Veterinary Parasitology 277 (2020) 109017

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Development of a copro-LAMP assay for detection of several species of *Echinococcus granulosus* sensu lato complex

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ARTICLE INFO

Keywords:
Echinococcus granulosus sensu lato
Loop mediated isothermal amplification
LAMP
Copro-diagnosis
Molecular epidemiology
Cystic echinococcosis

ABSTRACT

Cystic echinococcosis represents a significant problem in human and animal health and constitutes one of the most severe Neglected Tropical Diseases prioritized by the World Health Organization. The etiological agent is the complex *Echinococcus granulosus* sensu lato (s. l.), composed of several species/genotypes. Diagnosis in the definitive host and molecular epidemiology studies are important points for cystic echinococcosis control. Here we developed a new copro-LAMP assay, LAMP EGSL, for diagnosis in the definitive host for simultaneous detection of *Echinococcus granulosus* sensu stricto (s. s.), *Echinococcus ortleppi*, and *Echinococcus canadensis* species. Also, the analytical sensitivity, specificity and plausibility of performance in a rural context of a previously reported species-specific LAMP reaction, was evaluated. Both reactions showed high analytical sensitivity values (10 fg-100 fg DNA) and did not show cross reaction with DNA from host or other helminthic parasites. LAMP EGSL was performed with samples from an endemic area. In addition, the alkaline hydrolysis of one *E. granulosus* s. s. adult parasite followed by specific LAMP to *E. granulosus* s. s. was performed in a laboratory with low resources from another cystic echinococcosis endemic area. The results obtained suggest that LAMP EGSL represents a potential tool for canine diagnosis that could be useful for cystic echinococcosis control programs. In addition, we showed that LAMP reaction for *E. granulous* s. s., *E. ortleppi* and *E. canadensis* specific detection, could be useful for molecular epidemiology studies applicable to the definitive host. Both reactions were performed in endemic, rural areas without sophisticated equipment.

1. Introduction

Cystic echinococcosis, one of the neglected diseases prioritized by the World Health Organization (WHO, 2010), is an endemic zoonosis caused by *Echinococcus granulosus* sensu lato (s. l.) complex. *E. granulosus* s. l. complex is composed by *Echinococcus granulosus* sensu stricto (s. s.) (G1/G3 genotypes), *Echinococcus equinus* (G4 genotype), *Echinococcus ortleppi* (G5 genotype), *Echinococcus canadensis* (G6/G7/G8/G10 genotypes), and *Echinococcus felidis* (*E. felidis*). The disease is distributed worldwide, in communities based on rural activity especially in those with low economy standards (PANAFTOSA, 2015; Cucher et al., 2016; Ito et al., 2016; Casulli, 2017; Thompson, 2017). The study of molecular epidemiology of *E. granulosus* s. l. is important due to variations among the species of the complex, such as antigenicity (Alvarez Rojas et al., 2013), host specificity (Thompson, 2017) and pre-patent period (Soriano et al., 2016). In Argentina, the presence of *E. granulosus* s. s., *E.*

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https://doi.org/10.1016/j.vetpar.2019.109017
Received 25 December 2018; Received in revised form 19 December 2019; Accepted 20 December 2019

ortleppi, and *E. canadensis*, with the capacity to infect intermediate, accidental, and definitive hosts, was reported (Cucher et al., 2016; Avila et al., 2017; Debiaggi et al., 2017).

Diagnosis in the definitive host is an important step for beginning, evaluation, and completion of control programs of cystic echinococcosis. Two methods used for diagnosis in definitive hosts were necropsy and observation of adult worms in the dog´s small intestine (Craig, 1996), and arecoline purgation followed by visualization of released adult worms. Necropsy provides high specificity and sensitivity but it is laborious, and raises ethical issues (Pawlowski et al., 2001). Arecoline purgation is highly specific, but its sensitivity is variable, is time-consuming and is impractical for large-scale surveys (Craig et al., 2015). Microscopic examination is a classical method for the detection of eggs excreted in feces of the definitive hosts. However, the eggs of different species belonging to Taeniidae family cannot be differentiated due to their morphological similarity (Eckert and Deplazes, 2004). Methods based on copro-antigen detection have been described (Morel et al., 2013) but their sensitivity is relatively limited, resulting in an overall average test sensitivity of 60 % for natural canine *E. granulosus* s. l. infection (Craig et al., 2015). In addition, cross reactions with other helminths were reported (Craig et al., 2015). With the advent of molecular techniques, PCR and real time PCR were standardized for diagnosis. These techniques provide high sensitivity and specificity levels but require sophisticated equipment (Cabrera et al., 2002; Bell and Ranford-Cartwright, 2002; Abbasi et al., 2003; Bretagne, 2003;). In the last years, isothermal amplification of DNA was developed providing new tools for field implementation, such as Loop Mediated Isothermal Amplification assay (LAMP) (Notomi et al., 2000), Cross Priming Amplification assay (CPA) (Xu et al., 2012) and Recombinase Polymerase Amplification assay (RPA) (Piepenburg et al., 2006). Particularly, LAMP reaction is a powerful tool implemented for fast and easy diagnosis/identification of microbiological infections (Wong et al., 2017) specially in developing countries, due to its easy of performance without advanced equipment or trained personnel. LAMP reaction has been proposed as the ideal diagnosis method, since it meets all the criteria proposed by the WHO for ideal diagnoses (Mabey et al., 2004). Two LAMP reaction for copro-detection of *E. granulosus* s. s. were developed (Salant et al., 2012; Ni et al., 2014). Since the primers used in the mentioned assays were designed over *E. granulosus* s. s. (G1) genes, they are not expected to recognize the other species of the *E. granulosus* s. l. complex. In a study (Wassermann et al., 2014), LAMP reaction for specific detection of each species of *E. granulosus* s. l. complex was developed, but possible utility in *E. granulosus* s. l. detection in canine feces was not evaluated. In the present work, a new copro-LAMP assay, for simultaneous detection of *E. granulosus* s. s., *E. ortleppi*, and *E. canadensis* for definitive host diagnosis was developed. In addition, previously reported LAMP assays (Wassermann et al., 2014) for the specific detection of *E. granulosus* s. s., *E. ortleppi*, and *E. canadensis* were evaluated for molecular epidemiology studies to be performed in endemic areas.

2. Materials and methods

2.1. Parasite material

The isolates from intermediate and definitive hosts were conserved in 70 % ethanol. Each isolate refers to protoscoleces obtained from a single hydatid cyst or one adult worm. The parasite materials used in this study correspond to isolates of *E. granulosus* s. s. G1 (sheep and dog from Chubut Province, Argentina), *E. ortleppi* (cattle from Santa Fe Province, Argentina), and *E. canadensis* G7 (pigs from Córdoba Province, Argentina). For arecoline purgation, the procedure was performed according to Guidelines for Surveillance, Prevention and Control of Echinococcosis/Hydatidosis. FAO/UNEP/ WHO (Eckert, et al., 1981). Fecal samples were collected from the environment (squares, areas close to slaughterhouses and farms) in Chubut and Santa Cruz provinces, Argentina. Fecal samples were liquid, solid or semisolid and were collected in plastic flasks in 70 % ethanol.

2.2. Optical microscopy

Canine feces were processed either by Sheather method (Sheather, B.Sc.,1923) and modified Telemann method (Telemann, 1908). Each sample was examined under optical microscope at 100X and 400X amplifications. Identification of parasite eggs was performed by morphological characteristics.

2.3. DNA extraction

The genomic DNA extraction from isolates from intermediate hosts, small intestine dog, and other cestode parasites was made by DNeasy Blood & Tissue Kit® (QIAGEN), following the manufacturer's instructions. DNA concentration was determined using a Nanodrop 1000 and DNA integrity was assessed by electrophoresis in 1 % agarose gel stained with GelRed® (Biotium) and UV visualization. DNA extraction and purification from feces was performed using QIAamp DNA Stool Mini Kit® (QIAGEN), following the manufacturer's instructions. The DNA extraction from nematode parasites was performed according to Repetto et al. (2013), and DNA from *Escherichia coli* was extracted using the phenol–chloroform method (Sambrook y Russell, 2001). The DNAs were conserved at −20 °C.

2.4. Genotyping

Amplification of a fragment of the mitochondrial *cox* 1 gene was made by PCR based on Bowles et al. (1992), with minor modifications in primer set as described in Cucher et al. (2011). The reaction mixture was 5 mM dNTPS, 5 pmol of each primer, 1.2 mM $MgCl_2$, 1 U Pegasus Taq DNA polymerase® (EMBIOTEC, Argentina), 20 mM Tris−HCl (pH 8.4), 50 mM KCl, 10−50 ng of genomic DNA from *E. granulosus* s. l., in a total volume of 50 µl. The PCR conditions were: an initial denaturation step (95 °C for 5 min) followed by 35 cycles of 95 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 10 min. The PCR products were detected by 1 % agarose gel stained with GelRed® (BIOTUM) and UV visualization. The *E. granulosus* s. l. species/genotype determination was performed by sequencing a fragment of mitochondrial *cox* 1 gene. Sequencing by Sanger method was performed at Macrogen (Macrogen, South Korea). The alignment and species/genotype identification were performed as in Avila et al. (2017). Negative control of PCR was performed by adding ultrapure water instead of DNA (water control), and positive control was performed by adding 10 ng of genomic DNA from *E. granulosus* s. l. as template in the PCR reactions.

2.5. LAMP for simultaneous detection of E granulosus s. s., E. ortleppi, and E. canadensis

2.5.1. Primers design

The selection of the target gene for LAMP reaction useful for simultaneous detection of *E. granulosus* s. s., *E. ortleppi*, and *E. canadensis* (LAMP EGSL) was performed considering general rules, indicated by Notomi et al. (2000), and Primer V5 primers design guide (Eiken, 2018). The selected region for primer design was part of the *cox* 1 gene, due to the presence of few mismatches with other *E. granulosus* s. l. species/genotypes, and several mismatches with respect to the other parasites (Supplementary Fig. S1).

2.5.2. Master mix for LAMP EGSL

The LAMP-reactions were performed in a 12.5 µl reaction mixture containing: 20 mM Tris (pH 8.8), 50 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH4)_2SO_4$, 8 mM betaine, 1.4 mM dNTPs and 4 U *Bst* 2.0 polymerase® (New England Biolabs). The quantity of primers per reaction was 20

pmol of each FIP and BIP primer, 2.5 pmol of each F3 and B3 primer, and 5 pmol of LB primer. In all cases, 1 µl of DNA obtained from the 100 µl eluate from the QIAamp DNA Stool Mini Kit® (QIAGEN) was used as template. All components were keeped in an ice box during preparation of the LAMP reaction. The reaction mixture was incubated for 60 min at 54 °C. The results were visualized by adding 1 µl of 1000 X SYBER Green I, and by electrophoresis in 3 % agarose gel stained with GelRed® (Biotium) and UV visualization. The visual results were immediately checked.

2.5.3. Analytical sensitivity evaluation of LAMP EGSL

The analytical sensitivity was evaluated with serial 1:10 dilutions prepared from a 100 pg/µl solution of genomic DNA prepared from protoscoleces of *E. granulosus* s. s., *E. ortleppi*, and *E. canadensis,* obtained from hydatid cysts from sheep, cattle and pigs, respectively. Genomic DNA was obtained using DNeasy Blood & Tissue Kit® (QIAGEN), following the manufacturer's instructions. Additionally, sensitivity of LAMP EGSL was evaluated with serial dilutions of DNA extracted from 200 mg of feces, which were previously spiked with one adult worm of *E. granulosus* s. s. obtained by arecoline purgation. In each case, genomic DNA obtained was diluted in ultrapure water to obtain serial dilutions.

2.5.4. Specificity evaluation of LAMP EGSL

The specificity of LAMP EGSL assay was evaluated against genomic DNA from *Canis lupus familiaris* intestinal tissue and *E. coli*, since these DNAs are abundant in dog feces. Also, it was evaluated against genomic DNA from adult worm helminth parasites usually present in dog feces such as*: Dipylidium caninum, Toxocara canis, Toxascaris leonina, Ancylostoma caninum* and *Taenia hydatigena*. In addition, genomic DNAs from other helminthic parasites such as *Toxocara cati* and *Taenia crassiceps* were evaluated. Helminth parasites were obtained by arecoline purgation, except *A. caninum* that was obtained from a surgery of an infected dog and *T. crassiceps* cisticerci that were obtained from an experimentally infected mouse.

2.5.5. Samples from endemic areas

Four dogs from a house in Chubut where a pediatric patient (3 years old) was confirmed with three hydatid cysts (2 in the liver and 1 in the lung) were purged with arecoline bromhidrate, as previously described (Eckert, et al., 1981). Arecoline purgation was also applied to 1 dog from a slaughterhouse in Santa Cruz province. The samples were analyzed in black-blocked trays for visualization of adult worms, under optical microscope for identification of eggs from Taeniidae family, and by LAMP EGSL. Two hundred mg of feces from a parasite-free dog spiked with 2 *E. granulosus* s. s. adult worms and 50g of feces from another parasite-free dog spiked with 5, 15, or 20 *E. granulosus* s. s. adult worms were used as positive controls for LAMP and optical microscopy.

2.5.6. Environmental samples from endemic areas

Two hundred and forty-six fecal samples were collected from Chubut and Santa Cruz, Argentina, and analyzed by optical microscopy. All the samples with optical microscopy positive results were analyzed by LAMP EGSL. In addition, 10 % of negative optical microscopy samples from places likely to have infected dogs due to rural environment were also assayed by the LAMP EGSL reaction.

2.6. Species-specific LAMP for E. granulosus s. s., E. ortleppi, and E. canadensis detection

The reaction mix and incubation were performed according to Wassermann et al. (2014). The assay consists of a number of LAMP reactions, each one detecting one of the *E. granulosus* s. l. species. The target gene of the LAMP was the mitochondrial NADH dehydrogenase subunit 1 (*nad*1) gene. Here we used LAMP reaction for specific detection of *E. granulosus* s. s., *E. ortleppi* or *E. canadensis*, detection. Negative control of LAMP was performed by adding ultrapure water instead of DNA (water control).

2.6.1. Analytical sensitivity evaluation

The analytical sensitivity was evaluated with serial dilutions of genomic DNA of *E. granulosus* s. s., *E. ortleppi* and *E. canadensis*, obtained from protoscoleces from each species. Serial 1:10 dilutions were prepared by dilutions with ultrapure water starting with a solution of 1 ng/µl of each of the mentioned DNAs. DNA concentration of the original solution was determined by nanodrop. Additionally, the sensitivity of LAMP for specific detection of *E. granulosus* s. s. was evaluated with serial dilutions of genomic DNA extracted from 200 mg of feces, which were previously spiked with one adult worm of *E. granulosus* s. s. obtained by arecoline purgation. The DNA from feces was prepared as detailed above.

2.6.2. Specificity evaluation

The specificity of each primer set (Wassermann et al., 2014) against intestinal *C. lupus familiaris* genomic DNA, *E. coli* genomic DNA (both abundant in dog feces) and genomic DNA from other adult worm helminth parasites: *D. caninum*, *T. canis, T. hydatigena and T. crassiceps,* was evaluated.

2.6.3. Rural performance

A LAMP assay for *E. granulosus* s. s. detection with basic equipment (waterbath and micropipettes, see Fig. 5) of Hospital Barreal in San Juan, Argentina (31°38′38.8″S 69°28′18.7″W), was performed. The DNA template was prepared from alkaline hydrolysis of one adult of *E. granulosus* s. s., according to Nakao et al. (2003).

3. Results

3.1. LAMP for simultaneous detection of E granulosus s. s., E. ortleppi, and E. canadensis (LAMP EGSL)

3.1.1. Analytical sensitivity

The LAMP EGSL reaction was able to detect 10 fg of genomic DNA from *E. granulosus* s. s., 10 fg of genomic DNA from *E. ortleppi*, and 100 fg of genomic DNA from *E. canadensis* (Fig. 1). In each case, the results were visualized by electrophoresis in 3 % agarose gel and by direct visualization of green fluorescence in the reaction tube, using 1 µl of SYBR Green I®. The LAMP EGSL reaction showed positive results with DNA diluted up to 1:10,000 prepared from canine feces spiked with one *E. granulosus* s. s. adult worm (Supplementary Fig. S2).

3.1.2. Specificity

The specificity of LAMP EGSL was tested with 10 pg and 1 ng of DNA from intestinal *C. lupus familiaris* and *E. coli* (Fig. 2). In addition, the specificity was evaluated with DNA from parasite species commonly found in canine feces (*D. caninum*, *T. canis*, *T. leonina*, *A. caninum*, *T. hydatigena*) and DNA from other helminthic parasites (*T. cati*, *T. crassiceps*) (Fig. 2). No amplification products were observed with these genomic DNAs while the positive control (genomic DNA from *E. granulosus* s. s.) showed the expected amplification pattern.

3.1.3. Samples from endemic areas

In order to determine the usefulness of the LAMP EGSL assay for *E. granulosus* s. l. detection in stool samples from endemic areas, we obtained fecal canine samples from a house from Chubut Province in which a hydatid patient lives and from a dog from a slaughterhouse from Santa Cruz province. The results were compared to the results obtained with optical microscopy and arecoline purgation (Table 1, Supplementary Fig. S3). All Chubut samples but one were negative by arecoline purgation. One of the arecoline purgation negative samples was positive for LAMP EGSL, suggesting that LAMP EGSL could be more

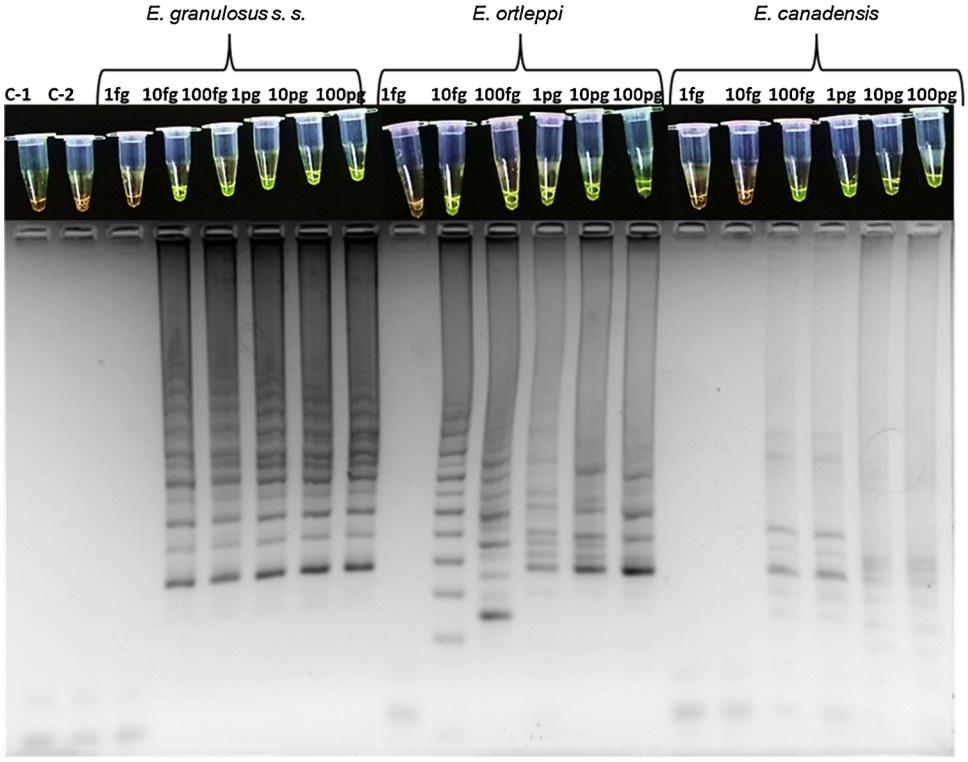

Fig. 1. Analytical sensitivity of LAMP EGSL: Analytical sensitivity of LAMP for simultaneous detection of *Echinococcus granulosus* sensu stricto, *Echinococcus ortleppi*, and *Echinococcus canadensis* (LAMP EGSL) was evaluated using serial dilutions (1 fg to 100 pg) of DNA extracted from protoscoleces of each species. C-1 and C-2: water controls. The LAMP results were analyzed by agarose gel electrophoresis and by visual detection with 1 µl of SYBR Green I® 1000x. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

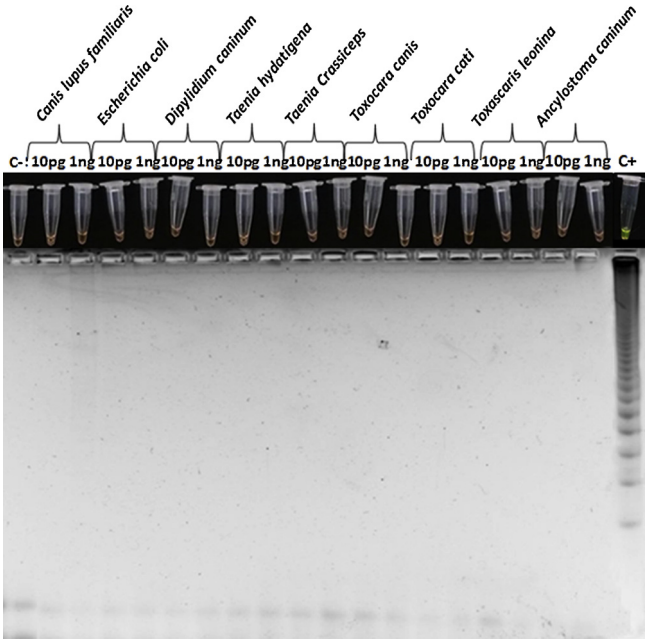

Fig. 2. Specificity of LAMP EGSL: The specificity of LAMP for simultaneous detection of *Echinococcus granulosus* sensu stricto (*E. granulosus* s. s.), *Echinococcus ortleppi,* and *Echinococcus canadensis* (LAMP EGSL) was evaluated using 10 pg and 1 ng of genomic DNA from intestinal *Canis lupus familiaris*, *Escherichia coli*, and the following parasites: *Dipylidium caninum*, *Taenia hydatigena, Taenia crassiceps, Toxocara canis, Toxocara cati, Toxascaris leonina* and *Ancylostoma caninum.* C-: water control, C+: 10 pg of DNA from *E. granulosus* s. s. protoscoleces. The LAMP results were analyzed by agarose gel electrophoresis and by visual detection with 1 µl of SYBR Green I® 1000x. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Samples from arecoline purgation of dogs: 4 samples from a house inhabited by a hydatid disease child (dog 1–4) and 1sample from a slaughterhouse (dog 5) were tested by optical microscopy and LAMP EGSL for simultaneous detection of *Echinococcus granulosus sensu stricto*, *Echinococcus ortleppi* and *Echinococcus canadensis*. Canine feces from a parasite-free dog were spiked with *E. granulosus* s. s. adult worms and used as positive controls.

Sample	Arecoline purgation	Optical microscopy	LAMP EGSL
Dog 1	–	–	–
Dog 2	–	–	–
Dog 3	–	–	–
Dog 4	–	–	+
Dog 5	+	+	+
Feces + 2 *E. granulosus* s. s. [a]	NA	+	+
Feces + 5 *E. granulosus* s. s.[b]	NA	+	+
Feces + 15 *E. granulosus* s. s.[c]	NA	+	+
Feces + 20 *E. granulosus* s. s.[d]	NA	+	+

[a] 200 mg of canine feces spiked with 2 *E. granulosus* s.s. adult worms, [b]: 50 g of canine feces spiked with 5 *E. granulosus* s.s. adult worms, [c]: 50 g of canine feces spiked with 15 *E. granulosus* s. s. adult worms, [d]: 50 g of canine feces spiked with 20 *E. granulosus* s. s. adult worms. NA: not applicable.

sensitive for *E. granulosus* s. l. detection in feces. With respect to the Santa Cruz sample from the slaughterhouse, it resulted positive by arecoline purgation and optical microscopy, in coincidence with LAMP EGSL result.

3.1.4. LAMP EGSL analysis of environmental samples from endemic areas

LAMP EGSL was applied to 61 fecal samples collected from Chubut and Santa Cruz, Argentina, in order to determine if this technique was useful for *E. granulosus* s. l. detection from feces collected from the environment. This group of samples was composed by all the samples

Table 2
LAMP EGSL analysis of environmental samples from endemic areas: environmental samples from endemic areas were tested by optical microscopy and LAMP EGSL for simultaneous detection of *Echinococcus granulosus sensu stricto*, *Echinococcus ortleppi* and *Echinococcus canadensis*.

Number of samples	Optical microscopy	Number (%) of LAMP EGSL positive samples
40	+	4 (10.0%)
21	–	2 (9.5%)

that showed optical microscopy positive results (40) and 21 samples that were negative by that technique but were taken from regions likely to have parasite circulation. Positive controls were feces from a parasite-free dog spiked with 2 *E. granulosus* s. s. adult worms. Negative controls were feces from the same parasite-free dog. LAMP positive control was DNA prepared from *E. granulosus* s. s. protoscoleces and LAMP negative control was water. As a result, it was observed that 6 samples (10 %) were LAMP EGSL positive (Table 2) and all the controls rendered the expected result. Samples that were positive by optical microscopy but LAMP negative, probably correspond to the presence of eggs of Taeniidae family not belonging to *Echinococcus granulosus* s. l. eggs from different species of this family cannot be differentiated by microscopy due to their morphological similarity (Eckert y Deplazes, 2004).

3.2. Species-specific LAMP

3.2.1. Analytical sensitivity

Species-specific LAMP was able to detect 10 fg of genomic DNA from *E. granulosus* s. s., 10 fg of genomic DNA from *E. ortleppi*, and 100 fg of *E. canadensis* DNA (Fig. 3). In each case, the results were analyzed by electrophoresis in 3 % agarose gel and by direct visualization of green fluorescence in the reaction tube using 1 µl of SYBR Green I® 1000x. We compared species-specific LAMP with *cox*1 PCR and found that the first was able to detect less quantities of DNA, suggesting that LAMP has higher analytical sensitivity. The LAMP for *E. granulosus* s. s. detection showed positive results with one *E. granulosus* s. s. adult worm spiked feces diluted up to 1:600 (Supplementary Fig. S4).

3.2.2. Specificity

The specificity of each species specific LAMP was tested with 10 pg and 100 pg of DNA from intestinal *C. lupus familiaris* and *E. coli* (Fig. 4A), taking into account that DNA from these species always are present in canine feces. In addition, the specificity was evaluated with DNA from species of parasites commonly found in dog feces (*D. caninum, T. canis, T. hydatigena*), and genomic DNA from other cestodes (*T. crassiceps*) (Fig. 4B). No amplification products were observed with these DNAs.

3.2.3. Rural performance

The alkaline lysis of one adult parasite of *E. granulosus* s. s., followed by species specific LAMP, was performed with the basic equipment of rural Hospital of Barreal, in San Juan Province, Argentina (Fig. 5A). The amplification products were visualized using 1 µl of SYBR Green I® 1000X (Fig. 5B). This result was later confirmed in our laboratory through electrophoresis in 3 % agarose gel (Fig. 5C).

4. Discussion

Due to its simplicity and extremely high sensitivity and specificity, LAMP assay has been implemented for the detection of protozoan (Singh et al., 2013) as well as helminthic parasites (Deng et al., 2019) and has already entered into the market and epidemiological surveys. Overall, it is considered that the methodology will be improved in the future, and the active role of LAMP in clinical and epidemiological practice is foreseeable (Deng et al., 2019).

Diagnosis of definitive hosts is an important tool for CE control programs, since a rapid detection and treatment of infected dogs can interrupt the life cycle avoiding contamination of intermediate and accidental hosts such as humans. In the present work, a novel LAMP reaction useful for simultaneous detection of *E. granulosus* s. s., *E. ortleppi* and *E. canadensis* (LAMP EGSL), was designed an evaluated for diagnosis in canine feces. In addition, the performance of a previously

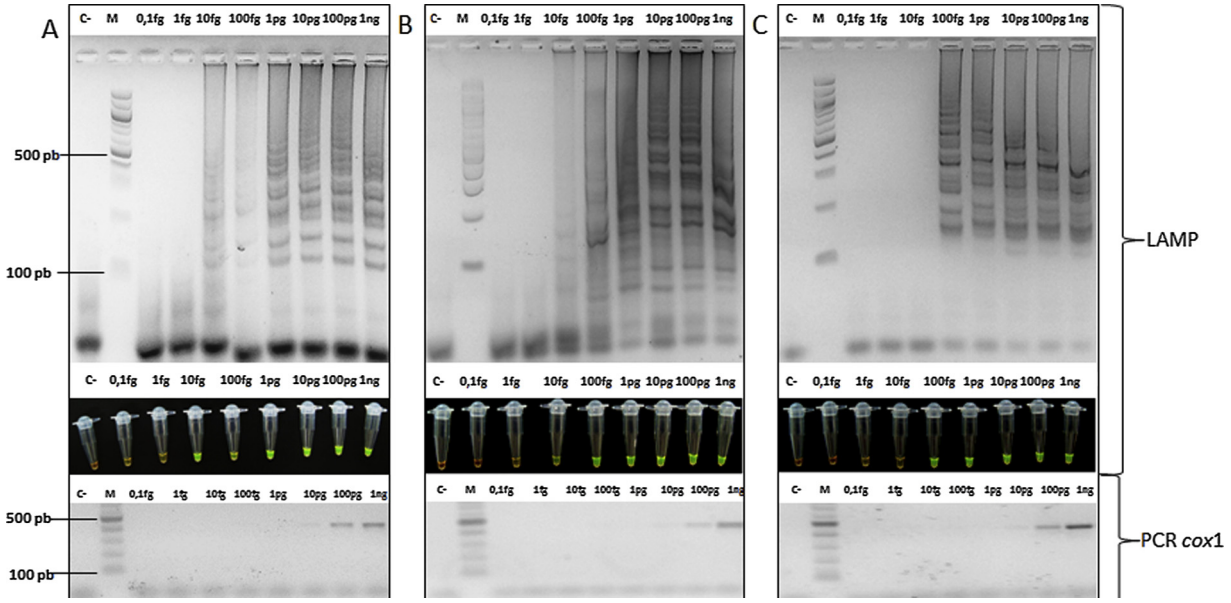

Fig. 3. Analytical sensitivity of species-specific LAMP reaction: The analytical sensitivity of each LAMP reaction was evaluated using serial dilutions (0,1 fg-1 ng) of genomic DNA. A: Sensitivity of LAMP reaction for *Echinococcus granulosus* sensu stricto (*E. granulosus* s. s.) detection and conventional *cox*1 PCR with *E. granulosus* s. s. DNA, B: Sensitivity of LAMP reaction for *Echinococcus ortleppi* detection and conventional *cox*1 PCR with *E. ortleppi* DNA **C**: Sensitivity of LAMP reaction for *Echinococcus canadensis* detection and conventional *cox*1 PCR with *E. canadensis* DNA. C-: water control. In each case, the LAMP results were analyzed by agarose gel electrophoresis and by visual detection with 1 µl of SYBR Green I® 1000x. M: 100 bp DNA Ladder®. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

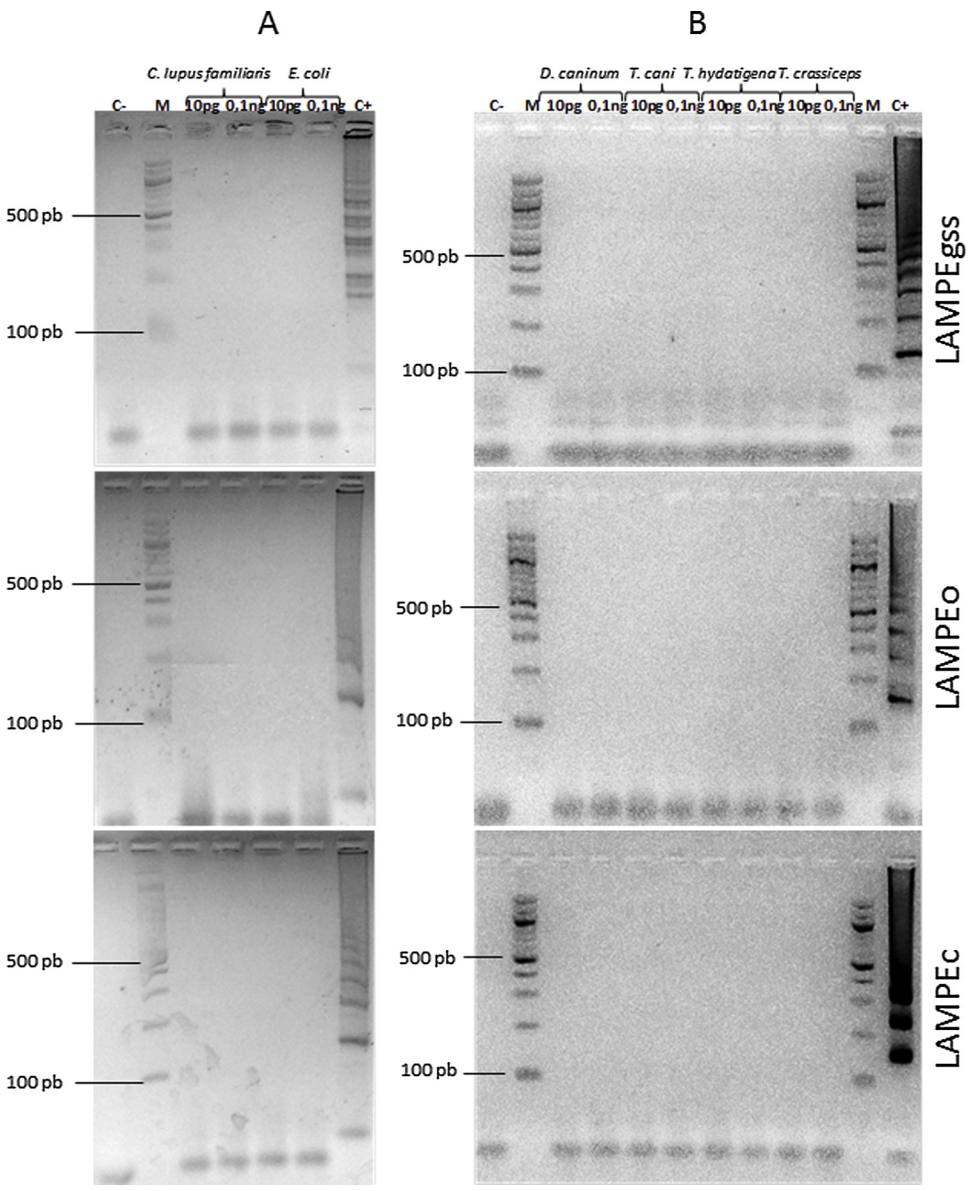

Fig. 4. Specificity of species- specific LAMP: specificity of each species-specific LAMP assay to detect *Echinococcus granulosus* sensu stricto (LAMP *E. granulosus* s. s.), *Echinococcus ortleppi* (LAMP *E. ortleppi*), and *Echinococcus canadensis* (LAMP *E. canadensis*) was evaluated with: (A) 10 pg and 100 pg of DNA from intestinal *Canis lupus familiaris* and *Escherichia coli;* and (B) 10 pg and 100 pg of DNA from *Dipylidium caninum, Toxocara canis, Taenia hydatigena and Taenia crassiceps.* C-: water control, C+: 10 pg of genomic DNA from *E. granulosus* s. s. M: 100 bp DNA Ladder®.

published LAMP reaction for differential detection of *E. granulosus* s. s., *E. ortleppi*, and *E. canadensis* (Wassermann et al., 2014) was evaluated in order to be used in molecular epidemiology studies using fecal samples.

The analytical sensitivity of LAMP EGSL was in the order of 10 fg to 100 fg of DNA from each *E. granulosus* s. l. species. These values are concordant with those reported by Salant et al. (2012) (100 fg of genomic DNA from *E. granulosus* s. s. G1 genomic DNA), and Ahmed et al. (2016) (10 fg of genomic DNA from *E. ortleppi* G5 genomic DNA). Also, LAMP EGSL showed higher sensitivity than the LAMP reaction reported by Ni et al. (2014) (10 pg of genomic DNA from *E. granulosus* s. s. G1). In comparison with copro-PCR reaction for *E. granulosus* s. l. detection, the LAMP reaction developed in this work showed higher analytical sensitivity than Stefanic et al. (2004) (1 ng of genomic DNA from *E. granulosus* s. s. G1), but lower than the PCR reaction previously reported by Abbasi et al. (2003), Cabrera et al. (2002) and Naidich et al. (2006). In those methods the analytical sensitivity was 1 fg of genomic DNA from *E. granulosus* s. s. G1.

The lower analytical sensitivity of LAMP EGSL observed for *E. canadensis* with respect to *E. granulosus* s. s. and *E. ortleppi* could be due to the mismatch present on 5´ end of F1c and B1c primers, and 3´ end of B3 primer (Supplementary Fig. S1). For LAMP EGSL primer design a mitochondrial DNA region from *E. granulosus* s. s. showing several mismatches with parasites from other genera but the lowest possible mismatches with other species of *E. granulosus* s. l. complex was chosen. However, some mismatches could not be avoided.

Importantly, LAMP EGSL technique was useful to analyze arecoline purgation samples as well as environmental samples collected from 2 endemic areas from Patagonia, Argentina, a region known to have *E. granulosus* s. l. circulation. The method could be applied in a field laboratory lacking sophisticated equipment.

Species-specific LAMP and LAMP EGSL for molecular epidemiology studies and diagnosis in dogs respectively showed specificity for *E. granulosus* s. s., *E. ortleppi* and *E. canadensis* species since no crossed reaction was observed with genomic DNA from other parasites commonly present in canine feces or host and bacterial genomic DNA, complying with one of the main requirements of diagnosis techniques

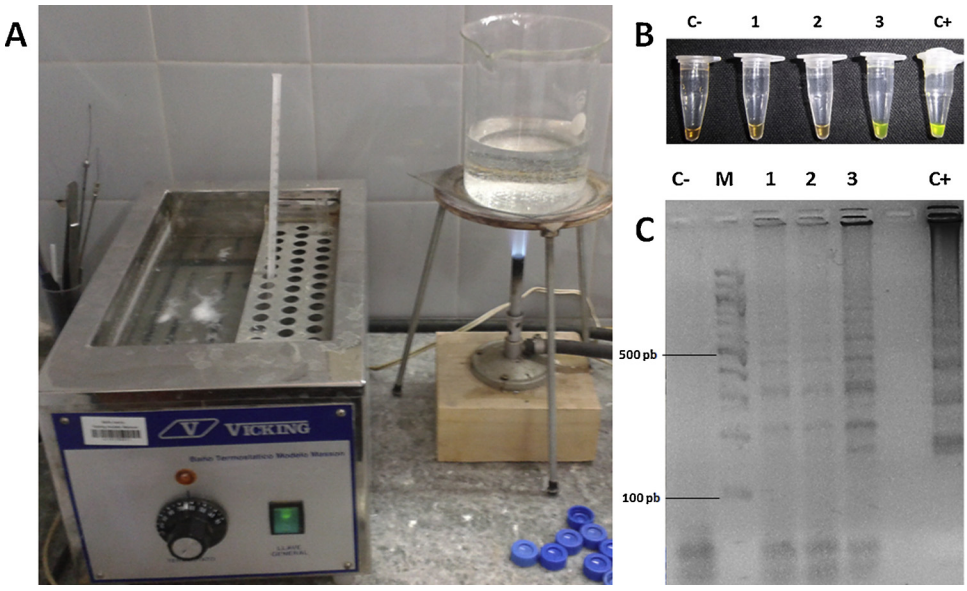

Fig. 5. Rural performance of specie specific LAMP: (A) Basic equipment employed in rural experience in Hospital of Barreal, San Juan province, Argentina. (B) The alkaline lysis of one adult parasite of *Echinococcus granulosus* sensu stricto, followed by LAMP, was performed with the basic equipment of rural Hospital of Barreal, the amplification products were visualized using 1 µl of SYBR Green I® 1000 × . 1: 1:100 dilution, 2: 1:10 dilution, 3: 1 µl of lysate. This result was later confirmed in our laboratory through electrophoresis in 3 % agarose gel (C). C-: Water control, C+: 10 pg of genomic DNA from *E. granulosus* s. s. M: 100 bp DNA Ladder®. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Mabey et al., 2004).

Although previous LAMP reaction were reported for *E. granulosus* s. s. G1 (Salant et al., 2012; Ni et al., 2014) and *E. ortleppi* G5 / *E. canadensis* G6 (Ahmed et al., 2016) detection, in no one of them, simultaneous detection of these 3 species/genotypes was evaluated. In fact, Ni et al. (2014) considered that the LAMP reaction that they developed could be unable to identify *E. canadensis*.

LAMP was also implemented to diagnose pathogens associated with food-borne diseases, such as *Salmonella typhi* (Abdullah et al., 2014), *Campylobacter jejuni* and *Campylobacter coli* (Pham et al., 2015), *Helicobacter pylori* (Bakhtiari et al., 2016) and *Listeria monocytogenes* (Wang et al., 2015) and for water control (Azizi et al., 2019; Wang et al., 2019). Water and food are nonliving reservoirs of *Echinococcus* and are major risk sources of infection. Due to its extremely high sensitivity and simplicity, LAMP could be useful to monitor water and food in order to avoid parasite transmission. Also, other parasites, such as *Cryptosporidium* spp, *Giardia lamblia*, *Plasmodium* spp, *Trypanosoma cruzi*and *Leishmania* spp, have been detected by LAMP method (Han et al., 2007; Karanis et al., 2007; Plutzer and Karanis, 2009; De Ruiter et al., 2014; Karani et al., 2014; Gallas-Lindemann et al., 2016; Besuschio et al., 2017; Nzelu et al., 2019).

5. Conclusion

We have developed a LAMP assay (LAMP EGSL) that can provide a specific and sensitive means to detect *E. granulosus* s. l. species that could be useful in areas where several species of the complex are circulating. In addition, we showed that an already reported species specific LAMP can be applied to fecal samples in order to conduct molecular epidemiology studies in the definitive host. The results obtained in this work provide new tools to improve control programs in endemic areas for cystic echinococcosis lacking sophisticated equipment.

Declaration of Competing Interest

The authors declare that no conflict of interest exists.

Acknowledgements

This work was supported by Fundación Mundo Sano; Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina, project PICT 2017 Nº 2966; Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET); Ministerio de Salud de la Provincia de San Juan and Ministerio de Salud de la Provincia del Chubut.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.109017.

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