

## Prevalence of *Toxoplasma gondii* oocysts through Copro-PCR in cats at Pet Center (UVAS), Lahore, Pakistan

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### Abstract

Toxoplasmosis is a major zoonotic disease of warm-blooded animals caused by *Toxoplasma gondii*. Cats are the only definitive host and they excrete environmentally resistant *T. gondii* oocysts in their faeces. Coproscopy was used to detect oocysts of enteric coccidians and then Copro-PCR was employed to test specifically for *T. gondii* in 470 cat samples. The prevalence of *T. gondii* oocysts was 2.3% (11/470) based on PCR. We observed 15 (3.2%) of 470 samples positive for coccidian oocysts by microscopy. The presence of Copro-DNA of *T. gondii* was found significantly higher ( $p < 0.05$ ) in males than females. We tested 11 samples of *T. gondii* oocysts in which 9 samples were from coccidian oocysts positive samples and 2 samples from negative faecal samples. Our results showed that PCR is the reliable method for the detection of faecal oocysts of *T. gondii* in cats as compared to microscopy. As per our knowledge, ours is first study for Copro-PCR prevalence of cats' *T. gondii* oocysts excretion in Pakistan.

**Keywords:** *Toxoplasma gondii*, Copro-PCR, Oocysts, Cat, Prevalence, Lahore, Pakistan

### Introduction

Toxoplasmosis is a parasitic zoonotic disease in humans and warm-blooded animals, caused by *Toxoplasma gondii*. Up to one-third of the human population worldwide is chronically infected with *T. gondii*.<sup>1</sup> Toxoplasmosis can be severe in the immunocompromised people. Reactivation of latent infection can result in severe encephalitis in up to 40% in AIDS patients resulting in 10-30% deaths in uncontrolled infections.<sup>2</sup>

Transmission to human occurs either by ingestion of *T. gondii* tissue cysts in inadequately cooked meat of infected animals or by accidental intake of sporulated oocysts in contaminated drinking water. Domestic cats

can excrete millions of *T. gondii* oocysts in their faeces and thus have an important role in spreading and maintaining the parasite in the environment. *T. gondii* oocysts in faecal samples are always difficult to identify microscopically since they are similar to other coccidian oocysts infecting cats like *Hammondia hammondi*, *Besnoitia* spp. and *Cystoisospora* spp.<sup>3</sup> PCR is being reliably used to detect *T. gondii* DNA. The highly conserved and repetitive (35-fold) B1 gene of *T. gondii* is often used for the molecular detection of infection.<sup>4</sup> The present study was done to determine the prevalence of *T. gondii* oocysts in the faeces of cats in Lahore, Pakistan using a Copro-PCR test.

### Methods and Results

This cross-sectional study was carried out on cats presented to the Pet center, University of Veterinary and Animal Sciences (UVAS) between June 2013 and May 2014. The sample size was calculated by the formula for the determination of sample size by using normal determination with confidence interval 95% and alpha value 5% with 50% assumed prevalence of toxoplasmosis in population.<sup>5</sup> The mean was calculated as 2.06 with margin of error ranging from 1.98 to 2.14.

A total of 470 faecal samples were collected by the authorized veterinarian from pet cats attended at the Pet center. Ethical approval was obtained from UVAS ethical committee. Faecal samples were collected from rectum by inserting thermometer. About 0.5 g faecal material could stick to the tip of the thermometer. Information on breed, age and sex were recorded for each cat. Faecal samples were processed using faecal flotation technique. The slides were examined for parasites under 400x magnification of light microscope. DNA was extracted from 200 mg faecal sample with AxyPrep™ DNA mini-prep kit (Axygen-Biosciences, USA) with minor modifications in the protocol. Briefly, minor modifications were done to freeze thaw the oocysts in liquid nitrogen twice to disrupt them as described elsewhere.<sup>6</sup> Primers were designed using Primer-Blast software. A set of two primers was used to amplify a 529 bp (Forward: 5'-CCTGGTGTCTCTCAAGCGT-3' and Reverse: 5'-AAAGGAGAATGAGCGCACGA-3').

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**Table:** Prevalence of faecal oocysts in cats.

Factor	Positive/total (prevalence in %±S.E)		Positive/total (prevalence in %±S.E)		
	Faecal microscopy for enteric coccidian parasites	P value	PCR for <i>T. gondii</i>	PCR for <i>H. hammondi</i>	P value
<b>Sex</b>					
Male	8/60 (13.3%±0.044)	0.001*	7/60 (11.6%±0.041)××	3/60 (5%±0.028)	0.001*
Female	7/410 (1.7%±0.006)##		4/410 (0.97%±0.005)	1/410 (0.24%±0.002)	
<b>Age</b>					
1-6 months	12/300 (4%±0.011)#	0.146	8/300 (2.67%±0.009)	3/300 (1%±0.006)	0.392
>6 months	3/170 (1.76%±0.010)#		3/120 (1.76%±0.010)××	1/120 (0.83%±0.012)	
<b>Breed</b>					
Persian	4/170 (2.35%±0.012)#	0.663	3/170 (1.76%±0.010)		0.795
Siamese	3/100 (3%±0.017)		3/100 (3%±0.017)		
Local/Crossed/Un-descript	8/200 (4%±0.014)#		5/200 (2.5%±0.011)××	4/200 (2%±0.010)	
Overall	15/470 (3.2%±0.008)##		11/470 (2.3%±0.0207) ××	4/470	

\* Statistically significant

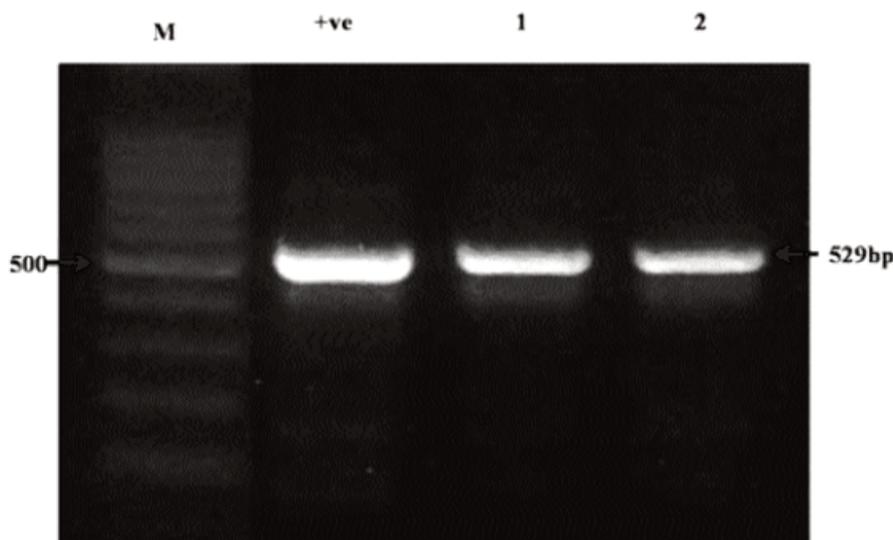
× Number of microscopically negative samples which are positive by PCR

# Number of *Cystoisospora* spp. positive samples.

For *T. gondii*, PCRs were carried out in a total volume of 25 µl of reaction mixture consisting of 0.1 µM of each primer (Eurofins, Canada), PCR buffer (Fermentas, USA), 200 µM of each dNTP, 2 U of Taq polymerase (Fermentas, USA) and 200-500 ng of DNA template. The PCRs were performed in a thermocycler (Robus tech. UK) under the following conditions: 2 minutes at 95°C for initial denaturation, followed by 40 cycles of 95°C for 30 seconds denaturation, 59°C for 30 seconds as annealing and 72°C for 2 minutes as extension and a last extension step at 72°C for 10 minutes. Positive control DNA (RH) of *Toxoplasma* was used to detect indigenous *toxoplasma* DNA. This reference DNA was provided by Dr. Henrik Vedel Nielsen (Statens Serum

Institute, Denmark) and Dr. Jorge Enrique Gomez Marin (Universidad del Quindio, COLOMBIA, South America). For *H. hammondi*, PCR was used as described elsewhere.<sup>7</sup> Briefly, *H. hammondi*-specific primer pair; Hham34F ATCCCATCCGGCTTCAGTCTTTC and Hham3R ACAGCGGAGCCGAAGTTGGTTT were used. Chi square test was performed with SPSS software version 20 (SPSS Inc., Chicago, IL, USA) at a threshold value of 5%. The concordance between the two techniques was measured by calculating Kappa value with SPSS.

We observed 15 (3.2%) samples positive for coccidian oocysts out of 470 samples through microscopy. With micrometry, small and large oocysts were differentiated. Oocysts measuring from 10 to 15 µm were considered *T. gondii* or *H. hammondi*. Large oocysts measuring from 20 to 40 µm were *Cystoisospora* spp. Two (0.4%) samples of oocysts out of 470 were found of *Cystoisospora* spp. confirmed through micrometry owing to its large size. Four (0.8%) and eleven (2.3%) samples of oocysts of *H. hammondi* and *T. gondii* respectively were confirmed through PCR as shown in Table. Eleven (2.3%) samples gave a species-specific product of 529 bp through PCR for *T. gondii* as shown in Figure.



1-2: Samples PCR Product; +ve: Positive control sample; M: 100 bp Ladder

**Figure:** PCR product of *Toxoplasma gondii* B1 multicopy gene.

All 470 samples were subjected to microscopy or PCR in our experiment. We found 15 samples

out of 470, positive for coccidian parasites through microscopy. Out of 15 coccidian parasites samples, 9 samples were confirmed through PCR for *T. gondii*. Other 2 samples from microscopically negative samples, were found positive for *T. gondii* through PCR. Thus we found total 11 samples of *T. gondii* oocysts. Two samples of oocysts out of 15 coccidian parasites samples were found of *Cystoisospora* spp. confirmed through microscopic micrometry. Four samples of oocysts out of 15 coccidian parasites samples were found of *H. hammondi* confirmed through PCR as shown in Table.

Kappa value was 0.721 between the two tests namely faecal microscopy and Copro-PCR. The prevalence of *Toxoplasma gondii* oocysts in cats through Copro-PCR was significantly higher in males than females ( $p < 0.05$ ) (Table), but there was no difference in prevalence of *T. gondii* excretion among either breeds or age groups ( $p > 0.05$ ).

## Discussion

Several studies highlighted the importance of *T. gondii* infection in humans and animals in Pakistan but none studied the prevalence of oocysts' faecal excretion through Copro-PCR. Previously in a study, the seroprevalence was found high in cats 26.43% (111/420) in arid region of Pakistan.<sup>8</sup> Contaminated soil and water are important sources of *T. gondii* oocysts for humans.<sup>9</sup>

Faecal microscopy can be done to detect coccidian oocysts; however, it is not able to differentiate between *T. gondii* oocysts from *H. hammondi* in cats because they are the same size. We developed a Copro-PCR to detect DNA of *T. gondii* in oocysts in feline faecal samples. A PCR was performed to identify *H. hammondi* using the primer pair: Hham34F-Hham3R and the amplicons with expected size of 283 bp<sup>7</sup> was detected (results not shown). Similarly, Salant et al., (2007; 2010) had developed and employed PCR to compare different techniques for the detection of faecal oocysts to toxoplasma.<sup>6,10</sup> In one experiment, they tested 122 stool samples from Jerusalem cats by PCR targeting B1 gene. Eleven samples were detected by PCR which were negative through microscopy.<sup>6</sup> In another experiment, they compared copro-PCR for detection of *T. gondii* infected cats with microscopy and a bioassay. They found copro-PCR at least as sensitive and specific as the bioassay and it was capable of detecting infective oocysts during cat infection so it can be used as new gold standard for determining potential cat infectivity.<sup>10</sup> It is superior to bioassay in term of less time consuming so it can be used for screening of cats at large scale or in the field condition.

We reported 2.3% prevalence which was higher than

those reported by several authors: 1.3% in Czech Republic,<sup>11</sup> 0.11 to 1.1% in Germany,<sup>12,13</sup> 0.3% in Netherland,<sup>14</sup> 0.23% in France and 0.4% in Switzerland,<sup>15</sup> 0.9 to 1.8% in USA<sup>16,17</sup> and 1.3% in Brazil.<sup>18</sup> The excretion rate varies with the life style, age, vicinity of the cats and the prevalence of *T. gondii* in intermediate hosts.<sup>19</sup>

Some studies reported that cats less than 1 year of age produced larger numbers of *T. gondii* oocysts, they become infected shortly after they were weaned. Most cats that had excreted oocysts once developed immunity and did not repeatedly excrete oocysts after challenge with *T. gondii*.<sup>20</sup> High excretion rates in young cats were reported in Costa Rica (23.2%),<sup>21</sup> Chile (4.3%),<sup>22</sup> Germany (12%),<sup>12</sup> Egypt (50%),<sup>23</sup> Qatar (10%)<sup>24</sup> and Australia (15%).<sup>25</sup>

As per our knowledge, our study is the first Copro-PCR prevalence estimation of *T. gondii* oocysts in Pakistan.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

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## References

1. Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: from animals to humans. *Int J Parasitol.* 2000; 30: 1217-58.
2. Antinori A, Larussa D, Cingolani A, Lorenzini P, Bossolasco S, Finazzi MG, et al. Prevalence, associated factors, and prognostic determinants of AIDS-related toxoplasmic encephalitis in the era of advanced highly active antiretroviral therapy. *Clinical infectious diseases.* 2004; 39: 1681-91.
3. Dubey JP, Jones JL. *Toxoplasma gondii* infection in humans and animals in the United States. *Int J Parasitol.* 2008; 38: 1257-78.
4. Kupferschmidt O, Kruger D, Held TK, Ellerbrok H, Siegert W, Janitschke K. Quantitative detection of *Toxoplasma gondii* DNA in human body fluids by TaqMan polymerase chain reaction. *Clin Microbiol Infect.* 2001; 7: 120-4.
5. Thrusfield M. *Veterinary epidemiology*: London. Elsevier Academic Press; 2013.
6. Salant H, Markovics A, Spira DT, Hamburger J. The development of a molecular approach for coprodiagnosis of *Toxoplasma gondii*. *Vet Parasitol.* 2007; 146: 214-20.
7. Schares G, Herrmann DC, Beckert A, Schares S, Hosseininejad M, Pantchev N, et al. Characterization of a repetitive DNA fragment in *Hammondia hammondi* and its utility for the specific differentiation of *H. hammondi* from *Toxoplasma gondii* by PCR. *Mol Cell Probes.* 2008; 22: 244-51.
8. Ahmad N, Ahmed H, Irum S, Qayyum M. Seroprevalence of IgG and IgM antibodies and associated risk factors for toxoplasmosis in cats and dogs from sub-tropical arid parts of Pakistan. *Tropical Biomedicine.* 2014; 31: 777-84.

9. Dumetre A, Darde ML. How to detect *Toxoplasma gondii* oocysts in environmental samples? *FEMS Microbiol Rev.* 2003; 27: 651-61.
  10. Salant H, Spira DT, Hamburger J. A comparative analysis of coprologic diagnostic methods for detection of *Toxoplasma gondii* in cats. *Am J Trop Med Hyg.* 2010; 82: 865-70.
  11. Svobodova V, Svoboda M. [Incidence of *Toxoplasma gondii* oocysts in cat faeces]. *Vet Med (Praha).* 1986; 31: 621-8.
  12. Barutzki D, Schaper R. Endoparasites in dogs and cats in Germany 1999-2002. *Parasitol Res.* 2003; 90: S148-50.
  13. Schares G, Vrhovec MG, Pantchev N, Herrmann DC, Conraths FJ. Occurrence of *Toxoplasma gondii* and *Hammondia hammondi* oocysts in the faeces of cats from Germany and other European countries. *Vet Parasitol.* 2008; 152: 34-45.
  14. Robben S, Le Nobel W, Döpfer D, Hendrikx W, Boersema J, Franssen F, et al. [Infections with helminths and/or protozoa in cats in animal shelters in the Netherlands]. *Tijdschrift Voor Diergeneeskunde.* 2004; 129: 2-6.
  15. Berger-Schoch AE, Herrmann DC, Schares G, Muller N, Bernet D, Gottstein B, et al. Prevalence and genotypes of *Toxoplasma gondii* in feline faeces (oocysts) and meat from sheep, cattle and pigs in Switzerland. *Vet Parasitol.* 2011; 177: 290-7.
  16. Dubey JP, Weigel RM, Siegel AM, Thulliez P, Kitron UD, Mitchell MA, et al. Sources and reservoirs of *Toxoplasma gondii* infection on 47 swine farms in Illinois. *J Parasitol.* 1995; 81: 723-9.
  17. Dabritz HA, Miller MA, Atwill ER, Gardner IA, Leutenegger CM, Melli AC, et al. Detection of *Toxoplasma gondii*-like oocysts in cat faeces and estimates of the environmental oocyst burden. *J Am Vet Med Assoc.* 2007; 231: 1676-84.
  18. Pena HF, Soares RM, Amaku M, Dubey JP, Gennari SM. *Toxoplasma gondii* infection in cats from Sao Paulo state, Brazil: seroprevalence, oocyst shedding, isolation in mice, and biologic and molecular characterization. *Res Vet Sci.* 2006; 81: 58-67.
  19. Dubey JP, Beattie C. *Toxoplasmosis of animals and man.* In: Dubey JP, Beattie C. Boca Raton USA: CRC Press Inc, 1988; pp-220.
  20. Dubey JP. Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. *J Parasitol.* 1995; 81: 410-5.
  21. Ruiz A, Frenkel JK. *Toxoplasma gondii* in Costa Rican cats. *Am J Trop Med Hyg.* 1980; 29: 1150-60.
  22. Lopez J, Abarca K, Paredes P, Inzunza E. Intestinal parasites in dogs and cats with gastrointestinal symptoms in Santiago, Chile. *Revista médica de Chile.* 2006; 134: 193-200.
  23. Awadallah MA. Endoparasites of Zoonotic Importance. 2010.
  24. Abu-Madi MA, Al-Molawi N, Behnke JM. Seroprevalence and epidemiological correlates of *Toxoplasma gondii* infections among patients referred for hospital-based serological testing in Doha, Qatar. *Parasit Vectors.* 2008; 1: 39.
  25. Meloni B, Thompson R, Hopkins R, Reynoldson J, Gracey M. The prevalence of *Giardia* and other intestinal parasites in children, dogs and cats from aboriginal communities in the Kimberley. *Med J Aust.* 1993; 158: 157-9.
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