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Research Article

MOLECULAR STUDIES AND PHYLOGENETIC CHARACTERIZATION OF *TOXOPLASMA GONDII* IN CATS AND DETECTION OF INFECTION BY COPRO-PCR ASSAY

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Abstract: Considering the zoonotic importance and economic significance of *Toxoplasma gondii* and to note the status of *T. gondii* infection in cats from Bengaluru city region, Karnataka, India the present study was carried out by the application of coprologic–polymerase chain reaction (copro-PCR) assay. A total of 80 faecal samples were screened from cat shelters, Veterinary clinics, cat-owning households and stray animals. Oocysts were disrupted by freeze thawing coupled with mechanical means and DNA extraction was subsequently accomplished. Polymerase Chain Reaction (PCR) was standardized to detect *T. gondii* oocyst DNA in cat faecal samples. Out of 80 faecal samples examined, five (6.25%) were positive for Toxoplasma DNA by PCR. The test based on amplifying a 529 bp sequence repeated 200 to 300 times in the Toxoplasma genome proved highly sensitive by detecting *T. gondii* oocysts DNA in faecal samples. The phylogenetic results showed that T. gondii isolates obtained during the study were genetically similar to one another and also with the *T. gondii* Iran isolates.

Keywords: *Copro-PCR, Prevalence, Toxoplasma gondii, Cat, Phylogenetic analysis*

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Introduction

Toxoplasma gondii a coccidian parasite of domestic cats and other felids has a world-wide distribution with extremely wide range of species that can act as intermediate host for the organism, encompassing virtually all warmblooded animals. The organism is zoonotically important and up to one-third of the human population in the world is chronically infected [1] and the impact through congenital infection and transplacental transmission leading to reproductive failure is of great concern. A recent national survey revealed that 24.3 percent of Indian population is exposed to this parasite [2]. The disease impact on immunosuppressed patients as an opportunistic parasite is also of great concern. It is estimated that over 500 million people are infected worldwide. Specifically, toxoplasmosis ranks high on the list of diseases that lead to death in patients with acquired immunodeficiency syndrome (AIDS); approximately 10 percent of AIDS patients in the United States and up to 30% in Europe are estimated to die from toxoplasmosis [3]. It causes economic losses in farm animals but infections are mostly subclinical and undetected. Copro-diagnostic methods for *T. gondii* infected cats have been traditionally based on the identification of oocysts by light microscopy or by bioassays [4]. The sensitivity of light microscopy is low and thus detection is problematic when smaller oocyst numbers are excreted [5, 6].

T. gondii oocysts cannot be precisely identified by microscopy in faecal samples because these oocysts are morphologically indistinguishable from other coccidian parasites appearing in cat faeces, such as those of *Hammondia hammondi*. Microscopic detection of oocysts can at best identify oocysts as Toxoplasma-like [7]. Hence, large-scale screening of oocyst shedding in cats cannot rely on microscopy. Similarly, 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 possible biohazard element, it is also expensive, requires animal facilities, and is time consuming. Since molecular Copro-PCR can be expected to prove superior for parasite detection [4,8], in the present study PCR was used for amplifying a 529 bp repeated sequence [9] to detect *T. gondii* in faeces, in search of an alternative method for detecting parasites in infective faeces with high sensitivity, specificity and reproducibility.

Materials and Methods

Total 144 faecal samples of cats were processed by the Sheather's floatation technique and screened copromicroscopy. Study was mainly focused on young cats (kitten) as they shed more oocysts in faeces hence only 80 samples from kittens processed to carry out PCR. Flotation of oocysts was used to concentrate oocysts and thereby presumably also to reduce the number of potential inhibitors of PCR found in faeces. Oocysts were successfully disrupted by freeze thawing coupled with mechanical means and DNA extraction was carried out by using QIAamp® DNA stool mini kit (CA, USA) according to the manufacturer's instructions with following modifications: proteinase K incubation was carried out at 600C for 1 hour instead of 10 min and DNA elution from spin columns was performed twice.Non-coding 529 bp DNA fragment that is repeated 200-300 times in the genome was amplified by PCR using the *T. gondii* specific primers [9] and cycling conditions [Table-2&3]. The published specific primers of the *T. gondii* were synthesized by Eurofins Biotech, Bengaluru. The PCR reactions were performed in a thermal cycler (MRC scientific instruments) in a total reaction mixture of 25 µl containing 5 µl of DNA from the sample, 1 µl each of forward and reverse primer (20 pm), 12.5 µl PCR master mix (0.4 mM dNTPs, 0.05U/ µl Taq

DNA polymerase, 4mM $MgCl_2$) and 5.5µl Nuclease free water. DNA sample procured from the Division of Veterinary Parasitology, IVRI, Izatnagar, India was used as a positive control. PCR reaction mixture without template DNA was used as a no template control (NTC). The amplicons were analyzed by gel electrophoresis in 1.5% agarose gel to assess the presence of specific bands indicative of *T. gondii* (529 bp).

Table-1 *Collection of samples from different sources*

Place of collection	No. of samples collected	Age of cats
Shelters	82	Could not be discriminated
Clinical cases	23	Less than a year
House hold cats	24	Less than a year
Outdoor cats	15	Less than a year
Total	144	

Table-2 *Nucleotide sequence of Toxoplasma gondii specific primers*

Nucleotide sequence
Forward TOX4 (5'CGCTGCAGGGAGGAAGACGAAAGTTG-3')
Reverse TOX5 (5'CGCTGCAGACACAGTGCATCTGGATT-3')

Table-3 *PCR cycling conditions for T. gondii*

Initial denaturation	Denaturation	Annealing	Extension	Final extension
94°C 7min	95°C 1min	60°C 1min	72°C 1min	72°C 10min
	Repeated for 35 cycles			

Two purified positive PCR products were sequenced at Bioserve, Bengaluru. Reference sequences downloaded from GenBank were included in the analyses of 529 bp gene regions of our isolates. Sequences were aligned with Clustal W Bioedit v.7.0.5.3 [10] and the alignment gaps were treated as missing data for each gene, then multiple sequence alignments *i.e.*, a combined dataset of all gene done by Clustal-W as implemented in MEGA v.5 [11] and manually done wherever necessary.

Result

The DNA extracted from 80 faecal samples from kittens was subjected to PCR using *T. gondii* specific primers, TOX4 and TOX5. The PCR amplification yielded the expected amplicon of 529 bp size [Fig-1]. Five (6.25%) of the 80 cats were found to be positive for Toxoplasma DNA by PCR, three (60%) of 5 cats in the age group of 6 weeks to 3 months indicating young cats were more prone to getting infected at the age of weaning. The entire collected faecal samples were screened by the same protocol. The amplified products were loaded on 1.5 percent of agarose gel electrophoresis and along with Toxoplasma positive and negative control in lane 1 and 2 respectively [Fig-1]. Thus, lanes 3, 4, 5, 6, and 7 showing the amplification of the PCR products of the five samples and confirmed the samples as positive for *T. gondii* [Fig-1].

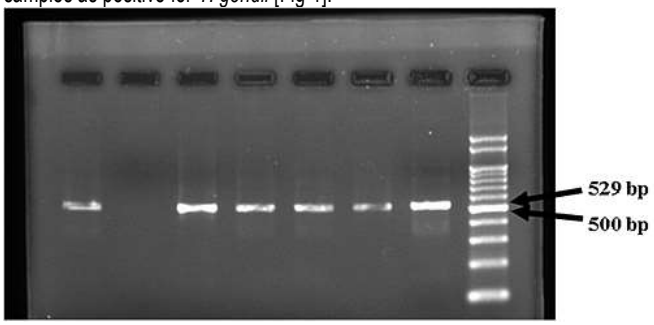

Fig-1 Copro-PCR for *T. gondii* detection

Lane 1: Positive control of *T. gondii* purified DNA. Lane 2: Negative control (no DNA added). Lanes 3 to 7: Field samples positive for *T. gondii*. L: 100 bp marker.

Two purified positive PCR products were sequenced at Bioserve, Bengaluru. The initial analysis of the partial sequence 529 bp gene of the 2 isolates revealed that they belonged to *T. gondii* with an out group DQ022686 *H. hammondi* (out group) were concatenated to form a super matrix of 628 bp. The dataset of gene comprised 628 characters including alignment gaps were processed, of which 248 characters were conserved, 287 characters variable and 102 characters were parsimony informative. For Bayesian analysis, a GTR+G+I model was selected for data analysis. The consensus tree obtained from Bayesian analyses confirmed the tree topology rapid bootstrapping estimations of RAxML [Fig-2]. The analyses resulted in the detection of two isolates included in the first clade which corresponded to *T. gondii* of Iran (HM569600 and HM569598) with a bootstrap support of above 89 percent and a Bayesian posterior probability value of 0.68 and the second clade had six isolates, from different countries with a bootstrap support/Bayesian posterior probability value of 52/0.91.

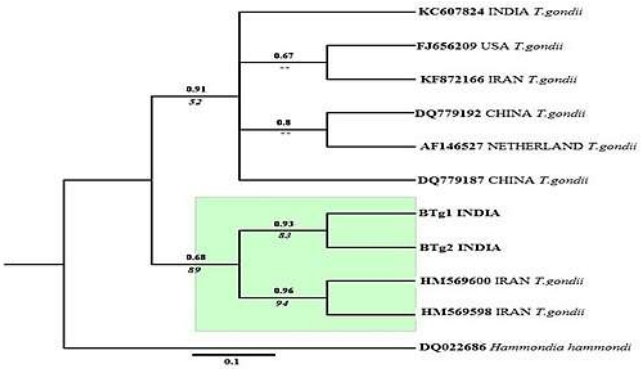

Fig-2 Phylogenetic tree constructed with concatenated sequences of 529 bp genes. Bayesian posterior probability values above 0.50 are shown at the nodes and bootstrap support values (500 replicates) above 50 percent below BPP values

Discussion

The present study was carried out to investigate the usefulness of the PCR technique in the detection of Toxoplasma oocysts DNA in cat faeces. Extraction of DNA from oocysts has been previously shown to be difficult due to the tenacious coccidian oocyst wall [12, 13]. The method used in the present study enabled disruption of oocysts using a combination of mechanical means (vigorous shaking with added glass beads) and freeze-thaw cycles prior to routine DNA extraction using a commercial kit. Given the required oocyst disruption step and the flotation applied at conditions known to concentrate oocysts prior to DNA extraction, the method can be used to detect oocyst DNA and therefore to identify potentially infective cats. Various methods for the removal of inhibitors from faecal samples to enable amplification have been used [14, 15]. The method used in the present study with the QIAamp-Stool kit was designed to overcome inhibition. Two (1.38%) of 144 cats found to be positive for Toxoplasma-like oocysts by copromicroscopy. Whereas, five (6.25%) of the 80 cats were found to be positive for Toxoplasma DNA by capro-PCR, three (60%) of 5 cats in the age group of 6 weeks to 3 months indicating young cats weremore prone to getting infected at the age of weaning. However, 52.9 (18/34) percent infection in cats up to 1 year old and 14.7 (5/34) percent infection in cats below the age of 6 months were reported from Germany [16]. Hence capro-PCR is highly sensitive and specific than copromicroscopy. The assay involved amplification of a 529 bp sequence represented by 200-300 copies in the Toxoplasma genome which enabled high detection sensitivity. The amplification of another repeated sequence, the B1 repeat, which was targeted for *T. gondii* PCR, was expected to be less sensitive because it is represented by only 35 copies [9]. In the present study, amplification of 529 bp gene sequence by copro-PCR assay confirmed the isolates were of *T. gondii*. This method helped to differentiate between coccidian parasites using coprodiagnostic PCR as in the present study. Similarly, same 529 bp gene sequence used for detection of Toxoplasma genome by copro-PCR assay [4, 17,18]. Moreover, strain identification by type is not possible using the 529 bp sequence given the highly conserved nature of this repeat and as we have found out [13]. In addition, *T. gondii* copro-PCR could be used for large scale epidemiological monitoring of any oocyst shedding host, whether mechanical oocyst sheddersor more specifically, definitive hosts. The phylogenetic results showed that *T. gondii* isolates obtained during the study were genetically similar to one another and also with the *T. gondii* Iran isolates (Accession number-HM569600 & HM569598). The nucleotide sequence analysis results further confirmed the isolates of present study as *T. gondii*.

Application of research: Application of coprologic-polymerase chain reaction (copro-PCR) assay for molecular studies and phylogenetic characterization of *T. gondii* in cats from Bengaluru city region, Karnataka, India.

Research Category: Veterinary Parasitology

Abbreviations: pm: pico moles, bp: base pair, mM: mili moles, µl: micro litre, copro-PCR: coprologic–polymerase chain reaction.

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Study area / Sample Collection: Faecal samples were collected from cat shelters, veterinary clinics, cat-owning households and stray animals in Bengaluru, India

Beed name: Cats

Conflict of Interest: None declared

Ethical approval: Ethical approval taken from Veterinary College, Bengaluru, 560024, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, 585401, Karnataka, India.
Ethical Committee Approval Number: Nil

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