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Loop-Mediated Isothermal Amplification (LAMP) assay for the identification of Echinococcus multilocularis infections in canids

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Abstract:	Background: Alveolar echinococcosis, caused by the metacestode larval stage of Echinococcus multilocularis, is a zoonosis of public health significance and is highly prevalent in northwest China. To effectively monitor its transmission, we developed a new rapid diagnostic method based on loop-mediated isothermal amplification (LAMP) to identify canine definitive hosts infected with E. multilocularis. Methods: The primers used in the LAMP assay were designed, based on the mitochondrial nad5 gene of E. multilocularis, using Primer Explorer V4 software. The developed LAMP assay was compared with a conventional PCR method, using DNA extracted from 189 dog fecal samples from three E. multilocularis-endemic regions in

	Qinghai province of the People's Republic of China and 30 negative control copro-samples from dogs taken from an area in Gansu province, and by light microscopy for detection of eggs. Results: The E. multilocularis-positivity rates obtained for the field-collected fecal samples were 16.4% and 5.3% by the LAMP and PCR methods, respectively, and all samples obtained from the control dogs were negative. Compared with conventional PCR, the LAMP assay provided 88.3% specificity and 100% sensitivity. The LAMP method was able to detect E. multilocularis DNA in the feces of experimentally infected dogs at 12 days post-infection, whereas the PCR assay was positive on the 17th day and eggs were first detectable by light microscopy at day 44 post-challenge. Conclusions: The earlier specific detection of an E. multilocularis infection in dog copro-samples indicates that the LAMP assay we developed is a realistic alternative method for the field surveillance of canines in echinococcosis-endemic areas.
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25/July/2013

The Editor,

PLoS Neglect Trop D

Dear Sir

We are submitting our MS entitled **'Loop-Mediated Isothermal Amplification (LAMP) assay for the identification of *Echinococcus multilocularis* infections in canids'** for re-consideration as a major article by PLoS NTDs. The paper (PNTD-12-01397) was originally submitted to PLoS NTDs and we have considered and modified the MS in line with the comments of the reviewers. This revised version of the paper comprises: abstract, author summary, article text, three Tables and one Figure.

All co-authors have seen the re-submitted paper and they are happy with its content and for Dr. YuRong Yang and me to act as joint corresponding authors.

In fact, the reviewers all suggested that the paper be resubmitted to after revision or just be improved instead. We had also received positive comments from them. Reviewer 1 commented that the specificity of the LAMP seems to be good according to our results. The second reviewer had his comment that information on a development of LAMP method for the detection of *Echinococcus multilocularis* in dog feces is interesting and the LAMP is an important tool for the control of alveolar echinococcosis. And the third one reviewed that the manuscript presents a very convincing study on the copro-detection of *E. multilocularis* in dog stools by using the LAMP method. By the way, our invention patent mentioned in the present study has been granted (ZL201110346474.8) by

the State Intellectual Property Office, PR China this year.

In order to be practicable and to define the accuracy of the LAMP method we have developed for the identification of canines infected with *E. multilocularis*, we have evaluated the assay using field samples from different endemic areas of western China. The results we have obtained indicate that the LAMP method provides exceptional performance in the detection of *E. multilocularis* in dog feces.

In response to the specific comments by the reviewers, the MS (PNTD-12-01397) has been revised extensively as follows:

The statistical methods and results for the comparison of the diagnostic tests have now been added in the new version. The details of the concentrations of the reagents used in each assay are now included. Explanation of the digestion and recognition of the LAMP products is now also included. The reason for our use of *nad*5 as a target gene in the LAMP assay is now clarified. Details of the specificity and sensitivity of the LAMP assay are clarified in the Results section, and to make the data more understandable, we have now included two new tables (Tables 2 and 3) in the revised version. We have also provided more details of the dog challenge infections and, with the help of a native English expert (Prof. McManus), we have thoroughly and carefully checked and edited the English in the manuscript. We now consider the new version is much improved in quality.

We believe our MS provided unique data, and the study is important for *E. multilocularis* control tool development and should stimulate significant interest for readers of PLoS NTDs.

We look forward to hearing from you in due course.

Sincerely yours,

Drs YuRong Yang & WanZhong Jia

Loop-Mediated Isothermal Amplification (LAMP) assay for the identification of *Echinococcus multilocularis* infections in canids

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Abstract

Background: Alveolar echinococcosis, caused by the metacestode larval stage of *Echinococcus multilocularis*, is a zoonosis of public health significance and is highly prevalent in northwest China. To effectively monitor its transmission, we developed a new rapid diagnostic method based on loop-mediated isothermal amplification (LAMP) to identify canine definitive hosts infected with *E. multilocularis*.

Methods: The primers used in the LAMP assay were designed, based on the mitochondrial *nad*5

gene of *E. multilocularis*, using Primer Explorer V4 software. The developed LAMP assay was compared with a conventional PCR method, using DNA extracted from 189 dog fecal samples from three *E. multilocularis*-endemic regions in Qinghai province of the People's Republic of China and 30 negative control copro-samples from dogs taken from an area in Gansu province, and by light microscopy for detection of eggs.

Results: The *E. multilocularis*-positivity rates obtained for the field-collected fecal samples were 16.4% and 5.3% by the LAMP and PCR methods, respectively, and all samples obtained from the control dogs were negative. Compared with conventional PCR, the LAMP assay provided 88.3% specificity and 100% sensitivity. The LAMP method was able to detect *E. multilocularis* DNA in the feces of experimentally infected dogs at 12 days post-infection, whereas the PCR assay was positive on the 17th day and eggs were first detectable by light microscopy at day 44 post-challenge.

Conclusions: The earlier specific detection of an *E. multilocularis* infection in dog copro-samples indicates that the LAMP assay we developed is a realistic alternative method for the field surveillance of canines in echinococcosis-endemic areas.

Author Summary

Echinococcus multilocularis (*E.m.*) is the causative agent of alveolar echinococcosis, a lethal parasitic disease of humans. The parasite is primarily maintained in a sylvatic life-cycle between foxes and rodents, or via semi-sylvatic cycles, usually involving dogs (either domestic or stray) and small mammals. As part of extensive surveillance of *E.m.* transmission we are undertaking in

hyper-endemic areas of Western China, we developed a loop-mediated isothermal amplification (LAMP) method to rapidly detect *E.m.* DNA in the feces of infected dogs. The LAMP assay was shown to have higher sensitivity (100%) and specificity (88.3%) than conventional PCR. Further, it is simpler and faster and able to detect *E.m.* DNA a week earlier in the feces of experimentally infected dogs. Therefore, it is an important new advance for early diagnosis of *E.m.* infection in dogs and for epidemiological surveillance, and is an attractive, affordable and easy to use routine diagnostic tool for use in resource-poor countries, where echinococcosis is prevalent and facilities are relatively undeveloped.

Introduction

Alveolar echinococcosis (AE) is a zoonosis caused by the metacestode stage of *Echinococcus multilocularis* (*E.m.*), the fox tapeworm. The disease is of significant public health relevance in the Northern Hemisphere, particularly in the northwestern parts of the Peoples' Republic of China (PRC). Human AE has similar features to cancer [1-3] due to its infiltration of the liver and its invasive growth in this organ, together with its metastasis via the blood or lymphatic systems to other tissue sites. Recent epidemiological studies of human AE have shown an increase in new cases in areas where *E.m.* transmission had not been recorded previously [4]. The natural transmission cycle of *E.m.* involves small mammal intermediate hosts which become infected after ingesting eggs released in the feces of infected foxes or other canines in a predator-prey relationship; in turn, canines become infected by ingesting tissues of infected small mammals [5]. Transmission to humans occurs when eggs are ingested accidentally so that infected canine

definitive hosts act as the source of disease transmission and human infection.

Prior to the development of PCR-based methods, the most reliable procedure for the diagnosis of *Echinococcus* spp. in definitive hosts, especially in developing countries, was necropsy; using this approach, worm burdens can be estimated and parasites collected for identification [6, 7]. However, necropsy usually results in biased sampling, as generally, only stray or unwanted dogs are necropsied. Despite the early promise shown by the use of copro-antigen detection of *E.m.* by capture-ELISA [1], the test shows low specificity compared with necropsy for diagnosis and surveillance purposes. There is a high rate of cross-reactivity with other helminth infections [2], particularly *E. granulosus* [1], and sensitivity is also highly dependent on worm burden [8]. Copro-DNA-based tests can provide an alternative method of diagnosis [9, 10], and since the first PCR-based method described by Bretagne *et al*. [3] for the detection of *E.m.* DNA in the feces of foxes, the technique has been improved. A conventional PCR-based test using the mitochondrial (mt) *12S rRNA* gene [11], a multiplex-PCR test [4] and a nested PCR assay [12] have been shown to be of diagnostic value for the detection of *E.m.* infections in canines [13, 14]. However, for routine laboratory diagnosis and surveillance these methods have a considerable drawback, in that the sensitivity of PCR can be severely affected by inhibitory factors present in fecal samples [5]. Furthermore, the expensive facilities and reagents and the relatively long time required for test completion are additional disadvantages [6]. Consequently, a more rapid, sensitive and specific diagnostic method for the identification of *E.m.* infections in canines would be of considerable value.

A loop-mediated isothermal amplification (LAMP) method, developed by Notomi *et al* [15, 16], has been shown to rapidly detect and differentiate pathogen species and has higher specificity and sensitivity than multiplex PCR for detecting DNA in fecal samples [17, 18]. A LAMP assay has recently been described for *E. granulosus* copro-detection [7] but it has not been tested on canine stool samples collected in the field. Moreover, to date, no such test is available for the identification of *E.m.* infections in canines. The present study describes the establishment of such a LAMP assay, and is based on using a fragment of the mitochondrial (mt) *nad*5 gene of *E.m.* for the detection of *E.m.* DNA in dog feces. We have evaluated its applicability for testing dog fecal samples collected during routine *E.m.* surveillance in China and compared its practical value with conventional microscopy and a traditional PCR-based assay.

Materials and Methods

Ethical statement

All experiments using mice and dogs were undertaken under strict Chinese experimental animal clearances and animals at all times were treated in agreement with animal ethics procedures and guidelines for animal husbandry of the Institutional Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The study and the use of animals were approved by this Committee (Approval No. LVRIAEC2010-005). Also, all animals were handled in strict accordance with the animal protection laws of the People's Republic of China (A Draft of an Animal Protection Law in China released on September 18, 2009).

E. multilocularis material

The larval isolate of *E.m.* used in all experiments was obtained originally in 2010 from a naturally infected plateau pika (*Ochotona curzoniae*) in Shiqu county, Sichuan province, P.R. China. It was subsequently routinely passaged in laboratory white mice by intra peritoneal (i.p.) inoculation of 0.1 ml of minced metacestode material in sterile phosphate-buffered saline (PBS) containing penicillin (100 U/ml) and streptomycin (100 µg/ml). For the current study, fifteen 8-week old white mice (15-20 g) (purchased from Lanzhou Institute of Biological Products Co., Ltd.) received 0.5 ml minced metacestode material injected into the abdominal cavity, and animals were humanely sacrificed four months later. The larval mass was rapidly removed from the abdominal cavity and immersed in PBS at 10°C. All subsequent procedures were carried out in a laminar flow sterile air chamber. Once sufficient material was collected, it was minced finely with a scalpel blade in a stainless steel sieve with an overall diameter of 6 cm and 1-mm mesh size. PBS was poured periodically through the sieve and parasite material collected into a 500-ml conical flask. The sediment was washed 4 times to remove host fibrous material and fatty tissue. Following washing, collected protoscoleces were checked by microscopy to determine viability by flame cell activity. Only parasites having at least 95% viability were used either to experimentally infect dogs or were stored immediately at -70°C for DNA isolation.

Experimental infection of dogs

Common breed puppies were born from two pregnant dogs, purchased at a local market, at

Lanzhou Veterinary Research Institute. The dogs were treated with albendazole (5 mg/kg body weight on three consecutive days) in order to remove cestodes and nematodes from their intestines two months prior to study commencement, and the dogs were certified helminth-free by routine microscopic examination of feces. The animals were kept in the experimental facility at Lanzhou Veterinary Research Institute for two weeks prior to commencement of the study to allow them to adapt to the living conditions and diet. All animals were fed a heat-treated meal once daily and examined weekly by a veterinarian. Six dogs (kept in individual cages; circa six months old; average weight 10 kg) each received orally approximately 10,000 protoscoleces of *E.m.*, administered in saline as part of their normal daily meal. Fecal samples were collected daily from the bottom of the individual cages, placed into sterilized 50-ml containers with tight fitting lids, and stored at -70°C until use. The six dogs were sacrificed humanely for determining their *E.m.* infections at day 50 post-challenge.

Collection of dog feces in the field

Fecal samples (n=189) were collected from individual dogs in three *E.m.*-endemic areas of Qinghai province, P.R. China. A further 30 fecal samples from unwanted domestic dogs were collected as negative-controls from an area of Gansu province, where mass dog treatment with praziquantel (10 mg/kg) had been carried out monthly for more than one and a half years, and where no human AE cases had been recently recorded. The 30 dogs were sacrificed humanely to confirm they were cestode-free. All collected fecal samples were stored at –70°C before examination by microscopy and for DNA isolation.

Microscopic examination of fecal samples for the presence of taeniid eggs

All dog fecal samples were subjected to a conventional saturated sodium chloride (NaCl) flotation method for isolation of eggs [19]. Briefly, 2 g feces were washed with distilled water and then sedimented by centrifugation at 2,500×g for 10 min with the supernatant being discarded. Then the sediment was suspended in saturated NaCl solution and any eggs present were observed by light microscopy.

Parasite and host DNA samples

Genomic DNA from *E.m.* protoscolex tissue, obtained from infected mice (t-g-DNA), and genomic DNA (f-g-DNA), isolated from fecal samples obtained from the experimentally infected dogs, were extracted using Axyprep^{TM} Multisource Genomic DNA Miniprep Kits (Axygen, CA, USA) and QIAamp DNA Stool Mini Kits (Qiagen, Germany), respectively. The t-g-DNA samples were used as positive controls for establishment of the sensitivity of the LAMP assay. Genomic DNA samples (g-DNAs) from *E. granulosus* (common sheep strain; G1 genotype), *E. shiquicus,* T. *hydatigena*, *T. pisiformis*, *T. taeniaeformis*, *T. multiceps* and *Dipylidium caninum* were used to determine the specificity of the *E.m.* LAMP method. Apart from the *T. taeniaeformis* DNA, which was kindly provided by Viktor Dyachenko, Institute for Infectious Diseases and Zoonoses, Ludwig-Maximilians-University of Munich, Munich, Germany and the *E. shiquicus* DNA, which was extracted from a cyst collected from a naturally infected plateau pika in Shiqu in 2011, all the other cestode DNA samples were obtained from experimentally infected dogs at Lanzhou Veterinary Research Institute. In addition, intestinal tissue and fecal samples (n-f-DNA) from

uninfected dogs were obtained from newly born pups and the DNAs were extracted to serve as negative controls.

Conventional PCR assay

Conventional PCR was carried out for comparative purposes. The PCR primers EMH17/EMH15 were used to amplify a 200 bp fragment of the mt *12S rRNA* gene of *E.m.* (GenBank accession No. AB031351) [8]: EmH17 (F): 5' -GTG AGT GAT TCT TGT TAG GGG AAG-3' (positions 12242-12265); EmH15 (R): 5' -CCA TAT TAC AAC AAT ATT CCT ATC-3' (positions 12439-12416) [9]. The PCR amplification reactions took place in a total volume of 50 μl consisting of 10 mM Tris-HCl (pH 9), 50 mM KC1, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM each primer, 3.1 U *Taq* polymerase (TaKaRa, Dalian, China). The thermal cycling conditions used were as follows 95°C for 4 min; 35 cycles at 94°C for 30 sec, 53°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 10 min.

LAMP assay

LAMP primers were designed based on the mt *nad*5 gene (GenBank accession No. AB031351) [8] using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html). The forward inner primer (FIP), backward inner primer (BIP), and two outer primers (F3 and B3) were specifically designed to recognize six separate regions within the *nad*5 gene. All the primer sequences are listed in Table 1. An *Eco*RI restriction site (*gaattc*) was introduced into the FIP and BIP primers for restriction enzyme digestion analysis of the LAMP products.

The LAMP reaction was performed in a 25 µl volume with 2 µl of *E.m.* g-DNA, 1.8 µl of primer mix (40 pmol each of FIP and BIP, 5 pmol each of F3 and B3), 1.0 µl of *Bst* DNA polymerase (8 U), 12.5 µl of reaction buffer (1.6 M betaine, 40 mM Tris-HCl [pH8.8], 20 mM KCl, 20 mM $(NH_4)_2SO_4$, 16 mM $MgSO_4$, 0.2 % (v/v) Tween 20 and 2.8 mM dNTPs) and 7.7 µl of ddH_2O. In order to determine the optimal reaction temperature and time, the reaction mixture was incubated at 60°C, 61°C, 62°C, 63°C, 64°C and 65°C, respectively, for 30 min and then heated at 80°C for 5 min to terminate the reaction; then six different reaction time periods (10, 20, 30, 40, 50 and 60 min) were compared at the optimal reaction temperature.

The specificity and sensitivity of the LAMP assay

To verify the specificity of the LAMP assay for detection of *E.m.* DNA, the LAMP primers were tested using g-DNAs from *E. granulosus* (G1 genotype), *E. shiquicus*, *T. hydatigena*, *T. pisiformis*, *T. taeniaeformis*, *T. multiceps*, *D. caninum*, and n-f-DNA (fecal samples from cestode-free dogs) and dog intestinal tissue as negative controls. To further confirm the specificity of the LAMP amplification, the sequences of the LAMP amplicons were determined using a modification of the method described by Nkouawa *et al*. [25]. Briefly, the LAMP products were digested for 4 h at 37°C with *Eco*RI (TakaRa, Dalian, China).. The digested products, purified using Axyprep^{TM} DNA Gel Extraction Kits, were then ligated into *Eco*RI-digested pMD-18 T vector at 4°C overnight. The ligation mixtures were used to transform *Escherichia coli* JM109 cells by incubating for 12 h at 37°C. Single colonies were cultivated in Luria-Bertani medium (LB) with Amp^+ for 12 h at 37°C and then were analyzed by PCR using vector primers (M13F/M13R). Positive colonies were

sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). In order to determine the sensitivity of the LAMP assay, *E.m.* t-g-DNA was diluted to 10 ng/µl and then successively diluted 10 times by the addition of 1 µl of a 1/10 dilution of the previous concentration. The same dilution procedure was also performed on DNA samples from dog-feces (f-g-DNA) obtained at different days post-experimental infection.

Examination of field obtained fecal samples

The 189 field-obtained copro-samples (collected from *E.m.*-endemic areas) were examined by the LAMP and PCR assays. The presence of taeniid eggs was confirmed by microscopy. The f-g-DNA extracted from the feces of an experimentally infected dog was used as positive control. The LAMP and PCR products were electrophoresed on a 1.5% (w/v) agar gel containing ethidium bromide and photographed using a gel documentation system.

Statistics

Differences in sensitivity between the LAMP test, PCR assay and microscopy were analyzed using a One-Way ANOVA with post hoc LSD tests and the Chi-square test using SPSS 11.5 [20].

RESULTS

The optimal reaction temperature and time for the LAMP assay

The optimal reaction temperature for the LAMP assay was determined using a range of temperatures from 60 to 65°C; as a result, 63°C was chosen as the optimal reaction temperature (Figure 1A). The optimal length of time for the assay reaction, determined using a range from 10 to 60 min tested at 63°C, indicated 30 to 40 min was optimal; thus, 40 min was chosen for all future assays (Figure 1A).

Analysis of the digested LAMP products

The LAMP products demonstrated typical patterns of ladder-like bands on agar gels and their *Eco*RI digestion products were as expected (Figure 1A). The LAMP products with the correct target gene and sequence were confirmed by DNA sequencing (data not shown).

Specificity and sensitivity of LAMP in experimental samples

E.m. t-g-DNA and f-g-DNA and g-DNAs extracted from the other parasites and dog host intestinal tissues were tested to determine the specificity of the LAMP assay for *E.m.* DNA. Only the target gene fragments in *E.m.* t-g-DNA and f-g-DNA produced amplified products (Figure 1B). The PCR and LAMP assays showed a similar level of sensitivity (detecting as low as 10^{-3} ng DNA) when using different concentration of *E.m.* t-g-DNA (data not shown). However, a positive result was obtained on day 12 post infection in the fecal samples of three of six dogs using the LAMP assay whereas a positive result was not obtained until day 17 post infection using the traditional PCR test, thereby indicating the former was more sensitive with a statistically significant difference ($p<0.05$) between the two methods. The LAMP and PCR assays were substantially more sensitive

than microscopy, as eggs were not detected visually in feces until day 44 post challenge ($p<0.01$) (Table 2). Fecal samples from three of the experimentally infected dogs were shown to be continuously positive by LAMP, PCR, and light microscopy until the dogs were sacrificed. The level of detection sensitivity for the LAMP assay was four *E.m.* eggs per gram of feces (Figure 1C).

Performance of the LAMP assay using dog fecal samples collected in the field

The highest positivity rate (31/189; 16.4%) was achieved using the LAMP assay with dog feces collected from *E.m.*-endemic areas [10] (Table 3). All 30 negative fecal control samples were negative by LAMP, PCR and microscopy. Some of the positive LAMP products were randomly selected for analysis by DNA sequencing, which confirmed their integrity. Twenty one fecal samples that were *E.m.* LAMP-positive but PCR-negative were shown to be negative again when subjected to a second round of the PCR assay. The outcomes of the field collected fecal samples using the LAMP and PCR methods and microscopy are summarized in Table 3. Overall, the specificity and sensitivity of the LAMP assay were calculated to be 88.3% and 100%, respectively, when the PCR assay was used as reference method. The positivity rate for the PCR assay and microscopy (10/189; 5.3%) was the same but some samples positive using one method were negative by the other and vice versa. The LAMP positive samples included all the positive samples determined by both the PCR assay and microscopy and some other samples that were negative by these two methods. Therefore, the LAMP assay was significantly more sensitive ($P<0.001$; Pearson chi-square test) than either of the other two methods used.

Discussion

With the increasing numbers of mitochondrial (mt) DNA gene sequences becoming available, mt genes are being increasingly applied in species identification, molecular taxonomy, evolutionary studies and diagnosis, and in molecular epidemiological investigations of the parasitic helminths [11, 21]. We recently sequenced full-length mt DNAs for several cestode species parasitic in the small intestine of carnivores [11, 21]. The *nad*5 gene is a protein-encoding gene with substantial nucleotide variability which makes it highly suitable for designing LAMP primers for the identification of related species [21]. At least five and up to nine species are accepted within the genus *Echinococcus* [22]; three species - *E. multilocularis*, *E. granulosus* and *E. shiquicus* – occur sympatrically on the Qinghai-Tibet Plateau of China [23-25].

LAMP is a novel nucleic acid amplification method involving the use of four primers that has been developed as a useful tool for the epidemiological surveillance of several parasitic infections [12-14, 26], and has value for the specific and sensitive identification of adult tapeworm infections in dogs and other canines. The LAMP primers used in this study amplified the *nad*5 target gene from the g-DNA of *E.m.*, but not from the g-DNAs of any other cestode tested, including the closely related *E. granulosus* and *E. shiquicus*. Therefore, the LAMP assay exhibited high specificity for application in the diagnosis of *E.m.* infection in canine hosts similar to that recently reported for the differential detection of *Taenia* species from humans using fecal specimens [27]. The assay can provide a very useful tool for differential diagnosis between co-endemic *E. granulosus* [7] and *E.m.* in canines, thereby providing an improved surveillance method for discriminating the two species in order to provide the accurate information required for the

implementation of echinococcosis control programs.

Whereas the PCR and LAMP methods employed in this study exhibited similar levels of sensitivity when tested with different concentrations of *E.m.* g-DNA, the latter displayed a higher sensitivity in the detection of f-DNA from dogs experimentally infected with *E.m.*, disclosing an infection in challenged dogs about a week earlier. A possible explanation for this may be the presence of inhibitors in the f-DNA templates, which can result in lower sensitivity and reproducibility of PCR assays [5, 12, 17, 27] so that more eggs or parasite DNA may be required to obtain a positive reaction. The *Taq* DNA polymerase used in PCR is more often inactivated and affected by these inhibitors than the *Bst* DNA polymerase used in LAMP [5, 27]. It is noteworthy that other studies have shown the LAMP method is also more sensitive in detecting other pathogens in fecal samples [18, 27-29].

The results of this study indicate that the LAMP method is much more sensitive than both conventional PCR and light microscopy for the identification of *E.m.* in dog fecal samples collected in the field. Furthermore, the LAMP amplification can take place at an isothermal temperature in a water bath or a heating block, and it requires one reaction of 40 min compared with PCR which generally requires two hours, or more, involving denaturation, annealing and extension reaction steps. Overall, therefore, the LAMP assay is simpler and faster than the PCR method and is an approach that has been applied successfully for the detection of a range of viral, bacterial, fungal, and parasitic infections [12, 16-19, 26-35].

Therefore, considering it is less expensive and more rapid than traditional PCR methods, LAMP is an attractive, alternative diagnostic tool for use in resource-poor countries, where parasites are

prevalent and facilities are relatively undeveloped [33, 34]. Furthermore, if the amplified mix is combined with SYBR Green [17, 35] or another dye such as hydroxynaphthol blue [36], the test can be immediately visualized to distinguish a positive LAMP reaction from a negative control, thereby providing ease of use in the field.

In summary, the LAMP method we have developed has significant potential for the diagnosis of *E.m.* infected canines in *Echinococcus*-endemic regions, particularly in underdeveloped countries such as China [37, 38]. It is an important new advance for early diagnosis and is a potentially useful epidemiological surveillance tool since it provides an accurate, sensitive, affordable, and easy-to-use method and a practical alternative to PCR for the routine diagnosis of *E.m.* infection in dogs, foxes and other canines.

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Competing interests: The authors have declared that no competing interests exist.

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Author Contributions

Conceived and designed the experiments: XWN DPM HBY JFY ZZL YRY WZJ. Performed the experiments: XWN HBY JFY ZZL HML WZJ. Analyzed the data: XWN DPM HBY YRY WZJ. Contributed reagents/ materials/ analysis tools: XWN HBY ZZL HML LL MTL JZC YLF YDZ BQF YRY WZJ. Wrote the paper: XWN DPM YRY WZJ.

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Figure Legends

Figure 1. Establishment of optimized incubation conditions for the LAMP assay

(**A: Left Panel**) Amplification of LAMP of f-DNA and restriction digestion of LAMP products at 63℃ for 40 min): Lane M, DNA marker; 1, LAMP products of f-DNA; 2, water control; 3, *Eco*RⅠ digestion of LAMP products. (**B: Right-top Panel**) Specificity of LAMP assay for *E. multilocularis* DNA: Lane M, DNA marker; Lanes 1 and 2, g-DNA from *E. multilocularis* larvae and f-DNA; Lanes 3 to 9, g-DNA of *E. granulosus* (G1 strain), *E. shiquicus*, *T hydatigena*, *T. pisiformis*, *T. taeniaeformis*, *T. multiceps*, *D. caninum* parasites; Lane 10, g-DNA from dog intestinal tissues; Lane 11, no f-DNA; Lane 12, water control. (**C: Right-bottom Panel**) Sensitivity of LAMP method using different numbers of *E. multilocularis* eggs per gram of feces: Lanes: M, DNA marker; Lanes 1 to 5, one to five eggs; Lane 6, ten eggs; Lane 7, fifteen eggs; Lane 8, twenty eggs; Lane 9, negative control.

Tables

Table 1. Nucleotide sequences of the LAMP primers (Licensed Patent No. ZL201110346474.8) targeting the mt *nad*5 gene

Primer name	Sequence (5' ⟶ 3')
FIP	TTAACCAACCAATAACAACCCAGT*gaattc*GTGGTGTTAGTTAT TTGGTTAGG
BIP	ATGTGACGTTTGGTGTGGTAGTTA*gaattc*AAGAACCACCAAAA TAATGTCT
F3	GTGTGTTGCTATATTGCTTGT
B3	AACTTTAACAACATACACCTAGT

Note: The lower case italicized *gaattc* in the primers FIP and BIP shows the position of the introduced *Eco*RI restriction site.

Table 2. Comparison of the earliest day when fecal samples of *E.m.*-experimentally infected dogs tested positive with the LAMP and PCR methods and by microscopy

Method	Earliest day for positivity			Mean of earliest day for positivity	*P value
	Dog 1	Dog 2	Dog 3		
LAMP	12	12	13	12.3	0.03[a]
PCR	17	17	18	17.3	0.0001[b]
Microscopy	42	44	46	44	0.0001[c]

Note: a, LAMP versus PCR; b, LAMP versus microscopy; c, PCR versus microscopy.

* Statistical analysis used one-way ANOVA with post hoc LSD tests.

Table 3: Number of field collected dog fecal samples shown to be positive or negative by the LAMP assay, PCR method and microscopy

Number of samples	Assay outcomes		
	LAMP	PCR	Microscopy
5	Positive	Positive	Positive
5	Positive	Positive	Negative
5	Positive	Negative	Positive
16	Positive	Negative	Negative
158	Negative	Negative	Negative
Total 189	*31 positive samples	10 positive samples	10 positive samples
	158 negative samples	197 negative samples	197 negative samples

* The LAMP assay exhibited a significantly higher level of sensitivity than either the PCR method or microscopy ($P<0.001$; Pearson chi-square test).

Figure
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