidian oocysts with very similar morphological and morphometric characteristics, e.g., *Hammondia hammondi*, a parasite commonly found in cats (Barutzki & Schaper, 2011; Lappin, 2010; Veronesi et al., 2017). In this context, molecular methods such as Copro-PCR stand out in the diagnosis of *T. gondii*, due to their high sensitivity and specificity, which enables them to differentiate between *T. gondii* and other coccids (Burg et al., 1989).

The main molecular markers used in Copro-PCR for *T. gondii* are the 529-bp repeat element (RE), with 200-300 copies (Costa & Bretagne, 2012) and the B1 gene, with 35 copies (Burg et al., 1989; Mousavi et al., 2016). Studies suggest that the Copro-PCR that amplifies the 529-bp RE is considered sensitive and specific for the detection of *T. gondii* in cat feces (Salant et al., 2007, 2010). However, there are few comparative analyses using different genetic markers for the diagnosis of *T. gondii* in fecal samples from cats (Veronesi et al., 2017; Chemoh et al., 2018). This study aims the detection of *Toxoplasma gondii* copro-prevalence by polymerase chain reaction using three different primers in feces of cats in Goiânia, contributing to the use of genetic markers for the diagnosis of *T. gondii* in fecal samples of cats.

## Material and Methods

### The study

This study was approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Goiás, under protocols no. 054/2013 and 024/2016. 149 cats of varying ages and both sexes were analyzed, from the city of Goiânia, state of Goiás. The animals were divided into two groups: domiciled cats and stray cats. Domiciled cats were considered those that lived in houses or apartments with their owners, without access to the street, and stray cats were captured by the Zoonosis Control Center (ZCC) of Goiânia and by a non-governmental organization (NGO) that protects animals. A total of 149 fecal samples were collected, 65 from stray cats and 84 from domiciled cats. The samples were collected between March 2015 and May 2016. Fecal samples from stray cats were collected directly from the animals' cages before they were wormed, and fecal samples from domiciled cats were collected by their owners. About 10 g of feces from each cat were stored in universal sterile containers, taking care to exclude samples contaminated with soil.

### Analysis of fecal samples

*T. gondii* oocysts were identified using the Sheather, Hoffman-Pons-Janer or Lutz (HPJL), and Faust and Willis techniques (Willis, 1921; Sheather, 1923; Hoffman et al., 1934; Faust et al., 1938). The oocysts of *T. gondii* were

identified using the measurement of oocyst diameter, according to the protocol described by Simamora et al. (2015). After a parasitology fecal test (PFT) performed in all 149 fecal samples, the same samples were subjected to Copro-PCR for *T. gondii*, with amplification of the B1 gene using primers B5 and B6, following the protocol proposed by Robert-Gangneux & Dardé (2012). Samples that yielded contradictory PFT and Copro-PCR (primers B5 and B6) results were analyzed by another Copro-PCR assay, amplifying the B1 gene with the 18S marker (Cazenave et al., 1992) and the 529-bp repeat element (Homan et al., 2000).

### Copro-PCR employed in the detection of *Toxoplasma gondii*

DNA was extracted from the fecal samples of the animals. The DNA extraction was performed following the instructions of the kit LightMix®, manufacturer ROCHE®, Mannheim, Germany. After adding the lysis buffer, the samples were boiled for 20 minutes and left to rest overnight at 37 °C to break the oocyst wall. The extraction protocol was then performed the next day.

The polymerase chain reactions (PCR) were carried out in a final volume of 25µL containing 10mM TRIS HCl (pH 9.0), 3.5mM MgCl<sub>2</sub>, 0.2U of Taq DNA Polymerase (Invitrogen), 0.5mM of each deoxynucleotide (dATP/ dTTP/ dGTP/ dCTP, Sigma Chemical Co., USA®), 50 pmols of each reaction primer (Invitrogen®) and 5µL of DNA template. The reactions were carried out in a MasterCycler Personal thermal cycler. The amplification program consisted of an initial denaturation at 94 °C (5 min), followed by 35 cycles of denaturation at 94 °C (1 min), annealing at 62 °C (1 min) and extension at 72 °C (1 min), ending with a final extension at 72 °C for 10 min.

The following primers were used: Toxo-B5 and B6 (Robert-Gangneux & Dardé, 2012), 18S (Cazenave et al., 1992) and 529 bp (Homan et al., 2000). Mouse peritoneal fluid infected with the RH and ME49 strains and positive stool samples were used as positive control, while previously confirmed negative fecal samples were used as negative control. The PCR amplification products (110 bp) were examined using silver-stained 6% polyacrylamide gel electrophoresis (Santos et al., 1993).

### Statistical analysis

The agreement between Copro-PCR and the other tests applied was calculated using the Cohen Kappa index ( $\kappa$ ), using 2 × 2 contingency tables. The  $\kappa$  values obtained were interpreted according to the following parameters: <0.2 = weak, 0.2 to 0.4 = poor, 0.4 to 0.6 = regular, 0.6 to 0.8 = good, > 0.8 = excellent and 1 = perfect agreement (Kraemer & Bloch, 1988; Thrusfield, 2007). Parasitological results were analyzed using the Minitab software.

## Results and Discussion

A total of 149 fecal samples were analyzed, of which 27.5% (41/149) were positive in one of the tests performed and 72.5% (108/149) were negative by all the techniques performed. Table 1 lists the positive results obtained by the conventional parasitological and molecular techniques.

In this study, *T. gondii*/H. *hammondi* oocysts were detected by means of conventional parasitology fecal testing (PFT) in 15.4% (23/149) of the samples. Using Copro-PCR (primers B5 and B6), it was found that 4% (6/149) of the

**Table 1.** Comparison of conventional parasitological methods and the different primers used in Copro-PCR for the detection of *Toxoplasma gondii* oocysts in fecal samples from cats in Goiânia, Goiás.

PFT	Copro-PCR	Copro-PCR	Positive
	(B5 and B6)	(18S and 529pb)	Samples
Positive	Positive	Positive	6
Positive	Negative	Positive	9
Positive	Negative	Negative	8
Negative	Positive	Positive	18
<b>Total</b>			<b>41</b>

PFT: Parasitology Fecal Test; Copro-PCR (B5 and B6): Polymerase chain reaction on fecal samples using primers B5 and B6; Copro-PCR (18S and 529pb): Polymerase chain reaction on fecal samples using the primer 18S and 529-bp repeat element.

oocysts were really *T. gondii*, while 11.4% (17/149) of the oocysts examined under optical microscopy were probably *H. hammondi*, a species morphologically similar to *T. gondii*, making it necessary to test the rest of the positive samples by PFT. The methods used here produced congruent positive results in 4% (6/149) of the samples (Table 1). These data underscore the importance of the differential diagnosis of *H. hammondi* and *T. gondii*, as these coccidia share the same definitive hosts (felids) and are morphologically very similar, so that differentiation depends mainly on molecular tools. However *H. hammondi* is not pathogenic in cats (Dubey & Sreekumar, 2003; Schares et al., 2008).

On the other hand, the use of Copro-PCR (primers B5 and B6) enabled us to identify the presence of genetic material from *T. gondii* in 16.1% (24/149) of the analyzed fecal samples. However, 75% (18/24) of the samples that tested positive by Copro-PCR (primers B5 and B6) tested negative in the conventional parasitology fecal tests (Table 1). Nabi et al. (2018), who analyzed cat feces by microscopy and Copro-PCR, also found discordant results in the methodologies, like this study. They reported that samples testing negative by microscopy were positive by the Copro-PCR technique, demonstrating that molecular tests are more sensitive than microscopy, which is considered the gold standard in the diagnosis of *T. gondii*, since molecular tests detect the parasite's DNA even not detecting oocysts in microscopy (Nabi et al., 2018).

In view of the detection of samples with contradictory results (positive by PFT and negative by Copro-PCR (primers B5 and B6)), these samples were subjected to a new Copro-PCR assay using the markers 18S and 529-bp repeat element. Of the 17 samples with discrepant results, 9 contained *T. gondii* genetic material were revealed by Copro-PCR using 18S marker and the 529-bp repeat element, unlike the markers used in the first Copro-PCR with B5 and B6 (Table 1). These data are in agreement with those reported by Veronesi et al. (2017), who detected positive samples by Copro-PCR using 529-bp as marker, even though they were considered negative by microscopy and by Copro-PCR using B1 as marker (Veronesi et al., 2017).

In this study, a comparison of the molecular methods and conventional parasitological techniques for the detection of *T. gondii* oocysts revealed that the former are more sensitive, detecting 22.1% (33/149) of the samples, while only 15.4% (23/149) of the samples tested positive by PFT, as has also been reported by other authors (Dubey, 2010; Salant et al., 2010; Veronesi et al., 2017).

The comparison of the PCR 18S and 529 using the 18S gene as a comparative test (standard) in the statistical analysis, showed an excellent agreement with  $k=0.809$  and when comparing the 18S with PTF the agreement was weak, with  $k=0.184$ , since that molecular techniques are more sensitive (Table 2). The comparison of PTF with PCR 18S, using PTF as a comparative test (standard) showed a weak agreement, with  $k=0.103$  and when comparing PTF and 529, the agreement was regular, with  $k=0.424$  (Table 3). These data demonstrate the importance of confirming the diagnosis of *T. gondii* by molecular techniques with different primer pairs in order to increase the sensitivity of the reaction.

**Table 2.** Comparison between Copro-PCR for *Toxoplasma gondii* with primers 18S and 529 in fecal samples from cats in Goiania-Goias, using 18S as a comparative test (standard).

	Sensitivity	Specificity	PPV	NPV	Kappa
18S with 529	100%	92.8%	72.7%	100%	0.809
18S with PTF	25%	86.4%	26.1%	85.7%	0.184

PTF: Parasitology Fecal Test; PPV: Positive Predictive Value; NPV: Negative Predictive Value.

**Table 3.** Comparison between PTF and Copro-PCR techniques for *Toxoplasma gondii* with primers 18S and 529 in fecal samples from cats in Goiania-Goiás, using PTF as a comparative test (standard).

	Sensitivity	Specificity	PPV	NPV	Kappa
PTF with 18S	26.1%	85.7%	25%	86.4%	0.103
PTF with 529	65.2%	85.7%	45.5%	93.1%	0.424

PTF: Parasitology Fecal Test; PPV: Positive Predictive Value; NPV: Negative Predictive Value.

## Conclusions

The results of this study demonstrate the high sensitivity and specificity of the Copro-PCR technique, mainly using the association of primers, and the importance of using molecular techniques in human and veterinary laboratories. Copro-prevalence of *T. gondii* in cats in Goiania by means of PCR using repetitive 529 bp gene is

around 64%. Copro-PCR was able to detect samples considered negative by PFT, the gold standard method for the diagnosis of intestinal parasites. Therefore, we propose that Copro-PCR be used as the new gold standard for diagnosing *T. gondii* oocysts in cat fecal samples.

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