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Parasitology

Development of LAMP assays for the molecular detection of taeniid infection in canine in Tibetan rural area

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ABSTRACT. For field-identification of taeniid cestodes in canine animals in Tibetan area, loop-mediated isothermal amplification (LAMP) assays for *Echinococcus multilocularis*, *E. shiquicus*, *Taenia hydatigena*, *T. multiceps*, *T. pisiformis* and *T. crassiceps* were developed and evaluated along with the reported assay for *E. granulosus*. The LAMP assays showed specific reaction with their corresponding target species DNA with the detection limit of 1 to 10 *p*g. Moreover, the assays for *E. granulosus*, *E. multilocularis*, *T. hydatigena* and *T. multiceps* could detect DNA extracted from 3 or more eggs of their corresponding target species. Then, the LAMP assays were applied on samples containing 3 to 35 taeniid eggs obtained from 61 field-collected canine feces in Qinghai, and the result was compared with a reported multiplex PCR and sequence analysis. The LAMP assays and the PCR detected single species DNA of *E. granulosus*, *E. shiquicus*, *T. hydatigena* and *T. multiceps* in 5, 2, 44 and 2 samples, respectively. In the rest 8 samples, DNA of both *E. granulosus* and *T. hydatigena* were detected by the PCR but the LAMP assays detected those DNAs in 2 samples and only *T. hydatigena* DNA in 6 samples. It was assumed that less than 3 *E. granulosus* eggs were mixed in the samples although the samples contained 21 to 27 eggs in total. In conclusion, the LAMP assays were less sensitive than the multiplex PCR, but would have adequate sensitivity for field use in Tibetan area.

KEY WORDS: canine, China, diagnosis, taeniid cestodes, zoonosis

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Several species of canine taeniid cestodes are sympatrically epidemic in Tibetan area, China. The area was reported as one of the most endemic areas of *Echinococcus* spp. in the world [6, 13, 14, 31, 36, 37]. The prevalence of cystic echinococcosis caused by *E. granulosus* and alveolar echinococcosis caused by *E. multilocularis* in herdsmen reached to even 5.5 and 8.5%, respectively, in some counties of Qinghai province [13]. Besides, *E. shiquicus* that is maintained by Tibetan foxes (*Vulpes ferrilata*) and pikas has been reported in this area [35], although the zoonotic status of this species is still unknown [3]. *Taenia multiceps* and *T. hydatigena* were also highly endemic in the area, where around 50% of sheep and 15% of yaks were infected with *T. hydatigena* and 5% of

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Table 1. Specimen used for developing LAMP assays

Species	Country	Host	Development stage
E. granulosus	Mauritania	Camel	Larva
E. granulosus	Uruguay	Sheep	Larva
E. granulosus	Mauritania	Camel	Larva
E. granulosus	Argentina	Sheep	Larva
E. granulosus	China	Dog	Egg
E. multilocularis	Japan	Human	Larva
E. multilocularis	France	Human	Larva
E. multilocularis	Japan	Dog	Egg
E. shiquicus	China	Fox	Adult
E. shiquicus	China	Fox	Adult
E. shiquicus	China	Fox	Adult
T. crassiceps	Japan	Gerbil	Larva
T. crassiceps	Switzerland	Gerbil	Larva
T. pisiformis	Japan	Dog	Adult
T. hydatigena	China	Dog	Adult
T. hydatigena	China	Dog	Adult
T. hydatigena	China	Fox	Adult
T .hydatigena	China	Dog	Egg
T. multiceps	China	Sheep	Larva
T. multiceps	China	Sheep	Larva
T. multiceps	China	Dog	Egg
T. taeniaeformis	France	Water vole	Larva
T. taeniaeformis	France	Water vole	Larva
Dipylidium caninum	Korea	Dog	Adult
D. caninum	Uruguay	Dog	Adult

All samples were collected from different individual host.

sheep with *T. multiceps* in some counties [34]. The former may cause a serious damage to the liver of lambs with heavy infection and the latter is the well-known causative pathogen of neurocoenurosis in sheep. Moreover, *T. pisiformis* and *T. crassiceps* have been found in red foxes (*Vulpes vulpes*) [17].

The eggs of those taeniid cestodes are excreted in the feces of canine animals, such as dogs, red foxes and Tibetan foxes, and contaminate the nomadic environment of herdsmen. Since funding and facilities are not adequate in Tibetan rural area, it is important to have an on-site diagnostic tool that does not require expensive equipment in order to evaluate the prevalence of the individual taeniid species in canine animals and conduct local control programs for the pathogenic taeniid species in this area.

Microscopic examination of feces is a classical method for the detection of eggs excreted in feces of the definitive hosts, however, the eggs of taeniid species cannot be differentiated due to their morphological similarity [9]. Coproantigen detection methods based on sandwich ELISA have been also developed for taeniid species. However, the diagnostic reliability was impaired by cross-reactions among the taeniid species [7, 8, 10, 29]. Furthermore, polymerase chain reaction (PCR)-based techniques have been developed for the detection of taeniid cestodes [3–5, 20]. However, the requirement of sophisticated equipment and relatively long time for test completion is disadvantage in application [25].

While, loop-mediated isothermal amplification (LAMP) technique that can be conducted at constant temperature without using a thermal cycler would be a specific and sensitive diagnostic method for detecting target DNA [27], and amplification can be confirmed by the formation of white precipitate of a side product, magnesium pyrophosphate, in the reaction mixture [21, 22]. Because of the practical and economical advantages, LAMP assays have been widely used for the detection of different microorganisms, especially pathogens [15, 18, 21, 25, 33].

In this study, we developed a LAMP assay-based on-site species-diagnostic tool for canine animals infected with taeniid cestodes of *E. multilocularis*, *E. shiquicus*, *T. hydatigena*, *T. multiceps*, *T. pisiformis* and *T. crassiceps* that are prevalent in Tibetan area. Along with a reported LAMP assay for *E. granulosus* [25], the LAMP assay system composed of 7 LAMP assays was applied in field samples collected from Tibetan rural area and the reliability of the system was evaluated.

MATERIALS AND METHODS

Parasites materials

The parasite materials used for developing an assay system were listed in Table 1. A part of the materials were the same used in the previous work [1]. Besides, two metacestodes of *T. multiceps* were collected from two sheep at a slaughterhouse in Haiyan County, Qinghai province, China. Adult worms of *T. hydatigena* and *E. shiquicus* were provided from the Zoonosis Laboratory at Qinghai University. Species of the adult or larvae samples were identified either by their morphology or partial DNA sequence

of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene [11]. Eggs of *T. hydatigena*, *T. multiceps* and *E. granulosus* were isolated from feces of farm dogs in Xinghai County, Qinghai province, China. Species of eggs were identified by a reported multiplex PCR assay that can detect *E. granulosus*, *E. multilocularis* and *Taenia* spp. from one egg DNA [32]. Species of *Taenia* was identified by sequencing and BLASTN analysis of the amplified product of *Taenia*-specific primers. Eggs of *E. multilocularis* were collected from an experimentally infected dog in Hokkaido Institute of Public Health, Japan.

DNA extraction

Genomic DNAs of the samples were extracted by QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacture's instruction. The extracted DNA were eluted in 50 μl TE buffer and stored at −30°C until use. The DNA concentration of each sample was measured with Qubit™ Fluorometer (Invitrogen, Carlsbad, CA, U.S.A.). The DNAs were used for determining the specificity and sensitivity of LAMP assays, and used as control in field evaluation.

Design of LAMP primers

The partial sequences of mitochondrial *cox1* gene or NADH dehydrogenase subunit 1 (*nad1*) gene of the target species prevalent in Tibetan area were retrieved from GenBank. Then species-specific LAMP primer sets for the target parasite species were designed using the software PrimerExplorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html). Finally, matching validity of each primer set was evaluated *in silico* with other sequences registered in GenBank (Table 2). Information of primer sets and their optimized amplification temperatures are also shown in Table 2. For *E. granulosus*, a reported primer set and temperature [25] were used in the following experiments.

LAMP assay

LAMP reaction mixture was consisted of 12.5 μl of 2 × reaction buffer (40 mM Tris-HCl pH 8.8, 20 mM KCl, 16 mM $MgSO_4$, 20 mM (NH_4) SO_4, 0.2% Tween-20, 1.6 M Betaine, 2.8 mM each of dNTP), 1.3 μl of primer sets (20 pmol of FIP and BIP primers and 2.5 pmol of F3 and B3 primers), 8 units of *Bst* DNA polymerase (large fragment) (New England Biolabs, Inc., Ipiwich, MA, U.S.A.), 1 μl of DNA extracted from cestodes adult or larva or 5 μl of DNA from taeniid eggs, and DDW to a final volume of 25 μl. The reaction mixture was incubated for 90 min at 60°C for *E. multilocularis* and *T. crassiceps*, 61°C for *T. pisiformis*, 62°C for *E. shiquicus* and *T. hydatigena* or 64°C for *T. multiceps*, followed by heating at 85°C for 10 min to inactivate the enzyme. The LAMP assays were performed using LA-500 Loopamp™ real-time turbidimeter (Eiken Chemical Co., Ltd., Tokyo, Japan). The threshold time that is the required time for the turbidity to exceed the threshold value (0.1) was recorded for each sample by the device.

Specificity test of the LAMP assays

To verify the specific reaction of the designed species-specific LAMP assays, each assay was performed with genomic DNA (0.2 to 10 *n*g) of the cestodes listed in Table 1. The assays were performed in duplicate, and DW was used as template for checking the formation of primer dimmers.

Sensitivity test of the LAMP assays

Sensitivity of the assays was determined using DNA of the corresponding target parasites with 10-fold serial amount of 0.1 to 100 *p*g. The assays were performed in duplicate.

In addition, in order to know how many eggs are required for the detection of egg DNA at minimum, the assays were performed with DNA extracted from 1, 2, 3 and 5 eggs of *T. hydatigena*, *T. multiceps*, *E. granulosus* and *E. multilocularis*. The assays were performed in duplicate. One, 2, 3 and 5 eggs were picked up from the cover slides under a stereo-microscope after processing the fecal samples by sucrose centrifugal flotation technique, and DNA was extracted using QIAamp DNA Mini Kit. For the samples of *T. hydatigena*, *T. multiceps* and *E. granulosus* whose eggs were isolated from field dogs, we performed a reported multiplex PCR assay [32] to confirm each sample contained only one species DNA. For the samples showing a specific band for *Taenia* spp., a chromatogram of direct sequence data of the amplified products was examined for wave overlapping due to the existence of different nucleotides.

Evaluation of LAMP assay system with field samples

From 2014 to 2016, 481 canine fecal samples were collected in Xinghai county. Feces were collected in the nomadic grassland and in the defecation area of tethered dogs. For the field-collected feces, their origin animals were identified following the protocol described by Nonaka *et al*. [26]. Briefly, fecal DNA was extracted from the surface washing of the frozen feces using QIAamp Stool Mini Kit (Qiagen). Then PCR was conducted on the DNA with the primers prL (5′-CACCATTAGCACCCAAAGCT-3′) and prH (5′-CCTGAAGTAGGAACCAGATG-3′) to amplify a part of the D-loop region present in all carnivores. Feces samples were stored at −80°C for at least 10 days to inactive the *Echinococcus* eggs. Then, one gram of feces was examined by the sucrose centrifugal flotation technique with specific gravity 1.27.

A LAMP assay system for identifying 7 taeniid species prevalent in Tibetan area was designed by testing a sample simultaneously by a set of the 7 species-specific LAMP assays. For field evaluation, 3 to 35 taeniid eggs were recovered from the flotation solution and concentrated in 1.5 m*l* tubes. DNA was extracted from the eggs using QIAamp DNA mini kit and applied to the LAMP assay system and the multiplex PCR.

Table 2. Designed LAMP primer sets and amplification temperature of LAMP assays

Species	Target gene	Sequences[a)] used to design primers	Primer	Sequence (5′→3′)	Temperature (°C)	Sequences[a)] used to evaluate primers	No. of mismatches
E. shiquicus	*cox1*	JF906137	F3	TGCTATGTTTTCTATAGTGTGTT	62	JF906138−146	0
			B3	ACACTATAAAAGAAACAACCCA			
			FIP	ACAGAACTAAAAAAAAACAGCCGTCTGAAGGAGAGTTTGAGGTCA			
			BIP	AGGAGTTCCAACTGGTATAAAGGTGCCACAAAACAGGATCACTC			
E. multilocularis	*cox1*	AB461417	F3	GCGTTTGGGTTTTATGGTT	60	AB461412−416, AB461418−420, AB477010−012, KC550007, KC550004, AB374425, AB385610	0–1
			B3	ACCACAAAATAGGATCACTCTT			
			FIP	CATCCAACCCAACAGTAAACATATGGTTTTCTATAGTGTGTTTAGGGA			
			BIP	GTTACGATGATTATAGGTGTTCCGACTAGAATTAAGCAACATATACAACC			
T. hydatigena	*cox1*	JN831296	F3	TTTTGTTGTTAGTTACTCTTCCT	62	JN831297−314, GQ228819	0–3
			B3	TCAGGACTCATACTTATTCTCAA			
			FIP	CTCCACCACCTAATGGATCAAAATTACAATGCTTTTGTTTGATCGT			
			BIP	TTCAGCATATGTTTTGGTTCTTTGGGACTAATAATTCCAAATCCAGGA			
T. multiceps	*nad1*	KC794809	F3	TTGTAGTTATAGTTTGGTGGATT	64	KC794810, KC794811, GQ228818	0–3
			B3	AAAACCATTCCTAACCATGAA			
			FIP	CAAACGGAGTACGATTAGTTTCACAGGTTGTCGTTTATGGTTTTTCC			
			BIP	GGTGAGGCTGAAAGCGAGTTACTCACACGCAAACAAGCA			
T. crassiceps	*cox1*	EU544546	F3	GAATTGTGATTCTTTTGGTT	60	EU544547−550, KF751222, KF751223, NC002547	0–1
			B3	CATATACAATCAAGTAAACACC			
			FIP	TGATGACCCCAAACACTCCTAATGGATTGTTATTTGCTATGTTC			
			BIP	ACGGTTGGTTTAGATGTTAAGACTGTTTATACCTGTAGGTACTCC			
T. pisiformis	*cox1*	JX677964	F3	CAGGGTTTGGTATAATTAGTCAT	61	JX677965−975, JN870125, JN870126, KC020690−698, KC020700, KC020701, KC020709, KC020710, NC013844, GU569096	0–1
			B3	AGCATATAAAGTCATGTAAAGACC			
			FIP	GACCTCATACACTTCTACCTAAACAAAAGTATGTGTTCAGATGCGT			
			BIP	ACTGTTGGATTAGATGTAAAGACCGATTCCAGTAGGTACTCCAATT			

a) Accesion number in GenBank.

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Table 3. Mean threshold time (min:sec) of the LAMP assays performed with different amount of template DNA of target species

LAMP assay for	Template DNA amount (*p*g)			
	100	10	1	0.1
E. granulosus	62:54	70:22	ND[a)]	ND
E. multilocularis	63:21	70:36	78:42	ND
E. shiquicus	46:27	64:42	ND	ND
T. hydatigena	49:21	60:15	ND	ND
T. multiceps	46:21	57:42	73:09	ND
T. pisiformis	56:00	64:33	ND	ND
T. crassiceps	65:24	69:43	ND	ND

a) Amplification was not detected by the Turbidimeter.

Table 4. Detection of DNA extracted from different numbers of taeniid eggs by LAMP assays

LAMP assay for	No. of eggs for DNA extraction			
	5	3	2	1
E. granulosus	4/4[a)]	4/4	3/4	1/4
T. hydatigena	4/4	4/4	3/4	2/4
T. multiceps	4/4	4/4	4/4	3/4
E. multilocularis	4/4	4/4	4/4	1/4

a) No. of positive samples / No. of examined samples.

RESULTS

Validity of the LAMP primers in silico

When the matching validity of the developed primers was evaluated with various sequences registered at GenBank, the developed 4 primers for each species were completely matched with some of the sequences. However, 1 to 3 internal mismatches were also found with other sequences depending on the target species (Table 2).

Sensitivity and specificity of the LAMP assays

When the designed LAMP assays were performed with a serial dilution of DNA of their corresponding target species, the LAMP assays for *E. granulosus*, *T. crassiceps*, *T. pisiforms*, *E. shiquicus* and *T. hydatigena* could detect DNA up to 10 *p*g, and those for *E. multilocularis* and *T. multiceps* up to 1 *p*g (Table 3). Threshold time was apparently elongated as the amount of template DNA decreased.

While, no products were produced when the LAMP assays were performed with DNA of non-target cestodes. Primer dimers were not either formed.

Detecting taeniid eggs by LAMP assays

By the multiplex PCR and chromatogram analysis, it was confirmed that all samples of *T. hydatigena*, *T. multiceps* and *E. granulosus* contained only one species DNA. When LAMP assays for *T. hydatigena*, *T. multiceps*, *E. granulosus* and *E. multilocularis* were performed on DNA extracted from eggs of their target species, the assays could constantly detect DNA extracted from 2 eggs of *T. multiceps* and *E. multilocularis*, and 3 eggs of *T. hydatigena* and *E. granulosus* (Table 4).

Identification of feces origin animals and fecal examination

In the survey, 123 feces were collected at the nomadic field and 358 feces were collected in the defecation area of the tethered dogs. DNA analysis of the 123 field-collected feces revealed that 3 were from dogs, 22 from Tibetan foxes, 25 from red foxes, and 1 from wolf (*Canis lupus*). Feces origin of the rest 72 feces could not be identified. Taeniid eggs were found in 81 feces of tethered dogs with the intensity from 1 to more than 1,000 eggs per gram of feces. Of the 51 origin-identified feces, taeniid eggs were found in feces of 1 dog, 5 Tibetan foxes and 1 red fox with the intensity from 1 to 63 eggs per gram of feces.

Species identification of taeniid eggs isolated from field samples

From feces of 58 tethered dogs and 3 Tibetan foxes, the following number of taeniid eggs were isolated and DNA was extracted: 3 to 9 eggs from 17 dogs and 3 foxes, 10 to 19 eggs from 18 dogs, and 20 to 35 eggs from 23 dogs. While less than 3 eggs were recovered from the other 27 samples, thus they were not used for the evaluation.

When the DNA was amplified by the LAMP assay system and multiplex PCR, single species of DNA was detected by both methods in 53 samples in which DNA of *E. granulosus*, *E. shiquicus*, *T. hydatigena* and *T. multiceps* was detected in 5, 2, 44 and 2 samples, respectively. In the rest 8 samples, DNA of both *E. granulosus* and *T. hydatigena* were detected by the PCR, but the LAMP assay system detected those DNAs in 2 samples and only *T. hydatigena* DNA in 6 samples, although the 6 samples contained 21 to 27 eggs in total (Table 5).

DISCUSSION

In the present study, each LAMP assay was constructed with 4 primers (F3, B3, FIP and BIP). For ideal LAMP assay, two extra primers calling loop primers that target the loop-formed regions would be necessary for securing the specificity and sensitivity of LAMP assays and for accelerating the LAMP reaction [23]. Actually, we first tried to design loop primers, however, we could not design functional primers that cleared the recommended conditions for melting temperature and GC content by the program.

Table 5. Comparison of results of species identification by LAMP assay and multiplex PCR

Detection method	No. examined	Species identified				
		E. granulosus & *T. hydatigena*	*E. granulosus*	*T. hydatigena*	*E. shiquicus*	*T. multiceps*
Multiplex PCR	61	8	5	44	2	2
LAMP assays	61	2	5	50	2	2

Accordingly, we used only 4 primers that are essential and minimal requirement for LAMP assay.

When the matching validity of the developed primers was evaluated *in silico*, the primers did not match perfectly with some haplotypes registered in GenBank. However, all the mismatches were internal ones and the number of mismatches was within 1–3 bases. It was reported that the primers were effective for the haplotypes with two to three internal mismatches and ineffective when there were four to six mismatches in LAMP assay [28]. The validity of the developed primers was also confirmed in our study that the primers developed for 6 species could produce the LAMP products with all target specimens listed in Table 1.

The developed LAMP assays showed specific amplification for their target species. The detection limit of the LAMP assays for the target species DNA (1–10 *p*g) was in agreement with the previous LAMP protocols performed without loop primers, in which the detection limit of the assays were 10 *p*g of *E. granulosus* genomic DNA [25]. The minimum number of eggs required for the developed assays for egg DNA (2–3 eggs) was also in agreement with the previous study in which the assay could detect DNA of five eggs of *T. asiatica* and *T. saginata* [24].

In surveys on definitive hosts of taeniid cestodes using field-collected feces, parasite DNA has been obtained either from eggs isolated from animal feces [26, 32] or directly from feces (copro-DNA) [3, 4]. In this study, the LAMP assays were applied to the DNA that was extracted from eggs isolated from fecal samples following the microscopic fecal examination. We have been using fecal egg examination technique as a primer diagnostic tool of field-collected feces for evaluating the parasite prevalence in Qinghai province [12, 17] and using a molecular technique to further differentiate the parasite species. This is because parasitological examination can provide the direct evidence of parasite infection and also screen the samples for the application of a molecular technique, thus we could reduce the cost for examination. While, a copro-DNA detection technique has an advantage of detecting prepatent infection and potentially patent period infection without egg excretion [16, 19], however, it requires higher cost for testing all samples by a molecular method and the consideration for removing PCR inhibitors in feces [3, 19] and the potential of detecting DNA from consumed diet [2] and of other organism in feces by cross reaction [19].

The developed LAMP assays required for a minimum of 2 to 3 taeniid eggs to produce the products. In this study, egg DNA was eluted in 50 μl of TE buffer, and then 5μl of eluted DNA was applied to the assay as template DNA. Since a taeniid egg was reported to contain 8 *p*g of DNA [4, 30], the amount of template DNA of the samples containing 2 to 3 eggs was calculated to be 1.6 or 2.4 *p*g. Therefore, this is consistent with our finding of the detection limit of the assays (1 to 10 *p*g).

A multiplex PCR assay used in this study has been reported to amplify the DNA of *E. multilocularis*, *E. granulosus* and *Taenia* spp. with different product sizes [32]. However, they did not evaluate *E. shiquicus* DNA by the assay. In order to apply the assay in Qinghai, we evaluated *E. shiquicus* DNA by the assay and found that the amplification product of *E. shiquicus* was the same size as that of *Taenia* spp. (data not shown). Therefore, *E. shiquicus* could be differentiated by the sequence analysis of the amplification product with *Taenia* genus-specific primers.

In the field evaluation, we evaluated the field reliability of the developed LAMP assays by comparing the results with those of the reported multiplex PCR [32]. Discrepancy in the results were detected in 6 samples in which both *E. granulosus* and *T. hydatigena* DNA were detected by the multiplex PCR, however, only *T. hydatigena* DNA were detected by the LAMP system. Because 3 eggs are required for the LAMP assays to detect *E. granulosus* egg DNA while only 1 egg for the multiplex PCR [32], the reason was supposed that less than 3 *E. granulosus* eggs were included in the 6 samples even though the samples contained 21 to 27 eggs in total.

Although, we could not evaluate the minimum number of eggs required for the LAMP assays for *E. shiquicus*, *T. crassiceps* and *T. pisiformis* due to a lack of samples, those assays showed a similar detection limit of DNA with other assays (Table 3). Therefore, it could be expected that those assays require the similar number of eggs for producing the products.

In conclusion, the LAMP assay system showed less sensitivity than the multiplex PCR. However, the sensitivity disadvantage could be overcome by examining more number of eggs. Considering the advantages of LAMP method, the developed system covering the 7 taeniid species prevalent in Qinghai province could be a useful on-site species-diagnostic tool for taeniid infection in Tibetan area.

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