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METHODS
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Development of a New LAMP Assay for the Detection of *Ancylostoma caninum* DNA (Copro-LAMPAc) in Dog Fecal Samples

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Ancylostoma caninum is a zoonotic nematode which is able to affect animals and humans. Diagnosis in the definitive host and environmental detection are key to prevent its dissemination and achieve control. Herein, a new coprological LAMP method for the detection of *A. caninum* (Copro-LAMPAc) DNA was developed. DNA extraction was performed using a low-cost method and a fragment of the *cox*-1 gene was used for primer design. The analytical sensitivity, evaluated with serial dilutions of genomic DNA from *A. caninum* adult worms, was 100 fg. A specificity of 100% was obtained using genomic DNA from the host and other pathogens. The Copro-LAMPAc was evaluated using environmental canine fecal samples. When compared with gold standard optical microscopy in epidemiological studies, it proved to be more sensitive. This new LAMP assay can provide an alternative protocol for screening and identification of *A. caninum* for epidemiological studies in endemic areas.

Keywords: loop mediated isothermal amplification, *Ancylostoma caninum*, copro-diagnosis, ancylostomiasis, molecular diagnosis

INTRODUCTION

Over one-third of the worldwide population harbors a parasitic helminth; these parasitic diseases generate millions of deaths each year (1) and produce a series of morbidities that affect mostly vulnerable populations from low and medium income countries (LMIC), causing millions of disability-adjusted life years (DALYs) (2). *Ancylostoma caninum* is a zoonotic nematode, proven to cause local lesions (e.g., papular/pustular eruptions) rather than

serpiginous tracks typical of cutaneous larva migrans. Other conditions occasionally attributed to *A. caninum* are myositis, unilateral subacute neuroretinitis, and eosinophilic enteritis (3). The filariform larvae stage penetrates the human epidermis but typically does not develop in the intestine, therefore, it becomes trapped in the skin and underlying muscles, causing irritation and itching (4–6). In some studies, few cases were reported where *A. caninum* was able to complete its migration to the human intestine (7), generating several eosinophilic gastroenteritis (8). Recent studies suggests that patent human infection is a possibility (9, 10).

Infection in dogs can occur through percutaneous penetration of third-stage larvae, orally or through lactogenic and transmammary route (11). Previously infected rodents and insects can act as paratenic and transport hosts, respectively. Canids ingesting such a host will develop patent infections due to reactivation of hypobiotic larvae in the prey (12). In puppies, symptoms can be extensive, including, anemia diarrhea, malnutrition and death. In older dogs, symptoms are mostly limited to anemia (13). Immature worms may still produce clinical disease (i.e., no eggs observed in feces). In Argentina, previous epidemiological studies showed a variable range of *A. caninum* prevalence in canine feces (14–26). A study performed in the central Buenos Aires province showed that 60.5% of dogs were parasitized with *A. caninum* (27). In contrast to the southern Patagonian region of the country, where the reported prevalence of *A. caninum* in canines was lower (0.41–6.2%) (14, 22).

The detection of parasitic structures in the feces is used for detection of infection, using different coproparasitological techniques to concentrate the sample and increase sensitivity (13). The most common coproparasitological techniques used for the detection of *A. caninum* eggs in canine feces is the standard fecal flotation technique with either saturated salt (28) or sucrose (29). The main disadvantages of these techniques, in epidemiological studies, is associated to false negative results due to low parasite burden, the biology of the parasite, and human resources that are not specifically trained or have little experience in the identification of parasitic structures under the microscope (30). Nonetheless, there are alternative coproparasitological methods that may be used to improve the sensitivity (31). Copro-antigen detection methods through Enzyme-Linked Immuno Sorbent Assay (ELISA) have shown a wide range of sensitivity and specificity values (32). Moreover, the detection of nucleic acids through the use of molecular techniques has generated improvements in sensitivity and specificity values (33–36). However, these techniques cannot generally be performed in endemic areas because they require sophisticated equipment and highly qualified personnel, complicating its implementation.

On the other hand, the isothermal amplification of nucleic acids has begun to be widely used since it can be carried out in laboratories without specialized equipment. Particularly, the LAMP (loop-mediated isothermal amplification) technique (37), uses 3 primer pairs that recognize a small DNA fragment, and generate looped structures that serve as a template to start a new polymerization cycle thus providing both higher specificity and sensitivity (38). The DNA polymerase I from *Bacillus stearothermophilus* (*Bst*) used in the technique causes DNA strand displacement and therefore does not require denaturing the double strand, thus the technique can be performed with any equipment that guarantees a constant temperature (37). The LAMP reaction characteristics (affordable, sensitive, specific, user-friendly, rapid, equipment-free) make it an attractive method for use in diagnosis and epidemiological surveillance (39).

Different protocols based on LAMP reactions, have been implemented for diagnosis of different parasites (30, 38, 40–49). Similarly, LAMP reactions have been shown to be useful for the detection of pathogens in food and surveillance of water quality (50–57). Nonetheless, this technique is not currently widely used in the diagnostic routine, probably due to the high cost of the visualization methods required and the cost of DNA extraction. Another complication is the contamination of the sample with unwanted amplification products which can be avoided by correcting the workflow.

In this work the development of an easy copro-LAMP reaction for *A. caninum* detection was performed. Furthermore, this reaction was evaluated using two different methods for DNA extraction in order to be able to implement it in laboratories without sophisticated equipment.

MATERIALS AND METHODS

Samples and Parasite Isolation

All canine fecal samples collected in San Juan and Corrientes Province, Argentina, were stored at −80°C for a week to ensure inactivation. *A. caninum* adult worms were kindly provided by Dr. Marcos Butti (National University of La Plata, Buenos Aires province, Argentina) and were preserved in 70% ethanol.

Optical Microscopy

Three standard concentration methods were employed for detection of hookworm eggs: two different flotation techniques, one with sugar and one with salt (28, 29), and the Telemann sedimentation technique (58). The techniques chosen for this study are standard concentration techniques that increase the chances of detecting parasitic structures, including nematode parasites such as *Ancylostoma* sp. Each sample was microscopically examined at 100 X and 400 X magnifications.

TABLE 1 | Copro-LAMPAc primer set: sequence data of the primer set designed for *Ancylostoma caninum* detection.

Primer	Sequence
FIP-Ac	CTGTTCAACTAGTACCACAACCTATGTT TTTGATTGTTACCTACTGCTA
BIP-Ac	ACCCAGAGTTCTTAAAGGAGGATATT GGCGATTTTTAGTTTACATTG
F3-Ac	CGGATATAAGTTTTCCTCGTTTA
B3-Ac	ACCTAAAATTGAACTCAAACCA
LF-Ac	AGATTCTTGTTTTGTTGAT
LB-Ac	CACCCGGGTAGAAGAGTGG

This primer set was designed for the specific recognition of a mitochondrial cox1fragment.

DNA Extraction

DNA extraction from host, bacteria and parasites was performed using different protocols. The adult nematode DNA was obtained using an already published protocol (59). The DNA from adult cestode parasites was obtained using the DNeasy Blood & Tissue Kit® (QIAGEN, Germantown, MD, USA). While DNA from *Escherichia coli* was extracted using the phenol-chloroform method (60). Finally, DNA from the intestinal tissue of the host was extracted following the manufacturer's instructions for the DNeasy Blood & Tissue Kit® (QIAGEN, Germantown, MD, USA).

The DNA integrity and concentrations were determined according to Avila et al. (38). The DNA extraction and purification from feces (fDNA) was obtained using a commercial kit (CKM) and an alternative low-cost method (LCM) based on mesh-filtration of stool, followed by alkaline hydrolyses, according to Avila et al. (38).

LAMP Assay

Primer Design

The selection of the target gene for the primer design was performed according to Avila et al. (38), using Primer V5 design software (61). The target for primer design was a 208 bp region of the mitochondrial gene *cox*-1 (Genbank accession number NC_012309.1, region: 293-501 bp). This selected region guaranteed the necessary specificity for primer generation, according to previous descriptions (38, 49, 62).

Master Mix

The Copro-LAMPAc reaction was performed in a 12.5 µl final reaction mixture containing: 20 mM Tris (pH 8.8), 50 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 8 mM betaine, 1.4 mM dNTPs and 4 U *Bst* 2.0 polymerase (New England Biolabs, Ipswich, MA, United States). The primer concentration was 0.02 nmol of FIP and BIP primer, 0.0025 nmol of F3 and B3 primer, and 0.005 nmol of LB, LF primer and 1 µl of DNA as template or water for negative controls. All reactions were performed on ice. The amplification conditions were evaluated using a temperature gradient (52–62°C) and different incubation times (15–120 min). The results were obtained using 1 µl of 1000X SYBR Green I® (Thermo Fisher Scientific, Waltham, MA, USA).

Analytical Sensitivity

Ten-fold ultrapure water serial dilutions of gDNA from *A. caninum* were used to measure the analytical sensitivity for detection.

Specificity Evaluation

The specificity of the Copro-LAMPAc was evaluated by using 10 pg of gDNA of canine and bacterial DNA, which are always present. Additionally, the specificity was evaluated with

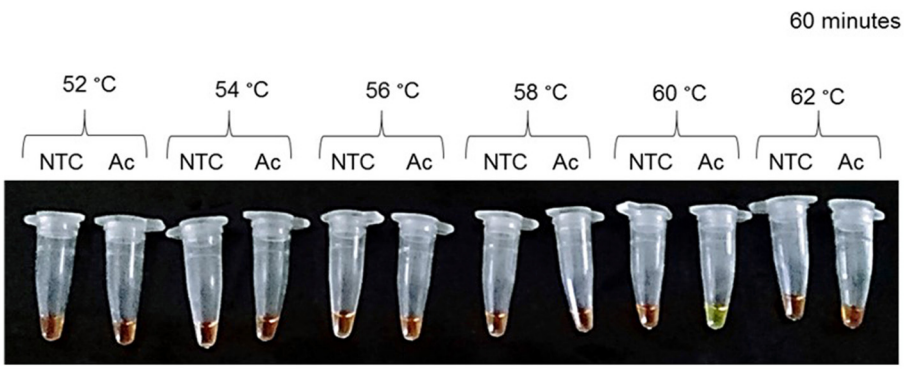

FIGURE 1 | Optimal conditions for *Ancylostoma caninum* genomic DNA amplification. Incubation temperature optimization at a range of 52–62°C. During a 60 min incubation, a temperature of 60°C was considered optimal. Results were visualized using SYBR Green I® 1000 X. NTC, negative control; Ac, *Ancylostoma caninum*.

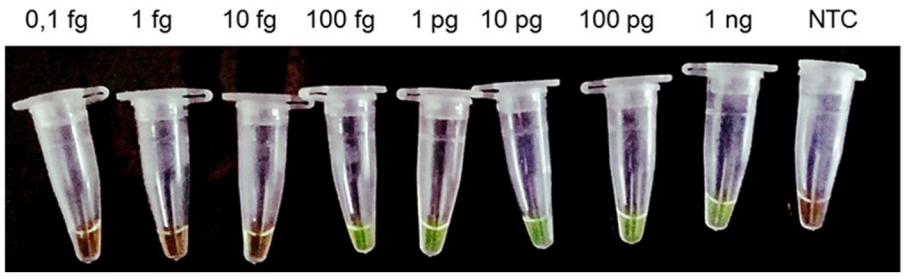

FIGURE 2 | Analytical sensitivity of the Copro-LAMPAc assay. Serial dilutions of *Ancylostoma caninum* genomic DNA (1 fg−1 ng) were used to determine the limit of detection. One microliter of SYBR Green I® 1000 X (Thermo Fisher Scientific, Waltham, MA, United States) was used view the results. NTC, negative control.

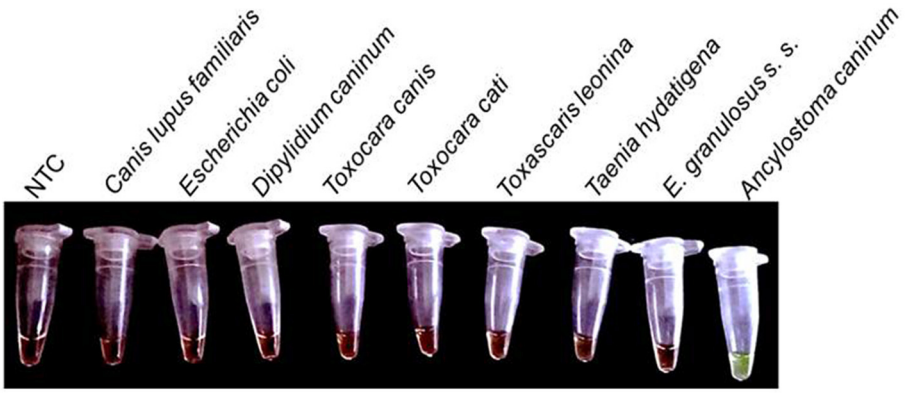

FIGURE 3 | Specificity of the Copro-LAMPAc assay. Cross reaction was not observed when 10 pg of DNA from *Canis lupus familiaris*, *Escherichia coli*, *Dipylidium caninum*, *Taenia hydatigena, Toxascaris leonina, Toxocara canis, Toxocara cati* and *Echinococcus granulosus sensu stricto*, were used. NTC, negative control; *A. caninum*, 1 pg of genomic DNA.

TABLE 2 | Detection of *Ancylostoma caninum* in environmental samples.

	Optical microscopy	LAMP-Ac + commercial kit	LAMP-Ac + low-cost method
Positive	12 (29.3%)	22 (53.7%)	22 (53.7%)
Negative	29 (70.7%)	19 (46.3%)	19 (46.3%)
Total	41 (100%)	41 (100%)	41 (100%)

Each positive result was considered according to the presence of hookworm eggs (optical microscopy) or visual orange-green conversion (LAMP). Identical results were obtained using DNA obtained by either a commercial kit or a low-cost method. Triplicates were used for each result.

DNA from other helminth parasites which are usually present in canine feces: *Toxocara canis, T. cati, Toxascaris leonina*, *Echinococcus granulosus sensu stricto, Dipylidium caninum*, and *Taenia hydatigena*. Particularly, the *T. hydatigena* cox-1 region, has high similarity with corresponding regions of other *Taenia* species, e.g., *T. pisiformis* (63).

Environmental Samples From Endemic Areas

Forty-one environmental fecal samples were collected from San Juan and Corrientes Provinces, Argentina. Samples were analyzed by optical microscopy and Copro-LAMPAc assay using triplicate DNA samples obtained by both CKM and LCM methods.

RESULTS

LAMP Design and Standardization

The primer set was designed using an *A. caninum cox*-1 fragment gene. The primer sets (**Table 1**) were selected according to Avila et al. (38). Optimal temperature incubation was 60°C for 1 h (**Figure 1**; **Supplementary Table 1**), with a final incubation at 80°C for 15 min.

The Copro-LAMPAc reaction was able to detect up to 100 fg of gDNA from *A. caninum* (**Figure 2**); cross reaction was not observed with either the host, bacterial DNA or DNA from the other parasites tested (**Figure 3**).

Environmental Samples Analysis

Forty-one canine fecal samples collected from the environment in Corrientes and San Juan provinces, were analyzed. Using optical microscopy methods, twelve samples (29.3%) were positive for hookworm eggs (**Table 2**). On the other hand, the Copro-LAMPAc assay was able to detect *A. caninum* DNA from 22 samples (53.7%). This result was not affected by the method of DNA extraction (CKM or LCM); this result was confirmed in triplicate.

DISCUSSION

Hookworms are nematodes that can affect human and animals (3, 64, 65). *Ancylostoma* diagnosis is not provided at the species level since the morphology of *Ancylostoma* egg species is often indistinguishable. The simplicity of copromicroscopy and its specificity makes it the gold standard method for the parasitic helminth egg detection. However, this copromicroscopy presents the disadvantage of low sensitivity in epidemiological studies, or in the identification of the hookworm species. These problems in sensitivity and specificity values can be resolved using molecular biology. Unfortunately, the high costs for its realization make it difficult to use in laboratories with poor resources or the lack of equipment specific for PCR. This is the reason why many PCR protocols specific for *A. caninum* detection that have been previously developed (33–35, 66, 67), are not currently available in some endemic areas.

Isothermal amplification techniques, such as LAMP, are able to specifically and sensitively amplify nucleic acids, without the need of expensive equipment. These characteristics make them an attractive alternative for molecular diagnosis in epidemiological studies (30, 68, 69). Different LAMP protocols for helminth DNA detection were developed. The LAMP assay for *E. granulosus* s. s. DNA detection developed by Ni et al. (70), was able to detect 10 pg of gDNA, and was more sensitive

than both copro-ELISA and copro-PCR. While Bucher et al. (71) developed an easy and cost-effective protocol for *E. multilocularis* copro-detection, some LAMP reactions for the detection of nematode DNA were not able to overcome the sensitivity values of gold standard techniques (72) or PCR methods (73).

Herein, we developed for the first time, a simple LAMP reaction for *A. caninum* copro-detection. The Copro-LAMPAc was able to detect 100 fg of gDNA from *A. caninum* adult worms, which is more sensitive than previously reported values for other PCR assays (33–35, 66). This analytical sensitivity value is included in the femtograms range, similar to what has been obtained in other studies (38, 49, 74). In future studies, the *A. caninum* egg limit of detection could be determined.

The Copro-LAMPAc assay proved to be more sensitive than optical microscopy for the identification of *A. caninum* (**Table 2**). As previously described (38), commercial kits for DNA extraction from stool can be replaced by more accessible methods. This significant cost-reduction in sample processing increases the possibility of the Copro-LAMPAc assay implementation in practically any laboratory. It could be compared to techniques that increase the sensitivity of conventional coprological methods, such as the FLOTAC or mini-FLOTAC (31), although these methods cannot be used to distinguish hookworm species.

Due to the zoonotic potential of *A. caninum*, which can cause skin damage, eosinophilic enteritis or patent infection in humans, it is important to be able to have a tool to monitor the environmental contamination by hookworms (and other nematodes, such as Ascarids), since free-roaming domestic animals are a primary source of contamination (75, 76). Additionally, recent studies have demonstrated the presence of drug resistance to anthelminthics in dog hookworms (*A. caninum*) (77–79), where the authors suggest the use of the fecal egg count reduction test to evaluate the efficacy of drugs used (79). In epidemiological studies, the efficacy of this technique is lower. Therefore, we believe that other important uses for the copro-LAMPAc test could be for epidemiological studies, screenings, and when a species-diagnosis for hookworms is needed in the context of a veterinary clinic.

The Copro-LAMPAc assay developed herein is promising in the light of the sensitivity/specificity values herein obtained for *A. caninum* detection. The Copro-LAMPAc protocol may be implemented for epidemiological purposes in low complexity laboratories. Nevertheless, given the results obtained here are preliminary, future wider field studies should be performed to validate these findings. If the larger scale is confirmed, this method could also be implemented to determine environmental contamination. Adaptations to this protocol could be performed in order to detect *A. caninum* contamination in other matrixes (i.e., food, water, sand, etc.), in order to prevent infection in both animals and humans.

CONCLUSIONS

The Copro-LAMPAc technique provides a specific and sensitive method for the *A. caninum* detection, a zoonotic parasite that affects both humans and animals. Moreover, this technique uses an accessible method for DNA extraction, providing an easy and low cost tool for diagnosis. This study provides a new protocol to improve hookworm screening for epidemiological studies in basic laboratories from endemic areas.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The parasite material was obtained from the cadaver of animals previously donated by their respective owners, to the University Hospital from the School of Veterinary of the National University of La Plata.

AUTHOR CONTRIBUTIONS

MP and HA conceptualized and designed the experiments. HA performed the experiments and wrote the manuscript. MR, MC, PR, SR, MB, MT, and GS contributed reagents, materials, and/or analytical tools, and wrote the manuscript. MP and VP critically revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.770508/full#supplementary-material

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