Development of a low cost copro-LAMP assay for simultaneous copro-detection of *Toxocara canis* and *Toxocara cati*

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Abstract

Toxocariasis is a zoonotic disease caused mainly by *Toxocara canis* and *Toxocara cati* and diagnosis in dogs and cats is an important tool for its control. For this reason, a new coprological loop mediated isothermal amplification (LAMP) assay was developed for the simultaneous detection of these species. The primer set was designed on a region of the mitochondrial *cox*-1 gene. Amplification conditions were evaluated using a temperature gradient (52ºC to 68ºC), different incubation times (15 to 120 min), and different concentrations of malachite green dye (0.004% w/v to 0.4% w/v). The analytical sensitivity was evaluated with serial dilutions of genomic DNA from *T. canis* and *T. cati* adult worms, and with serial dilutions of DNA extracted from feces using a low-cost in-house method. The specificity was evaluated using genomic DNA from *Canis lupus familiaris*, *Felis catus*, *Escherichia coli*, *Toxascaris leonina*, *Ancylostoma caninum*, *Echinococcus granulosus sensu stricto* and *Taenia hydatigena*. The LAMP assay applied to environmental fecal samples from an endemic area showed an analytical sensitivity of 10fg - 100fg of genomic DNA and 10^{-5} serial dilutions of DNA extracted from feces using the low-cost in-house method; with a specificity of 100%. Additionally, the total development of the assay was carried out in a basic laboratory and per-reaction reagent cost decreased by approximately 80%. This new, low-cost tool can help identify the most common agents of toxocariasis in endemic areas in order to manage prevention strategies without having to rely on a laboratory with sophisticated equipment.

Keywords

Loop Mediated Isothermal Amplification; Toxocariasis; *Toxocara canis*; *Toxocara cati*; Copro-diagnosis, Low-cost.

Introduction

Several worldwide studies have shown that dogs and cats play an important role in the transmission of zoonotic parasites (Torgerson and Macpherson, 2011; Otranto *et al.*, 2017). Domestic animals can act as definitive hosts for various helminth and protozoan parasites with zoonotic potential, e. g. *Toxocara canis*, *Toxocara cati, Taenia* spp., *Echinococcus* spp., *Dipylidium caninum*, *Ancylostoma* spp., *Giardia* spp., *Toxoplasma gondii*, *Cryptosporidium* spp. Particularly, toxocariasis in humans is a systemic larval parasitosis which is usually asymptomatic, but can sometime present respiratory symptoms, eosinophilia, fever, hepatomegaly, splenomegaly, hypergammaglobulinemia, lymphadenopathy, involvement of the central nervous system, myocardium and skin, and may even lead to death (Rostami *et al.,* 2019). Clinically, it can present as visceral larva migrans, ocular larva migrans, neurological toxocariasis and covert toxocariasis, being very common mainly in children (Fisher, 2003; Archelli and Kozubsky, 2008). Sometimes toxocariasis can be associated with complications, such as allergic and/or neurological disorders, possibly including cognitive or developmental delays in children (Rostami *et al.*, 2019). The literature published from Argentina in recent years reveals that the prevalence of toxocariasis infections in children is between 19.5%-38.3% (Archelli *et al.*, 2014; Ciarmela *et al.*, 2016).

In the canine host, heavy infections with *T. canis* via the transplacental route in neonates and puppies, may result in pneumonia and acute death owing to enteritis and gastrointestinal blockage as early as 10 days of age. Heavy burdens with *T. canis* in pups may produce ill thrift, stunting, abdominal discomfort (pups adopt a straddle-legged posture and a pot-bellied appearance), anorexia, diarrhea and vomiting (adult worms may be expelled). Occasional gastrointestinal obstruction and death may also occur (Dantas-Torres *et al*., 2020). In the feline host, clinical signs depend on the burden of infection, low burden *T. cati* infections may be subclinical in kittens but those infected with *T. cati*, especially by the trans-mammary route, may present with cachexia, pot-bellied appearance, respiratory disorders, diarrhea, vomiting, and other signs as early as 3 weeks of age. Heavy infections may cause intestinal blockage or intussusceptions in kittens, which are potentially fatal (Dantas-Torres *et al*., 2020).

Diagnosis of intestinal zoonotic parasites is based on the microscopic detection of cyst/oocysts/eggs in the feces (Dantas-Torres *et al.*, 2020). The eggs of *T. canis* and *T. cati* can be distinguished morphologically from each other in the definitive host (Warren, 1969; Uga *et al.,* 2000). However, it is known that the latter has low sensitivity, associated to low parasitic burden, intermittent egg output and the requirement of trained and experienced human resources for accurate identification of parasitic structures (Deng *et al*., 2019). Nonetheless, the sensitivity may be improved through the use of new copro-microscopic techniques with higher sensitivity, such as Mini-FLOTAC and FLOTAC, as described by Maurelli *et al*., (2014). Techniques based on the detection of antigens in feces have also been used and have shown a wide range of sensitivity and specificity (Deplazes *et al*., 1990; Sen *et al*., 2011; Christy *et al*., 2012; Shimelis and Tadesse, 2014; Luis *et al*., 2019). Due to the different sensitivity and specificity presented by both microscopic and antigen detection techniques and with the advent of molecular techniques based on the detection of nucleic acids, certain aspects of sensitivity and specificity were improved (Jacobs *et al.*, 1997; Li *et al.*, 2007; Pinelli *et al.*, 2013; Knapp *et al.*, 2016); however, supplies and equipment are usually expensive and the procedures require human resources with specific training which are not widely available in endemic areas, complicating its implementation. Nonetheless, techniques based on isothermal amplification of nucleic acids are promising for the transfer of molecular diagnostics to the field given their lower complexity, lower cost and the lack of specialized equipment for its implementation. Particularly, the loop-mediated isothermal amplification (LAMP) developed by Notomi *et al.* (2000) uses 2 or 3 pairs of primers that recognize different regions flanking a central DNA fragment thus providing higher specificity for this sequence since the primers generate looped structures that serve as a template to start a new polymerization cycle. The *Bacillus stearothermophilus* DNA polymerase I (*Bst*) used in the technique causes DNA strand displacement and therefore it does not require denaturing of the double strand, thus the technique can be carried out in a thermostatic bath without the need of a thermocycler. The LAMP reaction has been proposed as an attractive diagnostic method, since it meets all the criteria proposed by the World Health Organization (WHO) for an "ideal" diagnostic test: affordable, sensitive, specific, user-friendly, rapid, equipment-free and delivered to those who need it (Mabey *et al.*, 2004). A LAMP technique for the separate detection of *T. canis* and *T. cati* has been previously developed (Macuhova *et al.*, 2010), although it is not currently routinely used because two LAMP reactions are need for each environmental sample collected (either feces or sand / soil). Although the separate detection of *Toxocara* spp. may be useful for application in the definitive host, for epidemiological and environmental studies, simultaneous detection of *T. canis* and *T. cati* in the feces from definitive hosts could be more useful for wide scale implementation. Previous studies have shown the presence of *T. cati* in dog feces with a prevalence ranging from 7.3 to 34.5% (Fahrion *et al.*, 2011; Nagy *et al.*, 2011; Vienažindienė *et al.*, 2018; Maurelli *et al.*, 2019). The presence of *T. cati* in dogs could be mainly attributed to coprophagia of cat feces (Vienažindienė *et al.*, 2018).

Due to its simplicity and high sensitivity and specificity, LAMP assays have already been implemented for the detection of protozoan (Ikadai *et al.,* 2004; Poon *et al.,* 2006; Han *et al.*, 2007; Karanis *et al.*, 2007; Njiru *et al.,* 2008; Plutzer and Karanis, 2009; Singh *et al.*, 2013;

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De Ruiter *et al.*, 2014; Karani *et al.*, 2014; Gallas-Lindemann *et al.*, 2016; Besuschio *et al.*, 2017; Durand *et al.,* 2019; Nzelu and Kato, 2019), as well as helminth parasites and are even available in the market for epidemiological surveys (Kumagai, *et al.,* 2010; Deng *et al.*, 2019). LAMP has also been implemented for the diagnosis of pathogens associated with food-borne diseases, such as *Salmonella typhi, Campylobacter jejuni*, *Helicobacter pylori*, *Listeria monocytogenes*, *Leptospira interrogans*, *Bucella* sp, *Escherichia coli*, and for water control (Hara-Kudo *et al.,* 2012; Abdullah *et al.*, 2014; Pham *et al.*, 2015; Trangoni *et al.*, 2015; Wang *et al.*, 2015, 2019; Bakhtiari *et al.*, 2016; Loffler *et al.*, 2016; Azizi *et al.*, 2019). Nonetheless, this technique has some disadvantages, including the use of expensive commercial kits for DNA extraction as well as reagents for the detection and visualization of the amplified product. Another complication is the false positive reactions generated by contamination of the sample with unwanted amplification products. These disadvantages are probably the reason why this technique is not presently routinely and widely used.

In this study, a LAMP assay that was developed for the simultaneous detection of *T. canis* and *T. cati*, causal agents for human toxocariasis is described. An alternative in-house method for DNA extraction and basic reagents, widely available in most laboratories were used. This low-cost strategy aims at making this technique feasible and easier to implement in laboratories from endemic areas with scarce resources.

Materials and methods

Parasite material

All fecal samples collected from dogs and cats of San Juan Province, Argentina, were stored at -20 °C for one month. *T. canis* and *T. cati* adult worms were obtained from spontaneous

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elimination from their respective definitive hosts and were preserved in 70% ethanol.

Optical Microscopy

Feces were processed using two different flotation techniques, Sheather method (1.25 specific gravity) (Sheather, 1923) and Willis method (1.20 specific gravity) (Willis, 1921) as well as a sedimentation technique, Telemann method (Telemann, 1908). The techniques chosen for this study are standard concentration techniques that increase the chances of detecting intestinal parasitic structures, including helminth parasites such as *Toxocara* spp. Each sample was microscopically examined at 100 X and 400 X magnifications.

DNA extraction

Genomic DNA (gDNA) extraction from cestode parasites was performed using the DNeasy Blood & Tissue Kit® (QIAGEN), following the manufacturer's instructions. The extraction of gDNA from nematode parasites was performed according to Repetto *et al*. (2013), and DNA from *Escherichia coli* (abundant in canine and feline feces) was extracted using the phenol–chloroform method (Sambrook and Russell, 2001). In each case, DNA concentrations were determined using a Nanodrop® Nd 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and DNA integrity was assessed by electrophoresis in 1% agarose gel stained with GelRed® (Biotium®, San Francisco, California, United States) and UV visualization. The DNA extraction and purification from feces (fDNA) was performed using both a commercial kit (CK) (ADN Puriprep SUELO-kit® from Inbio-Highway, Tandil, Buenos Aires, Argentina) and an in-house method (IHM) adapted from Macuhova *et al* (2010). Briefly, each canine/feline stool sample was homogenized and filtered through 250 μm and 63 μm sieves. Filtered material was homogenized in 50% sodium hypochlorite diluted from commercially sold aqueous solution with 5% effective chlorine concentration. The material was then thoroughly mixed and incubated at room temperature for 10 min., followed by centrifugation at 800 rpm for 3 min. The supernatant containing the eggs was recovered in a separate tube. The eggs were washed with distilled water, followed by a centrifugation step (3000 rpm for 5 min.), and finally the supernatant was discarded. This procedure was repeated 3 times. DNA was extracted by incubating samples with 50 mM NaOH in a water bath at 95°C for 30 min while mixing thoroughly every 15 min. This solution was used as a template for the LAMP assay. The DNA obtained by CK was conserved at -20°C, while the DNA obtained by IHM was cooled and used as template for the LAMP reaction.

LAMP assay

Primer design

The selection of the target gene for the design of the LAMP assay for the simultaneous detection of *T. canis* and *T. cati* was based on the general rules indicated by Notomi *et al.* (2000), as well as the Primer V5 design software guide (Eiken, Eiken Chemical Co., Ltd.). The selected target for primer design was a 261 bp region of the mitochondrial gene *cox*-1 (Genbank accession number AM411108.1, nucleotides: 6046-7623 and AM411622.1, nucleotides: 6055-7632). This region was selected due to the high identity between the two *Toxocara* species (*T. canis* and *T. cati*) and the presence of several mismatches with respect to other parasites that might coexist in the same sample. This strategy is fully described in previous studies (Avila, 2019; Avila *et al.*, 2020).

Master Mix

The LAMP reaction was performed in a 12.5 µl final reaction mixture containing: 20 mM Tris (pH 8.8), 50 mM KCl, 8 mM MgSO4, 10 mM $(NH4)_2SO_4$, 8 mM betaine, 1.4 mM dNTPs and 4 U *Bst* 2.0 polymerase (New England Biolabs). The primer concentration was 20 pmol for each FIP and BIP primer, 2.5 pmol for each F3 and B3 primer, and 5 pmol for each LB and LF primer. In all cases, 1 µl of DNA was used as a template. All reactions were performed on ice. The amplification conditions were evaluated using a temperature gradient (since 52°C to 68°C), different incubation times (15-120 min) and different concentrations of malachite green dye (0.004% w/v to 0.4% w/v). The use of malachite green dye and the IHM were evaluated with the aim of obtaining a low-cost strategy for the detection of *Toxocara* spp.

Analytical Sensitivity

The analytical sensitivity was evaluated in two conditions: with 10-fold serial dilutions of gDNA from *T. canis* and *T. cati* (ranging from 10^{-12} to 10^{-15} g/µl), while the sensitivity of the LAMP reaction was evaluated with serial dilutions of fDNA (ranging from 10^{0} to 10^{-6}) extracted from feces using CK and IHM. In each case, DNA obtained was diluted in ultrapure water to obtain serial dilutions. The feces used were positive for *Toxocara* spp. eggs under microscopic observation.

Specificity evaluation

The specificity of the LAMP reaction was evaluated by using gDNA from *Canis lupus familiaris, Felis catus, Escherichia coli*, since these DNAs are abundant in canine and feline feces and also gDNA from other helminth parasites usually present in feces such as*: Toxascaris*

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leonina, Ancylostoma caninum, *Echinococcus granulosus sensu stricto and Taenia hydatigena*. Ten picograms of gDNA were used in all cases.

Environmental Samples from endemic areas

Thirty-eight environmental fecal samples were collected from San Juan Province, Argentina, and were analyzed by optical microscopy. Samples were considered positive when *Toxocara* spp. eggs were observed by at least one of the methods used (Telemann concentration technique, as well as Willis and Sheather flotation techniques). All samples were analyzed by the LAMP assay using triplicate DNA samples obtained by both CK and IHM. The final cost (USD $) of reagents per reaction for each method was estimated using the cost of each necessary reagent for both the LAMP reaction and the method for DNA extraction. This cost was obtained from each of the commercial providers in Argentina.

Results

Primer design

The *T. canis cox*-1 gene was selected as a target for the primer design. The region of the gene was chosen according to the number of mismatches between sequences (Fig. 1). The primer sets (Table 1) were selected according to the number of mismatches in the 3' and 5' ends, where each of the primers aligns (Table 2).

Amplification conditions

Optimal conditions for DNA amplification were 60 min at 58ºC with a final incubation at 80°C for polymerase inactivation (Supplementary table S1). The optimal malachite green dye final concentration was 0.016% w/v (Supplementary table S2).

Sensitivity

The LAMP reaction was able to detect 10 fg of gDNA from *T. canis* and 100 fg of gDNA from *T. cati* (Fig. 2). In each case, the results were obtained by direct visualization of green fluorescence in the reaction tube, using 1 µl of SYBR Green I®, and 0.016% w/v malachite green dye final concentration. The LAMP reaction showed positive results with fDNA prepared from canine feces positive for *T. canis* eggs by using CK (diluted up to 1/10,000 or 10^{-4}) and IHM (diluted up to 1/100,000 or 10^{-5}), prepared from canine feces positive for *T. canis* eggs (Fig. 3A).

Specificity evaluation

The specificity of the LAMP reaction was tested with 1 ng of DNA from *C. lupus familiaris*, *F. catus* and *E. coli*. In addition, the specificity was evaluated with gDNA from parasite species commonly found in canine and feline feces (*E. granulosus s. s.*, *T. leonina*, *A. caninum*, *T. hydatigena*) (Fig. 3B). No amplification products were observed with these gDNAs while the positive control (gDNA from *T. canis*) showed the expected amplification signal.

LAMP analysis of environmental samples from endemic areas

The LAMP reaction was applied to 30 canine fecal samples and 8 feline fecal samples in order to determine the usefulness of the technique for the simultaneous detection of *T. canis*-*T. cati* from feces collected from the environment in endemic areas in Argentina. Out of the 38 samples, 13 (34%) were positive for *T. canis*-*T. cati* eggs (Table 3) when using optical microscopy methods.

On the other hand, when using the LAMP assay, a total of 28 samples (74%) had detectable DNA of *T. canis-T. cati*, regardless of the extraction method used (CK of IHM). Moreover, the 13 samples detected as positive by microscopy were also detected as positive by the LAMP assay. LAMP determination using CK for DNA extraction and SYBR Green I dye for visualization resulted in a final cost of reagents per reaction of approximately USD$ 6.12. On the other hand, use of the IHM method for extraction and malachite green dye for visualization, the final cost of reagents was reduced to approximately USD$ 1.16 per reaction (Table 3).

Discussion

Pathogen´s diagnoses through techniques that detect nucleic acids are increasingly used. The analytical sensitivity and specificity values place them among the best options for diagnosis. However, their high economic value and complexity make them hard to implement in areas where laboratory equipment is basic and budget for reagents is low. Although the detection of *T. canis* and *T. cati* eggs by optical microscopy is the most widely used technique, due to its high specificity and low cost, its sensitivity can vary according to the parasite burden, egg shedding dynamics or sample size; the sensitivity also depends on the experience of the technician that performs the analysis. Although different PCR´s, with high sensitivity and specificity have been developed specifically for these parasites (Jacobs *et al.*, 1997; Li *et al.*, 2007; Pinelli *et al.*, 2013; Knapp *et al.*, 2016), they are not routinely and widely implemented due to its high cost and the need of sophisticated equipment which are not widely available in endemic areas. Nonetheless, with the advent of isothermal amplification techniques, such as the LAMP, which uses the same principle as a regular PCR but does not need specialized equipment, various researchers are studying the possibility of using it as a routine technique, even for point-of-care testing (Durant *et al.*, 2012; Njiru, 2012; Wong *et al.*, 2017; Deng *et al.*, 2019; Nzelu and Kato, 2019).

Specific LAMP primer sets for separate *T. canis* and *T. cati* detection have been previously designed using a partial sequence of the internal transcriber spacer 2 rRNA region (ITS-2), with an analytical sensitivity of 100 fg of gDNA (Macuhova *et al.*, 2010) and two to three eggs in 30 g of feces (Khoshakhlagh *et al.*, 2017). On the other hand, the LAMP assay developed herein was able to simultaneously detect *T. canis* and *T. cati* in a single reaction with analytical sensitivity of 10 fg for *T. canis* gDNA, which is more sensitive than previously reported values for both LAMP (Macuhova *et al.*, 2010) and PCR assays (Jacobs *et al.*, 1997; Pinelli *et al.*, 2013; Knapp *et al.*, 2016). The analytical sensitivity for *T. cati* detection was lower (100 fg) and could be related to the primer design, since they had two mismatches in the 3´ regions of the F3 and B3 primers (Table 1), this was previously observed for *Echinococcus* spp. (Avila *et al.*, 2020). These mismatches could influence initial LAMP reaction steps, due to an incorrect match with target sequence (Avila, 2019). The LAMP reaction developed is able to detect low concentrations of DNA from *Toxocara* spp in feces. Nevertheless, further studies are needed to evaluate the detection limit with known concentrations of *Toxocara* spp eggs.

The LAMP assay proved to be more sensitive than optical microscopy for the identification of *Toxocara* spp., as previously reported (Khoshakhlagh *et al.*, 2017; Avila *et al.*, 2020). Furthermore, in this study, the use of the IHM for DNA extraction adapted from (Macuhova *et al.*, 2010), was as efficient as CK, confirming that it could be used in laboratories that do not have funds to purchase a commercial kit, thus reducing costs. However, for the method to be effective it is necessary to use the entire sample in order to increase sensitivity. Moreover, the interpretation of results was performed with the commonly used malachite green dye which is usually available in parasitology labs since it is routinely used for the Kato-Katz thick smear method for helminths (Katz, *et al.,* 1972). This dye has been used before in other LAMP reactions (Nzelu *et al.*, 2014, 2016; Lucchi *et al.*, 2016; Chahar *et al.*, 2018; Kudyba *et al.*, 2019; Serra-Casas *et al.*, 2019) and it proved to be useful for the current study as well. The use of this dye also reduces the possibility of amplicon contamination, since tube opening after incubation is no longer necessary. The use of the IHM for DNA extraction and malachite green dye for visualization of the results guarantee their use in almost any laboratory since they reduce the cost of reagents for the reaction by 81%. The IHM along with the use of malachite green was applied in 38 environmental samples and the high sensitivity of the LAMP assay against conventional helminth egg concentration techniques was observed. The LAMP assay could be compared with copro-microscopic techniques with high sensitivity such as Mini-FLOTAC and FLOTAC (Maurelli *et al*., 2014).

Herein, a sensitive, specific, and economic LAMP assay was developed in order to simultaneously detect *T. canis* and *T. cati* in fecal material from definitive hosts. This method may be implemented in low complexity laboratories in endemic areas for epidemiological purposes. Wider field studies need to be conducted in order to corroborate its performance at a larger scale. In the future, this methodology could be tested in other matrixes such as food, water and sand, among others, in order to identify environmentally contaminated areas and prevent infection in humans, especially children.

Conclusions

The results of this study showed that the LAMP technique can provide a specific and sensitive method for the simultaneous detection of *T. canis* and *T. cati*, etiological agents of toxocariasis in humans. Detection in a single assay, using an IHM method for DNA extraction, and malachite green dye for visualization of results, make up an easy and low cost option for diagnosis, providing a new tool to improve toxocariasis diagnosis in facilities from endemic areas lacking sophisticated equipment.

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Ethical standards:

Not applicable

Conflicts of interest:

The authors declare none

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Table 1: Sequence data of primer set designed for simultaneous detection of a fragment of mitochondrial *cox*1 genes from *Toxocara canis* and *Toxocara cati* species.

Primer	*Sequence*
FIP-Tc	TCAAACGAGGAAACCTCATATCAGGGTTTTGGTAATTGGATATTACCTT
BIP-Tc	TTGGTTGTTGCCTACGGCTAAGTTCAACTAGTACCACACC
F3-Tc	GGTTATGCCTACTATGATTGG
B3-Tc	ATAGTCCTCAAAGGAGGATAC
LF-Tc	TGATGTTGGGGGCTC
LB-Tc	GGATGCTTGTTTTGTTGATATGG

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Table 2: Determination of the number of mismatches between LAMP primers and orthologous gene sequences. Mismatches in the 3' end from Forward Outer Primer (F3), F2 region from Forward Inner Primer (FIP), reverse complement sequence from F1c region from Forward Inner Primer (FIP), Backward Loop Primer (LB), Forward Loop Primer (LF) and mismatches in the 5' end from B1c region from Backward Inner Primer (BIP), reverse complement sequence from B2 region from Backward Inner Primer (BIP), reverse complement sequence from Backward Outer Primer (B3).

Species	F3 3´	F2 3´	F1c_rc 3´	B1c 5´	B2_rc 5´	B3_rc 5´	LB 3´	LF 3´
T. canis	0	0	0	0	0	0	0	0
T. cati	1	0	0	0	0	1	0	0
T. leonina	0	0	1	0	1	1	0	0
A. caninum	1	1	1	1	1	1	1	2
A. suum	0	1	1	0	1	1	0	1
E. granulosus s.s.	0	1	0	3	2	1	4	3
T. hydatigena	1	2	2	2	3	1	4	3
D. caninum	1	2	1	1	2	1	4	4
C. lupus familiaris	1	2	3	3	4	2	3	1
F. catus	1	2	2	3	4	2	3	1
H. sapiens	2	3	3	3	4	2	3	2

Table 3: Detection of *T. canis* and *T. cati* in environmental samples by optical microscopy and LAMP assay. Each sample (N=38) was considered positive if *Toxocara* spp. eggs were observed by at least one of the methods (8–10). LAMP assay was performed using DNA obtained from commercial kit and in-house method. All results were confirmed in three independent assays. The final cost of reagents per reaction (USD$) was estimated according to quotations from each commercial provider.

	Optic microscopy	*LAMP using commercial kit*	*LAMP using In-house method*
Positive (canine+feline)	13 (12+1) (34%)	28 (23+5) (74%)	28 (23+5) (74%)
Negative (canine+feline)	25 (18+7) (66%)	10 (7+3) (26%)	10 (7+3) (26%)
Final cost per reaction (USD$)	-	6.12 (100%)	1.16 (19%)

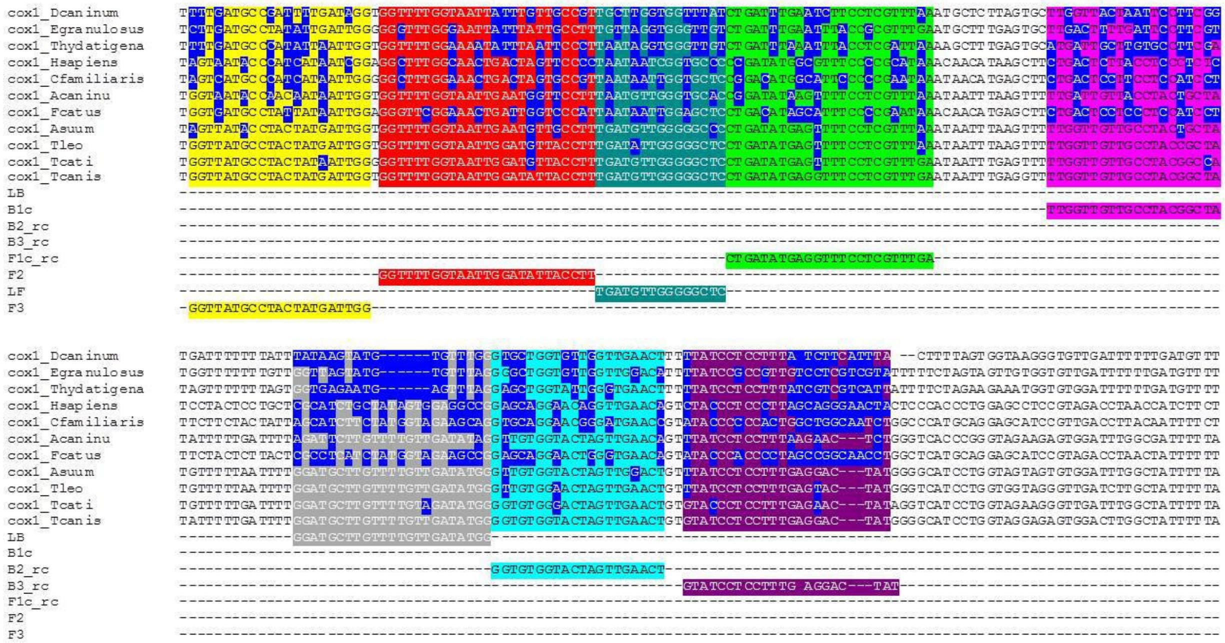

Fig. 1: Design of LAMP primers: Multiple alignment of target sequences for the design of LAMP primers for *Toxocara canis* and *Toxocara cati* simultaneous detection, using sequences of orthologous genes of *T. canis*_dCOX1 (AM411108.1:6046-7623) *T. cati*_dCOX1 (AM411622.1:6055-7632), *Echinococcus granulosus sensu stricto* G1_dCOX1 (AF297617.1:6760-8367), *Toxascaris leonina* (NC_023504.1:1-1578), *Ancylostoma caninum* (NC_012309.1:1-1578), *Ascaris suum* (NC_001327.1:8771-10347), *Dipylidium caninum* (AB732959.1:9743-11485), *Taenia hydatigena* (GQ228819.1:6831-8450), *Felis catus* (U20753.1), *Canis lupus familiaris* (U96639.2), and *Homo sapiens sapiens* (J01415.2). Mismatches are highlighted in blue.

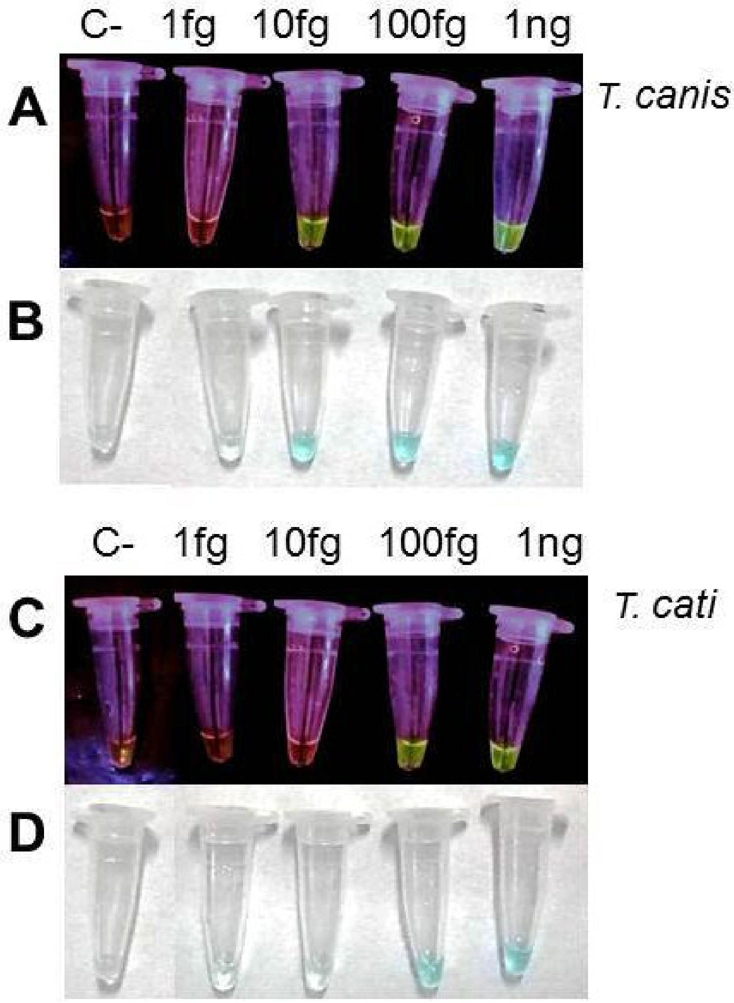

Fig. 2: Analytical sensitivity of the LAMP assay. The simultaneous detection of *Toxocara canis* and *Toxocara cati* was evaluated by using serial dilutions (1 fg to 1 ng) of genomic DNA extracted from adult worm parasites of each species. These results were visualized using SYBR Green I® 1000 X (A and C) and malachite green dye 0,016% w/v (B and D). C-: water control.

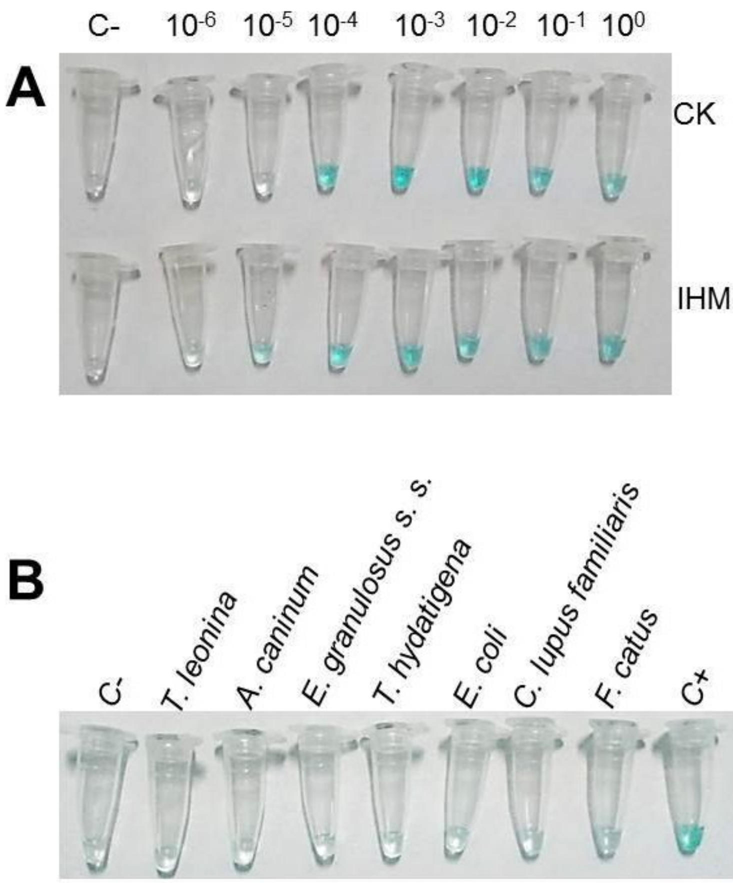

Fig. 3: (A) Sensitivity of the LAMP reaction with serial dilutions of DNA (10^{0} to 10^{-6}) extracted from feces by using a commercial kit (CK) and an in-house method (IHM). The feces used were positive for *Toxocara canis* eggs by optical microscopy. C-: water control. (B) Specificity of the LAMP assay: The specificity of LAMP for simultaneous *T. canis* and *T. cati* detection was evaluated using 10 pg of genomic DNA from *Canis lupus familiaris*, *Escherichia coli*, *Felis catus*, and the following parasites: *Dipylidium caninum*, *Taenia hydatigena, Toxascaris leonine,* and *Ancylostoma caninum.* C-: water control, C+: 1 pg of genomic DNA from *T. canis*.