The Development of a Loop-Mediated Isothermal Amplification Method (LAMP) for Echinococcus granulosus Coprodetection

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Abstract. We have previously developed a polymerase chain reaction (PCR) assay for detection of Echinococcus granulosus infection, which proved very sensitive and specific for identification of infected dogs. We have now developed a loop-mediated isothermal amplification (LAMP) assay, which amplifies the same genomic repeated sequences of E. granulosus for coprodetection. This assay enabled detection of a single egg in fecal samples and showed high species specificity for E. granulosus with no cross-amplification of DNA from closely related helminths, including Echinococcus multilocularis. Because the method does not require thermocycling for DNA amplification, or electrophoresis for amplicon detection, it can potentially be used for premortem identification of E. granulosus-infected dogs to enable large-scale surveys in endemic countries where highly specialized equipment to undertake PCR analysis is rare.

INTRODUCTION

Cystic echinococcosis or cystic hydatidosis is a chronic helminthic zoonotic disease caused by the larval stage of the tapeworm, Echinococcus granulosus. The disease has a cosmopolitan distribution,1,2 and is especially prevalent in sheep-raising countries;3 the life cycle of this parasite is perpetuated between definitive hosts, the domestic dog and a wide range of ungulates, like herbivorous livestock, which serve as intermediate hosts. Humans become infected by the accidental ingestion of eggs that give rise to hydatid cysts mainly in the liver and lungs. In view of the great economic importance of this disease in the livestock industry and the impact it has on human health, it is very important to be able to assess disease transmission dynamics in the canine population and to evaluate hydatid control programs.

Gold standard detection of E. granulosus in dogs has always been at post mortem on necropsy material. Although this method is highly sensitive, it is laborious, raises ethical issues, and is biohazardous;4 unfortunately, simple microscopical detection of worm eggs in fecal samples by routine coprological flotation is not useful because eggs of all species of the family Taeniidae (genera Echinococcus and Taenia) are morphologically indistinguishable from one another. Coproantigenic diagnosis for canine echinococcosis have been described by several groups.4–8 However, sensitivity of this method is relatively limited, resulting in an overall average test sensitivity of ~60% for natural canine E. granulosus infection.

Echinococcus DNA from proglottids, eggs, or sequestered infected cells excrated in feces can be detected after amplification by polymerase chain reaction (PCR) using primers that amplify regions of the 12S ribosomal RNA (rRNA) gene9,10 or, as was previously done, by detection of a tandem repeat sequence (the Hae III repeat) within the parasite genome.11 Although copro-PCR detection of parasitic material is generally sensitive and/or specific for Echinococcus diagnosis,12 many countries that are endemic for E. granulosus do not possess elaborate, well-equipped laboratories for PCR and gel analysis. Thus, it is extremely important that alternate, user friendly, robust, and accurate detection methods be developed for parasite detection. These should be accessible to medical and veterinary personnel to enable their widespread use for disease surveillance and control.

The loop-mediated isothermal amplification (LAMP) method is a nucleic acid amplification test used in various fields, including infection diagnosis to identify organisms. This assay uses a DNA polymerase called Bst polymerase, which has strand displacement activity and a set of four (or six) specially designed primers that recognize a total of six distinct sequences of the target DNA.13 It has been used to perform highly specific and sensitive amplifications of DNA to detect pathogens including viruses, bacteria, protozoa, helminths, and fungi. Recently, this technique has proven to be very useful in the diagnosis of parasitic infections, such as malaria, trypanosomiasis, dirofilariasis, schistosomiasis, and babesiosis.14–19 Because this method has been able to amplify DNA as soon as 1–2 hours at a single incubation temperature, with only a water bath and ice needed as equipment, this method would appear potentially ideal for field purposes.

The authors of a recent comparative analytical study that evaluated the three previously mentioned copro-PCR methods concluded that the copro-PCR based on a tandem repeat target sequence, which we had previously described,11 was the best method for species differentiation of E. granulosus from E. multilocularis and other taeniids.12,13 We have now developed a sensitive and specific LAMP based on PCR primers derived from the same repeated sequence.11

MATERIALS AND METHODS

Feces and parasites. Negative fecal samples used in spiking experiments were taken from an indoor housed domestic dog that had been dewormed on 3 biweekly separate occasions with a commercially available broad spectrum anthelminthic containing praziquantel, pyrantel pamoate, and febantel (Drontal Plus, Bayer, Pittsburgh, PA). A subsequent fecal flotation examination for helminth eggs after the deworming program was also negative from the dog. Fecal samples were stored at −20°C until used.

Echinococcus granulosus eggs were isolated from worms collected from infected dogs, and E. granulosus DNA was extracted from protoscolecies recovered from affected livers of locally slaughtered sheep. The strain of the parasite we have studied is assumed to be the common sheep strain (G1),

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as clarified in the Discussion. These were used for sensitivity and specificity spiking experiments.

Worms from Spirocerca lupi, Taenia spp., Fasciola spp., Toxocara canis, and Dipyldium caninum (kindly donated by Dr. Marcovics, Kimron, Veterinary Institute, Bet Dagan, Israel), and DNA from Neospora caninum (kindly donated by Dr. Schares, Institute of Epidemiology, Friedrich-Loeffler-Institut, Wusterhausen, Germany) and E. multilocularis (kindly donated by Dr. Craig, University of Salford, UK) were obtained for specificity tests.

DNA extraction methods. DNA from parasites provided for specificity tests was extracted using the Qiagen DNeasy Blood and Tissue extraction kit according to the manufacturer’s instructions (Qiagen, Valencia, CA).

For free DNA and egg spiking into fecal material, the PSP Spin Stool DNA Plus extraction Kit (Invitek, Berlin, Germany) was used according to the manufacturer’s instructions. Two hundred milligrams of fecal material was included in each sample.

Primers. Primers for the LAMP were designed by using the Eiken Genome site (http://primerexplorer.jp/elamp3.0.0/index.html). Sequences of the four primers used for the LAMP are shown in Table 1. The LAMP primers were designed according to E. granulosus repeat region sequence, accession no., DQ157697.

LAMP assays. The reaction mixture of 25 μL contained primers (40 pmol of FIP1ech and BIP1ech and 10 pmol of Eg121la and Eg122a outer primers), 8 units of BstI large fragment, 0.4 mM dNTPs, 1M Betaine; 1 x reaction buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM [NH4]2 SO4, 8 mM MgSO4, and 1% Tween 20), and 5 μL of target DNA from E. granulosus, other parasites or sterile double distilled water (ddw) for negative controls. The reaction was incubated at 60°C in a water bath or thermocycler (MJ Research Inc., Waltham, MA) for 2 hours.

Amplicon detection. Detection of LAMP-amplified products was performed by using SYBR Green I stain (Invitrogen, Carlsbad, CA). One microliter of 1:10 diluted SYBR Green I was added to the reaction mixture (25 μL). When using SYBR Green at a dilution of 1:10, amplicons could be detected directly by the unaided eye because the color of the reaction solution changed from orange to green in the presence of LAMP amplicon. Amplicon analysis by standard gel electrophoresis on 1.1% ethidium bromide agarose gels alongside a pUC Mix Marker 8 (Fermentas, Lithuania), and a positive and negative control was carried out in parallel with SYBR Green staining for quality control.

RESULTS

LAMP amplification of E. granulosus DNA for determining detection sensitivity. Serial 10-fold dilutions of E. granulosus genomic DNA were amplified by LAMP using LAMP primers (Table 1). The results of this amplification experiment are shown in Figure 1A and B after amplicon examination by agarose gel electrophoresis and staining with SYBR Green. Detection sensitivity of this assay was 100 fg DNA/200 μL in double distilled water (ddw).

Detection specificity of the LAMP assay in ddw. All the DNA extracts from the various parasites tested were initially shown to contain a sufficient amount of DNA for PCR by spectrophotometry (NanoDrop-ND1000, Wilmington, DE). Furthermore, DNA analysis was shown to contain a minimal amount of salts before specificity testing was undertaken. Species specificity of the LAMP primers was shown by successful amplification of E. granulosus and a failure of amplification of Fasciola spp., Toxocara canis, E. multilocularis, Spirocerca lupi, Taenia spp., N. caninum, and Dipyldium caninum, on agarose gel electrophoresis and staining with SYBR Green (Figure 2A and B). The capacity of LAMP to specifically detect E. granulosus was thus shown. This substantiates preliminary specificity of the test.

LAMP amplification of E. granulosus for determining DNA detection sensitivity in spiked feces. Serial 10-fold dilutions of E. granulosus genomic DNA in Echinococcus-negative fecal material were amplified by LAMP. The results of this amplification experiment are shown in Figure 3 after amplicon examination by agarose gel electrophoresis. Detection sensitivity of this assay was 1 pg/200 mg feces.

Table 1

<table>
<thead>
<tr>
<th>LAMP Primer</th>
<th>Sequence (5’ 3’)</th>
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<tr>
<td>Eg121la</td>
<td>GAA TGC AAG CAG CAG ATG</td>
</tr>
<tr>
<td>Eg122a</td>
<td>GAG ATG AGT GAG AAG GAG TG</td>
</tr>
<tr>
<td>FIP1ech</td>
<td>CTT TTC CGG ATG GGT AGG CAT CTT</td>
</tr>
<tr>
<td>BIP1ech</td>
<td>CGT GCT GTG GAG GTA GTT TCG TTT</td>
</tr>
</tbody>
</table>

## Figure 1

**A** Agarose gel electrophoresis of loop isothermal amplified products (LAMP) from different amounts of Echinococcus granulosus genomic DNA added to 200 μL of double distilled water. L = pUC molecular mass marker; Lane 1 = 1 ng; Lane 2 = 100 pg; Lane 3 = 10 pg; Lane 4 = 1 pg; Lane 5 = 100 fg; Lane 6 = 10 fg; Lane 7 = 1 fg; Lane 8 = negative control.

**B** Fluorescence of LAMP products as indicated in A using SYBR Green I stain. SYBR Green I stains vary from darker grey color in negative samples to a lighter grey color on positive samples.
LAMP amplification of *E. granulosus* for determining egg detection sensitivity in spiked feces. Figure 4A and B show the LAMP detection results of spiking experiments after which high and low numbers of eggs, consecutively, were spiked per fecal sample. As few as 1 *E. granulosus* egg could be detected in 3 out of 6 (50%) 200 mg fecal spikes by the LAMP method. It should be emphasized that egg numbers were determined by dilution without confirming the actual number per tube.

**DISCUSSION**

Although the gold standard for *E. granulosus* copro-detection in dogs has been traditionally based on parasitological detection of adult worms at necropsy or after arecoline purgation, this method is too laborious, expensive, bio-hazardous, and may cause ethical issues. Furthermore, the simple test of light microscopy following fecal flotation is unable to morphologically distinguish between eggs of all species of the family Taenidae. Although coproantigen tests have been developed, their sensitivity is somewhat limited...
and they are only genus specific. In addition, the Echinotest is not commercially available caused by a shortage of high quality antigen (Veermeier E, personal communication, EMEA). Copro-PCR methods are available and are quite sensitive and/or specific. However, laboratories need specialized equipment for their testing and at least 3 hours is required for completion of DNA amplification and electrophoresis agarose gel analysis. Thus, the requirement for a simpler, cheaper, yet sensitive and specific E. granulosus coprodetection method is necessary, especially in regions that have concurrent E. multilocularis or other taenid infections.

The E. granulosus strain from which the Hae III repeat was isolated11 is believed to be the common sheep strain. Because the LAMP is based on the Hae III repeat sequence described by Abbasi and others,11 which is shared by DNA from which worms were recovered, were PCR positive by amplifying the Hae III repeat.12,20 This test, which was not designed to differentiate between various E. granulosus genotypes, can be expected to be suitable for large-scale testing of dogs for wide coverage of E. granulosus infection. This notion is further strengthened by the finding that all tested dogs from Jordan, from which worms were recovered, were PCR positive by amplifying the Hae III repeat.11 Furthermore, in a work by Boufani and others,12 the said PCR assay was determined to identify the common sheep strain.

The LAMP method can be performed within 2 hours under isothermal conditions by using a simple incubator, such as a water bath or a block heater. There is no need for agarose gel electrophoresis for visual evaluation of the test results. Results can be immediately visualized and examined visually judged by adding SYBR Green I stain to the amplified mixes to distinguish a positive LAMP reaction from a negative reaction.

Our test can have great potential in developing countries, where the disease is endemic and equipment and expert technical staff is scarce. It should be recalled in this context that LAMP has already been established in field laboratories for tuberculosis15 and for Schistosomiasis (Hamburger and others, unpublished data). Its application in large-scale epidemiological studies and surveillance of echinococcosis in canine deworming control programs should be significant.

The sensitivity, specificity, and operational simplicity of LAMP could make it easily applicable for a clinical practice and for epidemiological surveys in areas of disease endemicity, especially in developing countries. It is hoped that further work in the search for cheaper DNA extraction methods from fecal material may allow this test to be a more cost-effective assay for widespread usage,

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