Table 1 Summary of clinical, parasitological, serological and molecular data of 26 people diagnosed as suffering from paragonimiasis.

Patient code	Age/gender	Eggs in sputum	Serology		Copro-DNA	Clinical signs and symptoms/history of eating crabs				
			ELISA	IB		Cough	Headache	Epilepsy	Chest pain	Crabs eaten
B07	5/M	−	+	+	−	+*	+	−	+	+
B08	6/F	−	+	+	−	+	+	−	+	+
ET56	8/F	+	+	+	−	+*	−	−	−	+
T76	8/F	+	+	+	+	+*	−	−	−	+
T85	11/M	−	+	+	ND	+	−	−	+	+
T86	8/F	+	+	+	+	+*	+	−	+	+
T92	12/M	+	+	+	+	+*	+	−	+	+
T95	9/F	−	+	+	ND	+	−	−	−	+
8	14/F	+	+	+	ND	+*	+	−	+	+
10	8/F	+	+	+	ND	+	+	−	+	+
12	24/F	+	+	+	ND	+*	+	−	−	+
14	23/F	+	+	+	ND	+	+	−	+	+
16	13/M	+	+	+	ND	+*	−	−	+	+
39	5/F	+	+	+	ND	+	+	−	+	+
46	6/F	+	+	+	ND	+	−	−	+	+
49	7/M	+	+	+	ND	+	−	−	+	+
E35	10/M	−	+	+	ND	+	+	−	−	+
ET60	10/M	−	+	+	ND	+	+	−	−	+
ET62	11/F	−	+	+	ND	+	+	−	−	+
ET73	12/F	−	+	+	ND	+	+	−	−	+
72	9/M	−	+	+	ND	+	+	+	+	+
31	8/F	+	−	+	ND	+	+	−	−	+
65	12/M	+	+	+	ND	+	−	−	+	+
66	10/M	−	+	+	ND	+	+	−	+	+
61	12/F	+	+	+	ND	+	+	−	+	+
E20	18/M	+	+	+	+	+*	+	−	+	+

IB: immunoblot; +: positive; −: negative; +*: cough with haemoptysis; ND: not done. Note: A further 16 faecal samples from egg-negative individuals tested by copro-PCR are not included in this table.

2.4. Morphological observation of the lung flukes

Adult lung flukes recovered from experimentally infected cats were originally preserved in RNAlater (Qiagen, Hilden, Germany) for RNA preparation. Some were later stained with acetocarmine for morphological observation.

2.5. Molecular identification and phylogenetic analysis of the lung flukes

DNA was extracted from adult worms using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The internal transcribed spacer 2 region (ITS2) of rDNA was amplified by PCR using a forward primer 5′-CGGTGGATCACTCGGCTCGT-3′ (3S) and a reverse primer 5′-CCTGGTTAGTTTCTTTTCCTCCGC-3′ (A28).[16,17] A portion of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) was also amplified using a forward primer 5′-TGGTTTTTTGTGCATCCTGAGGTTTA-3′ (pr-a) and a reverse primer 5′-AGAAAGAACGTAATGAAAATGAGCAAC-3′ (pr-b).[18] DNA sequencing was carried out using an ABI PRISM BigDye Terminator v 3.1 Cycle Sequencing Ready Reaction Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

DNA sequence data were aligned using the CLUSTAL W computer program.[19] Pairwise evolutionary distances were computed by Kimura's two-parameter method,[20] and the phylogenetic tree was constructed by the neighbour-joining method[21] implemented in MEGA version 3.1.[22]

2.6. Detection of *Paragonimus* DNA in faeces of paragonimiasis patients

DNA was extracted from 23 human faecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany): five (codes ET56, T76, T86, T92, E20 in Table 1) were from individuals who were both sputum egg-positive and seropositive and 18 were from egg-negative individuals. Among the egg-negative samples, two (B07, B08 in Table 1) and 16 (not included in Table 1) were seropositive and seronegative, respectively. PCR amplification and DNA sequencing of ITS2 and *cox1* genes were performed according to the above-mentioned methods.

2.7. Serodiagnosis

Crude antigens from *P. africanus*, *P. westermani* and *P. miyazakii* were used for both ELISA and immunoblots. ELISA was performed on 168 samples, as reported in previous studies on paragonimiasis[12,13] with slight modifications. Briefly, 96-well microtitre plates (Maxisorp; Nunc, Roskilde,

Denmark) were coated with each of the crude antigens at a concentration of 5 μg/ml protein in PBS and incubated at 4 °C overnight. The microtitre plates were probed with 1:200-diluted human serum samples in blocking buffer. Peroxidase-conjugated rec-Protein G (Zymed, San Francisco, USA) diluted in 1:4000 was added into each well. Peroxidase activity was revealed by adding 0.4 mmol/l 2,2-azino-bis 3 ethylbenz-thiazoline-6-sulphonic acid in 0.1 mol/l sodium citrate buffer, pH 4.7, containing 0.003% H_2O_2 at room temperature (RT). The optical density (OD) was monitored at 405 nm on a microplate reader (ImmunoMini, model NJ-2300; Nalgene Nunc International, Tokyo, Japan). The cut-off value was calculated for each antigen based on the means of pooled serum samples from healthy donors.

Immunoblot assay was performed (on 64 samples, including all samples positive or borderline by ELISA and samples lying just below the ELISA cut-off line) as described previously[13,23] with modifications. The antigens (80 μg/mini slab gel) from *P. africanus*, *P. westermani* and *P. miyazakii* were separated on 15% polyacrylamide gels containing 0.4% SDS and transferred onto an Immobilon-P transfer membrane (pore size 0.45 μm, Millipore, Tokyo, Japan) overnight. The membrane was cut into strips and probed with 1:50-diluted human serum in blocking buffer at RT for 1 h. The strips were incubated for 1 h in 1:1000-diluted peroxidase-conjugated rec-Protein G. The substrate, 4-chloro-1-naphthol (Nacalai Tesque, Tokyo, Japan) was added for colour development. The reaction was stopped with excess distilled water.

In order to evaluate the specificity of *P. africanus* antigens, ELISA and immunoblot were performed using serum samples from patients with other helminthic infections described above.[15]

3. Results

3.1. Morphological and molecular identification of the *Paragonimus* species used for antigen preparation

The morphology of immature and mature lung flukes obtained from experimentally infected cats was observed using acetocarmine-stained specimens. It was difficult to confirm critical morphological differences between *P. africanus* and other species due to poor initial preservation of the specimens in RNAlater. However, five uterine eggs from five adult worms averaged about 90 μm in length (data not shown), a size typical of *P. africanus*.

Unambiguous sequences of 445 bp spanning the ITS2 region were obtained from three adult worms. Two individuals had identical sequences (GenBank accession no. AB298779) that differed at two nucleotide positions from the third sequence (GenBank accession no. AB298780). The last was identical to the ITS2 region from *P. africanus* (D. Blair et al., unpublished data). The phylogenetic tree of *Paragonimus* spp. (including *Euparagonimus*), inferred from ITS2 sequences showed that our newly acquired sequences clustered unambiguously with *P. africanus* (data not shown). Unambiguous sequences of 391 bp of the *cox1* gene were also obtained from adult worms. Again, two individuals had identical sequences, but the third was slightly different (99.3% similarity) (not yet submitted to GenBank). All differences in the *cox1* sequences were synonymous substitutions, supporting the view that all came from the same species.

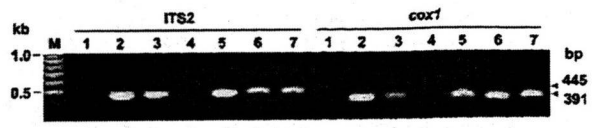

Figure 1 PCR-amplification of the ITS2 region (445 bp) and partial *cox1* gene (391 bp) from *Paragonimus* adult worms and from human faecal materials from Cameroon. M: 100 bp DNA ladder marker; lane 1: negative control without template DNA; lane 2: DNA from adult *P. africanus* from an experimentally infected cat; lanes 3–7 (E20, ET56, T92, T86 and T76): copro-DNA from sputum egg-positive patients (Table 1). An additional 18 samples from sputum egg-negative individuals, including two seropositive and 16 seronegative individuals, were PCR-negative (not shown).

3.2. Molecular diagnosis

Figure 1 shows the results obtained by PCR using copro-DNA. The ITS2 region and partial *cox1* gene could be amplified and sequenced from only four of the five samples from seropositive individuals with eggs in their sputum (Figure 1, lanes 3, 5, 6 and 7). The ITS2 sequences of all four were identical with AB298779 and the *cox1* sequences with the two identical sequences obtained from adult worms. The faecal sample of one serologically positive individual with eggs in the sputum failed to yield amplifiable copro-DNA (Figure 1, lane 4 and Table 1, code ET56).

3.3. Serological, parasitological and clinical evaluation

Figure 2 illustrates ELISA results using *P. africanus* antigens. Out of 168 serum samples examined, 25 (14.8%) were seropositive, and a further four were regarded as borderline. An additional sample lying just below the cut-off line gave

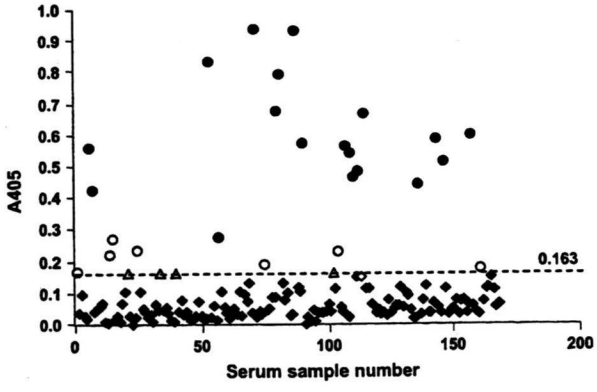

Figure 2 ELISA using *Paragonimus africanus* antigens. The broken line denotes the cut-off value. ●: strong positive reactions with the major bands of 33 and 35 kDa in immunoblot; ○: weak positive reactions with 16–18 or 27 kDa bands in immunoblot; □: ELISA-negative but weak positive reactions in immunoblot (patient 31 in Table 1); ■: ELISA- and immunoblot-negative; △: samples on the cut-off line for ELISA.

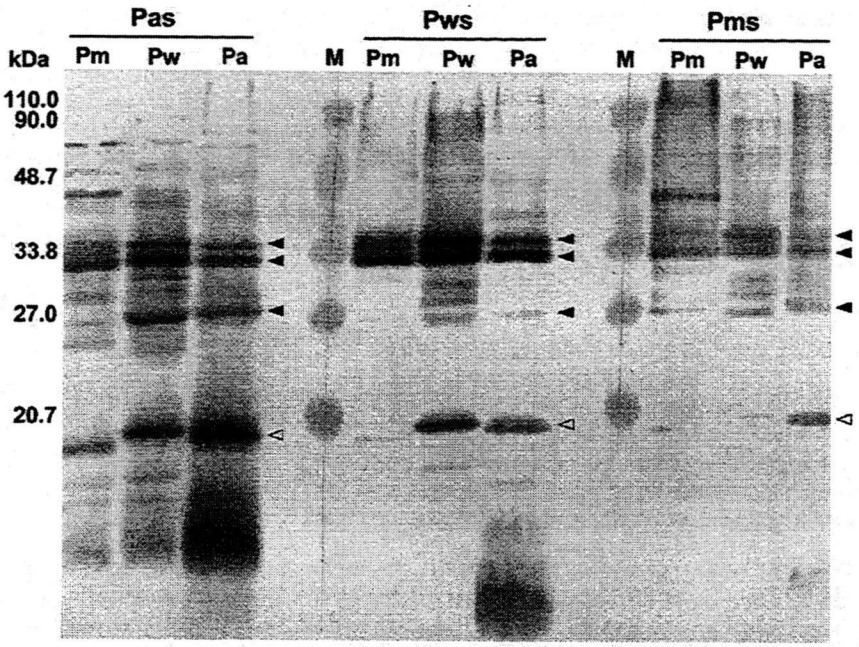

Figure 3 Immunoblot results using antigens from three *Paragonimus* species and serum sample from paragonimiasis patients. Pas: serum sample from a Cameroonian patient infected with *P. africanus*; Pws: serum sample from a *P. westermani*-infected patient from Japan; Pms: serum sample from a *P. miyazakii*-infected patient from Japan; Pm: *P. miyazakii* antigens; Pw: *P. westermani* antigens; Pa: *P. africanus* antigens. Closed arrow heads indicate 35, 33, and 27 kDa specific bands for paragonimiasis, whereas an open arrow head denotes low-molecular specific bands (16–18 kDa). Molecular size markers (M) are shown on the left side.

a positive result in the immunoblot test. Figure 3 shows immunoblot patterns of *P. africanus*, *P. westermani* and *P. miyazakii* antigens with serum samples from confirmed single-infection cases. All the sera cross-reacted with the antigens of the three species, although the serum of *P. miyazakii* cases appeared to be relatively weakly immunoreactive. The antigens from Japanese *P. westermani* and *P. miyazakii* provided similar results in ELISA and immunoblot with the Cameroon serum samples. Similarly, serum samples from confirmed *P. westermani* and *P. miyazakii* cases in Japan showed very similar results in both ELISA and immunoblot using crude antigens of *P. africanus* (data not shown). The antigens from the three species showed basically similar banding patterns, with particularly pronounced bands having molecular weights of approximately 35, 33, 27 and 16–18 kDa (Figure 3). The immunoblot assay using *P. africanus* antigens was carried out to confirm the results of ELISA (Supplementary Figure 1). In total, 64 samples were examined by immunoblot, but Supplementary Figure 1 illustrates all 25 ELISA-positive samples and five samples on the ELISA cut-off line (triangle and open square in Figure 2) only. Positive immunoblot results included 18 samples responding strongly to both the 33 and 35 kDa components and eight samples giving a weak response only to other components (Supplementary Figure 1, lanes a–z). Seventeen of the 18 strongly positive samples had OD values higher than 0.4 in the ELISA test. Seven of the samples weakly positive by ELISA (open circles in Figure 2) and one sample falling below the cut-off value (open square in Figure 2) were weakly positive by immunoblot, reacting only with the 16–18 kDa band. Samples from 10 sputum egg-negative individuals and from all egg-positive individuals were immunoblot-positive (Table 1). The specificity of *P. africanus* antigens was evaluated by ELISA and immunoblot with serum samples from patients with other helminth infections. Sera from a patient with sparganosis and a patient with fascioliasis cross-reacted weakly with *P. africanus* antigens by ELISA, but not by immunoblot (data not shown).

The parasitological, clinical and serological data of the people diagnosed as suffering from paragonimiasis are summarized in Table 1. The people enrolled in this study (78 males and 90 females) suffered from cough (80.3%), haemoptysis (11.3%), chest pain (47.6%), epilepsy (8.3%) and headache (63.0%). They ranged in age from 4 to 78 years (15.2 ± 8.2 years in males and 12.9 ± 5.9 years in females). All the immunoblot-positive patients had histories of eating raw or undercooked crabs and were suffering from cough (Table 1). No *Paragonimus* eggs were detected microscopically in any faecal samples using the formalin-ether concentration method. According to these results, 26 (15.5%) people were diagnosed as positive for paragonimiasis.

Paragonimiasis cases were confirmed from all four villages. Seroprevalence differed greatly among the four villages: 25.0% (7/28, Bulutu), 7.7% (4/52, Ebonji), 16.4% (9/55, Etam) and 18.2% (6/33, Teke).

4. Discussion

We reported the results of a survey for paragonimiasis in South West Province, Cameroon, and the identity of

the causative species based on molecular sequences. Of 168 persons with appropriate symptoms and a history of eating raw or undercooked crabs, 26 (15.5%) were seropositive for paragonimiasis, all but one both by ELISA and immunoblot. Among these seropositive individuals 16 were found to have eggs in their sputum. Four (of five tested) of these were positive for copro-DNA using PCR. It is known that *P. africanus*, *P. uterobilateralis*, *P. westermani*-like and *Euparagonimus* spp. occur in Cameroon.[24,25] However, *P. africanus* is the only species confirmed from humans in Cameroon so far,[7] and our molecular analyses are in agreement with that.

Serology had much higher sensitivity for diagnosis of African paragonimiasis (26 positive) than did examination of sputum for eggs (16 positive). This is in agreement with findings from other parts of the world.[13,26] Examination of faeces for eggs is the least sensitive method.[11] Our examination failed to detect eggs in any of the faecal samples, although the copro-DNA approach did detect DNA from eggs or other products of lungworms in four of five individuals with eggs in their sputum. Failure in this one case could be because some people do not swallow sputum. Similar methods to detect copro-DNA have been developed for schistosomiasis[27] and cestode infections.[28,29] Intapan and others (2005) have demonstrated that the approach is effective in diagnosing *P. heterotremus* in cats. We speculate that PCR using DNA from sputum might prove to be an ideal tool for detection of pulmonary paragonimiasis cases, as sputum might contain products of developing lung flukes that might allow detection of prepatent cases.

The antigens of three *Paragonimus* species reacted with serum samples from Cameroon and from Japan, exhibiting very similar sensitivity and specificity by ELISA and immunoblot. The three species showed similar immunoblot patterns, with four major bands. Among them, both 33 and 35 kDa bands were recognized most strongly by sera showing OD values higher than 0.4 by ELISA. Similar 33 and 35 kDa proteins in crude antigens of *P. westermani* adults were shown to elicit the most specific and strongest reactions with IgG4 of paragonimiasis patients from the early stage to chronic infection.[13] Clearly, it is difficult to differentiate the *Paragonimus* species by immunoblot. The immunodominant antigens with utility for serodiagnosis of paragonimiasis appear to be genus-specific.[13]

To our knowledge, this is the first report confirming paragonimiasis in humans using a copro-DNA test. This approach should be evaluated further using more faecal samples and highly specific and sensitive primers.[30] Serology, the copro-DNA test and detection of eggs in sputum (or faeces) are essential for differentiation of paragonimiasis from other diseases. Of the 168 symptomatic people we evaluated, 26 were confirmed as paragonimiasis cases. The seronegative people should be followed-up to exclude TB or other diseases. A previous study conducted in the same area failed to find any TB cases among those enrolled.[7] Individual no. 72 in Table 1 is interesting, as he was seropositive but eggs were not found in his sputum. His reported history of epilepsy might indicate a case of cerebral rather than pulmonary paragonimiasis. Further studies using modern tools are necessary to understand the epidemiology of paragonimiasis in Cameroon and other African countries.

Authors' contributions: RMS and AI conceived and designed the study; AN, MO, HY and MN carried out mitochondrial analysis; AKM, EE, PE and RMS carried out epidemiological studies, collection of the specimens and experimental infections in Cameroon; AN, YS and AI carried out serology; MO, KN and TS carried out morphological analysis; AN, MO, DB and TA carried out molecular phylogenetic analysis; AN, MO, DB and AI drafted the manuscript. All authors read and approved the final manuscript. RMS and AI are guarantors of the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.trstmh.2008.09.011.

Funding: The Leadership in Science and Technology in Asia from Ministry of Education, Culture, Sports, Science and Technology, Japan; International Collaboration Research Fund from the Japan Society for the Promotion of Science (JSPS) (14256001, 17256002) and JSPS-Asia/Africa Science Platform Fund to AI.

Conflicts of interest: None declared.

Ethical approval: The National Ethics Committee of Cameroon, Yaounde, Cameroon.

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JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2009, p. 168–174 Vol. 47, No. 1
0095-1137/09/$08.00+0 doi:10.1128/JCM.01573-08

Loop-Mediated Isothermal Amplification Method for Differentiation and Rapid Detection of *Taenia* Species[▽]

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Received 13 August 2008/Returned for modification 14 October 2008/Accepted 6 November 2008

Rapid detection and differentiation of *Taenia* species are required for the control and prevention of taeniasis and cysticercosis in areas where these diseases are endemic. Because of the lower sensitivity and specificity of the conventional diagnosis based on microscopical examination, molecular tools are more reliable for differential diagnosis of these diseases. In this study, we developed and evaluated a loop-mediated isothermal amplification (LAMP) assay for differential diagnosis of infections with *Taenia* species with cathepsin L-like cysteine peptidase (clp) and cytochrome *c* oxidase subunit 1 (cox1) genes. LAMP with primer sets to the cox1 gene could differentiate between three species, and LAMP with primer sets to the clp gene could differentiate *Taenia solium* from *Taenia saginata*/*Taenia asiatica*. Restriction enzyme digestion of the LAMP products from primer set Tsag-clp allowed the differentiation of *Taenia saginata* from *Taenia asiatica*. We demonstrated the high specificity of LAMP by testing known parasite DNA samples extracted from proglottids (n = 100) and cysticerci (n = 68). LAMP could detect one copy of the target gene or five eggs of *T. asiatica* and *T. saginata* per gram of feces, showing sensitivity similar to that of PCR methods. Furthermore, LAMP could detect parasite DNA in all taeniid egg-positive fecal samples (n = 6). Due to the rapid, simple, specific, and sensitive detection of *Taenia* species, the LAMP assays are valuable tools which might be easily applicable for the control and prevention of taeniasis and cysticercosis in countries where these diseases are endemic.

Cestode parasites *Taenia solium*, *Taenia saginata*, and *Taenia asiatica* are the causative agents of taeniasis. Although taeniasis is relatively innocuous, cysticercosis caused by *T. solium* larvae is one of the most severe diseases in humans and remains a complicated health problem in many areas around the world, especially in developing countries (4, 15). Therefore, differentiation of *Taenia* species becomes significant for epidemiological studies and for control of these diseases. Furthermore, it is expected that there are much wider areas in the Asia-Pacific region where the three taeniid species occur sympatrically (3, 5, 23, 24). Diagnosis is mainly performed by microscopic observation of eggs in feces and/or by comparative morphology of proglottids or scolices, but these methods lack both sensitivity and specificity. In order to overcome the lower sensitivity of microscopic diagnosis, various immunological or molecular approaches, including coproantigen and copro-DNA detection methods, have been developed (2, 6, 12, 18, 25). The coproantigen detection method has been shown to be more sensitive than the microscopy method, but it cannot differentiate between *Taenia* species because of it is genus specific, not species specific (2). By contrast, various copro-DNA detection methods using PCR have been developed for sensitive differential detection of taeniid cestodes (6, 12, 18, 25). Although these techniques provide sensitive and reliable diagnostic results, it is not easy to exploit in the laboratories of developing countries where these diseases are endemic, because PCR requires sophisticated equipment, such as a thermal cycler. Furthermore, *Taq* DNA polymerase is often inactivated by inhibitors present in biological samples, which sometimes cause problems for sensitivity and reproducibility (1, 13).

Recently, a novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) has been developed (17). LAMP employs a DNA polymerase with strand displacement activity and four primers that recognize six sequences on the target DNA. This method amplifies DNA with high specificity, sensitivity, and rapidity under isothermal conditions. Since LAMP is done under isothermal conditions (60 to 65°C), simple incubators, such as a water bath or a block heater, are sufficient for DNA amplification (17). Moreover, a large amount of white precipitate of magnesium pyrophosphate is produced as a byproduct, which enables the visual judgment of amplification by a naked eye (14). Hence, LAMP is a highly sensitive and specific DNA amplification tool suitable for the rapid diagnosis of infectious diseases, including parasitic diseases (9, 20, 22), in a well-equipped laboratory and/or small-scale clinical laboratories and is expected to be highly useful and feasible in the field.

In the present study, we developed and evaluated the LAMP assay with a cathepsin L-like cysteine peptidase (clp) gene of nuclear DNA and a cytochrome *c* oxidase subunit 1 (cox1) gene of mitochondrial DNA for differentiation between and rapid diagnosis of infection with *Taenia* species.

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▽ Published ahead of print on 12 November 2008.

TABLE 1. *Taenia* species DNA samples used in this study

Taenia species (no. of samples)	Locality	Developmental stage (no. of samples analyzed)
T. saginata (78)	Cambodia	Proglottid (3)
	China	Proglottid (5)
		Cysticercus (1)
	Indonesia	Proglottid (36)
		Cysticercus (12)[a]
	Nepal	Proglottid (2)
	Thailand	Proglottid (9)
	Cameroon	Proglottid (1)
	Ethiopia	Proglottid (2)
	Brazil	Cysticercus (2)
		Proglottid (1)
	Ecuador	Proglottid (1)
	Belgium	Proglottid (2)
		Cysticercus (1)[a]
T. asiatica (43)	China	Cysticercus (1)[a]
		Proglottid (10)
	Indonesia	Cysticercus (2)
		Cysticercus (16)[a]
	Korea	Cysticercus (2)[a]
	Taiwan	Cysticercus (1)[a]
		Proglottid (1)
	Thailand	Proglottid (6)
	Philippines	Proglottid (4)
T. solium (47)	China	Cysticercus (2)
		Proglottid (11)
	India	Cysticercus (11)
	Indonesia	Proglottid (1)
	Nepal	Cysticercus (5)
	Thailand	Proglottid (5)
	Vietnam	Cysticercus (1)
	Cameroon	Cysticercus (3)
	Mozambique	Cysticercus (2)
	South Africa	Cysticercus (1)
	Tanzania	Cysticercus (1)
	Brazil	Cysticercus (1)
	Ecuador	Cysticercus (2)
	Mexico	Cysticercus (1)

[a] The cysticercus was developed in NOD/shi-*scid* mice.

MATERIALS AND METHODS

Parasite materials. Cysticerci of *T. solium*, *T. saginata*, or *T. asiatica* were obtained from nonobese diabetic/severe combined immunodeficiency (NOD/shi-*scid*) mice (7, 16) infected by intraperitoneal cavity injection with oncospheres prepared from gravid proglottids of each parasite isolated in Thailand. Genomic DNA was extracted from one cysticercus by using a DNeasy tissue kit (Qiagen, Hilden, Germany).

DNA samples. For evaluation of the LAMP assay, a total of 168 DNA samples extracted from proglottids (n = 100) and cysticerci (n = 68), including 47 *T. solium* samples, 78 *T. saginata* samples, and 43 *T. asiatica* samples, were examined (Table 1). We used the stored DNA samples previously analyzed by multiplex PCR (25).

Cloning and sequencing of clp genes. The clp gene of each *Taenia* parasite was cloned by PCR with forward primer 5′-ACATTTTCGTTTCGATCGGTCAT G-3′ and reverse primer 5′-TGAACACATGGTTTAAACGTATGG-3′. Primers used were designed from conserved nucleotide sequences between *Echinococcus multilocularis* (21) and *T. solium* (10) clp genes to amplify almost the entire gene region. PCR was carried out using high-fidelity polymerase PrimeStar (Takara, Kyoto, Japan) in a final volume of 25 μl reaction mixture containing 0.2 μM of each primer, 200 μM each of deoxynucleoside triphosphate (dNTP), 0.625 units of PrimeStar DNA polymerase, and genomic DNA, as described above. Amplification was performed with 35 cycles of 94°C for 30 s, 60°C for 15 s, and 72°C for 4 min, followed by a final extension at 72°C for 10 min. A single band of approximately 3 kbp was excised from agarose gels by using a NucleoSpin ExTract kit (Macherey-Nagel, Düren, Germany) according to the instruction manual and was cloned into a pGEM-T vector (Promega, Madison, WI) after the addition of adenine to the ends of the PCR products. The plasmid clone was sequenced on an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA) with BigDye Terminator version 1.1 (AB Applied Biosystems) and was used as a standard plasmid for determining the specificity and sensitivity of LAMP.

Preparation of standard plasmids for cox1 genes. The cox1 gene of each *Taenia* parasite was amplified by PCR using forward primer 5′-ATGAATGTC AAATATTTGT-TAAGTT-3′ and reverse primer 5′-CTAAAAGACCATTTC ACACGCGAAT-3′ for *T. solium*, forward primer 5′-ATGAGTGTTAAATTT TTATTAAGTT-3′ and reverse primer 5′-TTAAACTAAAAAACCACGGGC AGGC-3′ for *T. saginata*, and forward primer 5′-ATGAGTGTTAAATTTTTA TTAAGTT-3′ and reverse primer 5′-TTAAACTAAAAAACCACGAGCAAA C-3′ for *T. asiatica*. PCR was carried out as described above, except the annealing temperature was at 58°C and the elongation time was 90 s. The amplified products of each *Taenia* species were cloned into a pGEM-T vector and used as a standard plasmid after being confirmed by sequencing.

LAMP primers. LAMP primers were designed using PrimerExplorer V4 software (http://primerexplorer.jp/). The following four oligonucleotide primers were specifically designed to amplify six distinct regions on the target gene: forward inner primer (FIP), backward inner primer (BIP), and two outer primers, F3 and B3 (Table 2). FIP consists of the sense sequence of F2 at the 3′ end and the F1c region at the 5′ end that is complementary to the F1 region. BIP consists of a B2 region at the 3′ end that is complementary to the B2c region and the same sequence as the B1c region at the 5′ end (Fig. 1A and 2A).

LAMP reaction. LAMP was carried out in a 25-μl volume of reaction mixture containing 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3, an 8-U large fragment of *Bst* DNA polymerase (New England Biolabs, Beverly, MA), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine (Sigma, St. Louis, MO), and 1.4 mM each of dNTP and the template DNA. The reaction mixture was incubated for 60 min at 63°C for the clp gene and at 60°C for the cox1 gene and heated at 80°C for 5 min. The LAMP products from primer set Tsag-clp were digested with the HinfI restriction enzyme (New England Biolabs) for 2 h at 37°C. The LAMP products and restriction enzyme-digested products were electrophoresed on a 2.0% agarose gel and detected by staining with ethidium bromide.

Sensitivity analyses. The sensitivities of the LAMP and PCR methods were assessed using a standard plasmid diluted from 10^4 copies/reaction to 1. LAMP was performed as previously mentioned. PCR was carried out by using F3 and B3 primers of each LAMP primer set. PCRs were performed in a 25-μl volume of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 μM of each primer, 0.2 mM each of dNTP, diluted standard plasmid, and 0.5 units of *Taq* DNA polymerase (ExTaq; Takara), and cycling conditions were 30 s at 94°C (first cycle, 2 min at 94°C), 30 s at 60°C (clp gene) and 58°C (cox1 gene), and 30 s at 72°C for 30 cycles. The PCR products were electrophoresed on a 2.0% agarose gel and detected by staining with ethidium bromide.

DNA extraction from fecal samples. In order to evaluate the detection limit of taeniid DNA in fecal samples, 1 gram of feces from a noninfected volunteer was mixed with 5, 10, 20, 30, 40, or 50 *T. asiatica* or *T. saginata* eggs, and DNA was extracted by using a QIAamp DNA stool mini kit (Qiagen) after egg disruption treatment with glass beads (19). In addition, taeniid egg-positive or -negative fecal samples obtained in Indonesia and Thailand were used to assess LAMP, and DNA samples were extracted by the same procedure. LAMP was performed as previously described except that the reaction was performed for 90 min.

Nucleotide sequence accession numbers. The nucleotide sequence data of the clp genes cloned in this study are available in the GenBank, EMBL, and DDBJ databases under accession numbers AB441815 (*T. solium*), AB441816 (*T. saginata*), and AB441817 (*T. asiatica*).

RESULTS AND DISCUSSION

The standard plasmid of each gene was constructed to facilitate initial evaluation and optimization of LAMP. The LAMP products are detected as a ladder of multiple bands on the gel due to the formation of a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures, with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (17). Application of the LAMP assay with each specific primer set to each

TABLE 2. LAMP primer sets

Species	Primer set	Primer type[a]	Sequence (5' to 3')	Target
T. saginata	Tsag-cox1	F3	TCGGCAAATATTTAATTCCTTTG	cox1 gene
		B3	AAATTCTAGACGCACCCG	
		FIP	GCCATCGAAGGAATCAATAACCAATCTGATTTGAATTTACCCCG	
		BIP	GACTTTTTATCCGCCTTTGTCGTCAATGCAACGAAAACATCAAGA	
T. asiatica	Tasi-cox1	F3	GATTTTCTTTTTTTTGATGCCCA	
		B3	TTGAAAATAATGACGACGACA	
		FIP	AGGCAAGTTTAAATCAGATAACCCATTTTGATAGGTGGTTTTGGTAA	
		BIP	GTGGTTGTTGATTCCTTCAATAGTTTAAGGCGGATAAAAAGTTCAC	
T. solium	Tsol-cox1	F3	CCTATTTTAATTGGAGGTTTTGG	
		B3	CTACCCCACTTCCTCTTGA	
		FIP	CAACCATGCACTTAAAGCATTCAAATTCCATTGATAAGAGGATTATCGG	
		BIP	GGATGTGTTTAGGCGCTGGTACAACGAAGATGATAAAGGTG	
T. saginata	Tsag-clp	F3	GGAAGTCAAAAATCAGGTGAG	clp gene
T. asiatica		B3	CGCTGATAGCTAGGCTAAC	
		FIP	CTCAGTCCCACCCAATCCATTTCAAATCTTCATTATGCTGCGTTAC	
		BIP	GCACCGTGTCATTGGTAAATTTGGTGGAGCTTACTAAGCTCTATCG	
T. solium	Tsol-clp	F3	GAGGTCAAAAATCAGGTGAGAT	
		B3	AATGCTCCTGACTTGGTT	
		FIP	AGGTGCTTTCACAATAGTCCCCTGCGTCATAGGTCTTGC	
		BIP	TAGTCGTTGCTTCGATAGAGCTCGCTGATATCTAGGCTAATGCTG	

[a] FIP consists of the sense sequence of F2 at the 3' end and the F1c region at the 5' end that is complementary to the F1 region. BIP consists of a B2 region at the 3' end that is complementary to the B2c region and the same sequence as the B1c region at the 5' end.

Taenia cox1 gene resulted in successful amplification of the target gene from the respective parasite genomic DNA (Fig. 1B). On the other hand, because of high nucleotide sequence similarity between the clp genes of *T. saginata* and *T. asiatica*, we could not design a specific primer set to differentiate between these two *Taenia* species but could design specific primer sets Tsol-clp and Tsag-clp to differentiate *T. solium* from *T. saginata* and *T. asiatica* and differentiate *T. saginata/T. asiatica* from *T. solium*, respectively. However, the recognition site GAGTC for the restriction enzyme HinfI in the amplified region of the *T. asiatica* clp gene (Fig. 2A) enables us to differentiate *T. asiatica* from *T. saginata*. In this case, restriction enzyme digestion of the LAMP products from *T. asiatica* genomic DNA with primer set Tsag-clp produces three bands with the predicted sizes of 179, 217, and 255 bp, because, unlike the PCR products, the LAMP products are characteristic structures with inverted repeats of the target sequence. In fact, three bands that agreed with the predicted size in the restriction enzyme digestion of the LAMP products from *T. asiatica* genomic DNA with primer set Tsag-clp were detected (Fig. 2B, lane 6).

Analytical specificity of the LAMP assays. In order to evaluate the specificity of the LAMP assays, the known parasite DNA samples prepared from proglottids and cysticerci were examined. Because LAMP requires four primers that recognize six different sequences on a target sequence, the target sequence specificity of the LAMP reaction appears to be high. Indeed, LAMP with primer sets to the cox1 or clp gene specifically amplified each respective target gene, with a species-specific detection. The results obtained by LAMP with cox1 primer sets were consistent with those obtained by multiplex PCR with the cox1 gene, whereas two DNA samples extracted from proglottids, which were identified as being *T. saginata* by both LAMP and multiplex PCR with cox1 primer sets, were detected as *T. asiatica* by LAMP with clp primer sets (Table 3). The size of bands produced by restriction enzyme digestion indicated that these two LAMP products were specifically derived from the *T. asiatica* clp gene (data not shown). After cloning of the clp gene from these two DNA samples by PCR with primer set Tsag-clp F3 and B3 and sequencing, we found a single nucleotide substitution, T to C, as indicated in Fig. 2A, which leads to the appearance of the HinfI recognition site in the region between B1c and B2 and is identical to the nucleotide substitution in *T. asiatica*. Thus, the nucleotide substitution in the clp gene of these two *T. saginata* samples may lead to the different result from the diagnosis based on the cox1 gene. Alternatively, the possibility that these two DNA samples were obtained from a hybrid parasite having *T. asiatica* nuclear DNA and *T. saginata* mitochondrial DNA could not be ruled out, because samples of these parasite materials were collected from areas where both parasites exist sympatrically in China and Thailand, respectively, and these materials were identified by only mitochondrial DNA (3, 8). Exactly two tapeworms obtained in Thailand where three human *Taenia* species were confirmed to be sympatrically occurring (3) are concluded to be the hybrids of *T. saginata* and *T. asiatica* (M. Okamoto, M. Nakao, M. T. Anantaphruti, J. Waikagul, and A. Ito, unpublished data). More-detailed characterizations of these two DNA samples must be performed using another nuclear DNA marker, in addition to analyses of longer nucleotide sequences of the clp genes. These results demonstrated that the LAMP methods with each primer set designed in this study can specifically

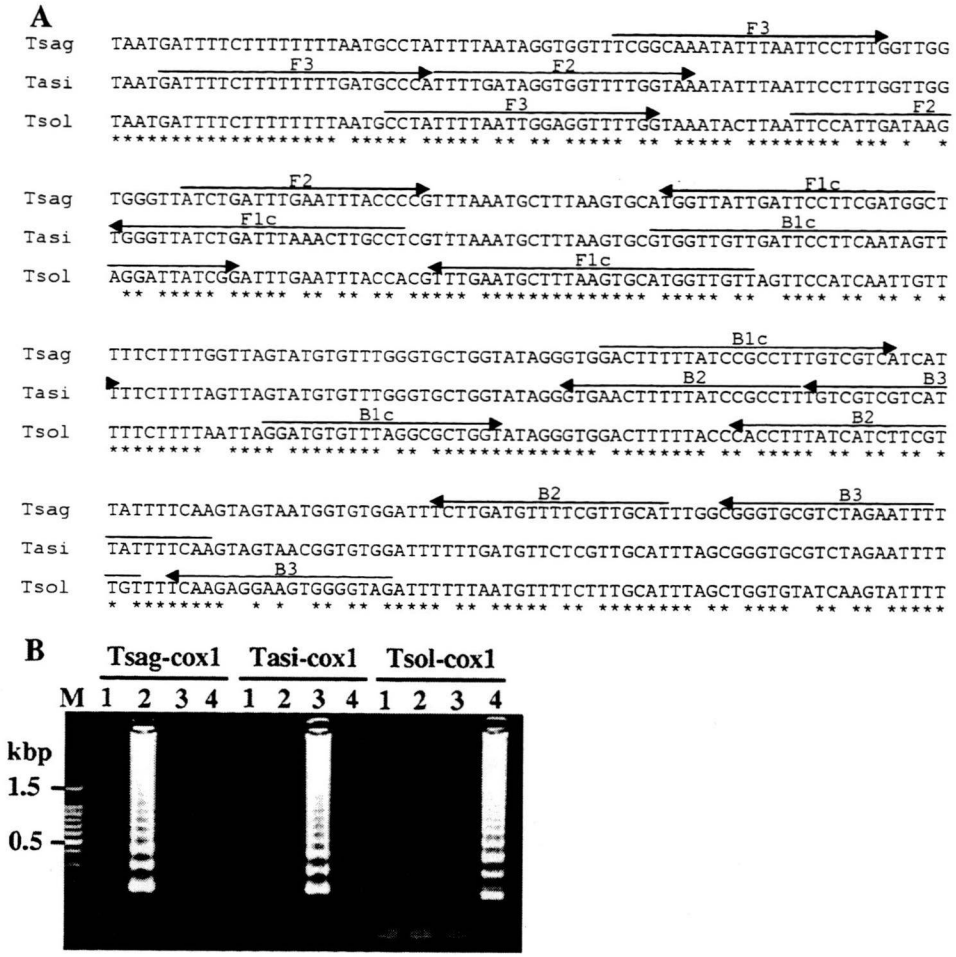

FIG. 1. Nucleotide sequence alignment of the target region of cox1 genes (A) and LAMP results (B). (A) The locations of the primer recognition sites are indicated by arrows. (B) The LAMP products were run on a 2% agarose gel. Lane M, 100-bp DNA ladder marker (Promega); lane 1, negative control; lane 2, *T. saginata* genomic DNA; lane 3, *T. asiatica* genomic DNA; lane 4, *T. solium* genomic DNA; Tsag-cox1, results of LAMP with primer set Tsag-cox1; Tasi-cox1, results of LAMP with primer set Tasi-cox1; Tsol-cox1, results of LAMP with primer set Tsol-cox1.

amplify the target gene and are applicable to a differential diagnosis of infections with *Taenia* species.

Analytical sensitivity of the LAMP assay. Tenfold serial dilution of each standard plasmid was used to determine the lower detection limits for LAMP and PCR, and the sensitivities of the two methods were compared. In this study, F3 and B3 primers of each LAMP primer set were utilized for PCR. Figure 3A shows results of LAMP and PCR with primer set Tsol-clp. Both LAMP and PCR detected up to one copy of target gene/reaction, which indicated no difference in sensitivity between the two methods. LAMP and PCR with other primer sets provided the same results as LAMP with primer set Tsol-clp (data not shown).

Next, the detection limit of taeniid eggs in feces was evaluated using *T. asiatica* and *T. saginata* eggs (Fig. 3B). At least five eggs per gram of feces was sufficient for taeniid eggs to be detected by LAMP with primer sets Tasi-cox1 and Tsag-cox1, whereas more than 10 eggs/g of feces was needed to be detected by LAMP with primer set Tsag-clp (data not shown). The differences between the cox1 gene and the clp gene in the detection sensitivity may be responsible for the number of copies of each target gene within the samples, since a large number of mitochondrial DNA exists in a cell, one feature to be selected as a target DNA for detection. Several detection methods for *Taenia* species in feces based on PCR techniques have been reported. The multiplex PCR method with mitochondrial DNA (25), the PCR-restriction fragment length polymorphism method with mitochondrial DNA (18), and the nested-PCR method with the Tso31 gene encoding the *T. solium* oncosphere-specific protein (12) have been reported to show detection limits of 5 eggs/g of feces, 17 eggs/g of feces, and 40 eggs/g of feces, respectively. It seems that more eggs are needed for detection when the nuclear gene is chosen as a diagnostic gene marker, although the amplification efficiency of each PCR method varies. In this study, *T. solium* eggs were not available, but a similar sensitivity might be expected because all LAMP primer sets could amplify one copy of the target gene when using pure plasmid DNA samples.

Differential detection of *Taenia* species in fecal samples. Furthermore, in order to assess the LAMP method for the detection of copro-DNA, we investigated fecal samples that

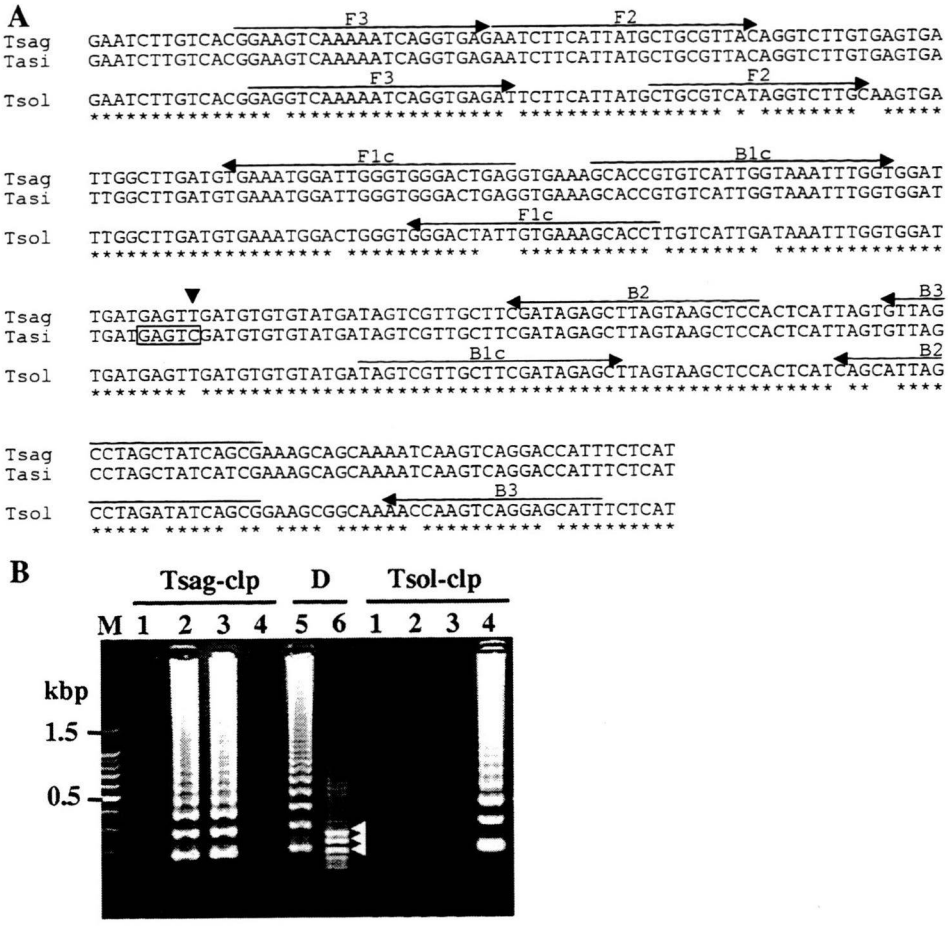

FIG. 2. Nucleotide sequence alignment of the target region of clp genes (A) and LAMP results (B). (A) The locations of the primer recognition sites are indicated by arrows and the restriction enzyme HinfI recognition site in the LAMP products of *T. asiatica* clp gene is boxed. The single nucleotide substitution, C to T, in the *T. saginata* sequence is indicated by an arrowhead. (B) The LAMP products and HinfI digestion products were run on a 2% agarose gel. Lane M, 100-bp DNA ladder marker (Promega); lane 1, negative control; lane 2, *T. saginata* genomic DNA; lane 3, *T. asiatica* genomic DNA; lane 4, *T. solium* genomic DNA; lane 5, HinfI digestion of LAMP products from *T. saginata* genomic DNA with primer set Tsag-clp; lane 6, HinfI digestion of LAMP products from *T. asiatica* genomic DNA with primer set Tsag-clp; Tsag-clp, results of LAMP with primer set Tsag-clp; D, results of HinfI digestion; Tsol-clp, results of LAMP with primer set Tsol-clp. The DNA fragments generated after digestion with HinfI are indicated by arrowheads.

were positive (n = 6) or negative (n = 10) for taeniid eggs by microscopy and collected in Indonesia and Thailand (Fig. 4). Out of six taeniid egg-positive fecal samples, three specimens from Indonesia tested positive with primer sets Tsag-cox1 (Fig. 4A, lanes 15 to 17) and Tsag-clp (data not shown), and the remaining specimens from Thailand were positive with primer sets Tasi-cox1 (Fig. 4B, lanes 12 to 14) and Tsag-clp (data not shown). No differences in detection be-

TABLE 3. Analytical specificity of the LAMP assays[a]

Taenia species (no. of samples)	No. of samples (%) detected by LAMP with indicated primer set	
	cox1	clp
T. saginata (78)	78 (100)	76 (97.4)[b]
T. asiatica (43)	43 (100)	43 (100)
T. solium (47)	47 (100)	47 (100)

[a] *Taenia* species were confirmed by multiplex PCR with cox1 genes (25).
[b] Two samples were detected as *T. asiatica* by LAMP.

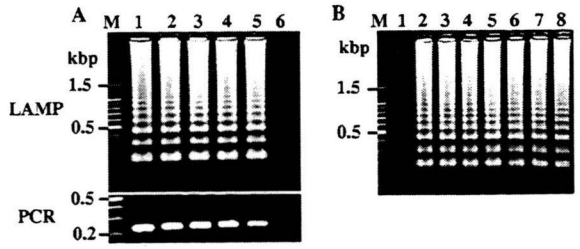

FIG. 3. (A) Comparison of detection sensitivities of LAMP and PCR with Tsol-clp primer set. The standard plasmids were serially diluted from 10^4 copies per reaction to 1 copy per reaction and amplified by LAMP (upper panel) and PCR (lower panel). The F3 and B3 primers of the Tsol-clp primer set were used in the PCR. The LAMP reactions were carried out for 60 min. Lane M, 100-bp DNA ladder marker (Promega); lanes 1 to 5 represent 10^4, 10^3, 10^2, 10, and 1 copy(ies)/reaction, respectively; lane 6, negative control. (B) Detection limits of target genes by LAMP with Tasi-cox1 primer set against DNA samples prepared from feces containing various numbers of *T. asiatica* eggs. The LAMP reactions were carried out for 90 min. Lane M, 100-bp DNA ladder marker; lane 1, negative control; lane 2, *T. asiatica* genomic DNA as a positive control; lanes 3 to 8 represent 5, 10, 20, 30, 40, and 50 *T. asiatica* eggs/g of feces, respectively.

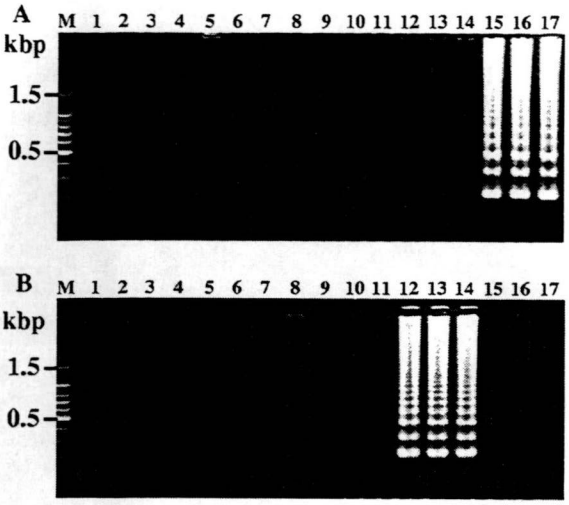

FIG. 4. Differential detection of *Taenia* species in fecal samples. Each genomic DNA extracted from taeniid egg-negative fecal samples collected in Indonesia (lanes 2 to 11) and taeniid egg-positive fecal samples collected in Thailand (lanes 12 to 14) and Indonesia (lanes 15 to 17) was examined by LAMP. The LAMP reactions were carried out for 90 min. The LAMP results with primer sets Tsag-cox1 (A) and Tasi-cox1 (B) are shown. Lane M, 100-bp DNA ladder marker (Promega); lane 1, negative control.

tween nuclear DNA and mitochondrial DNA were observed. The results obtained by LAMP were consistent with those of multiplex PCR. No positive results were observed with the taeniid egg-negative samples.

Our results demonstrated that the LAMP method has high sensitivity and specificity for differential detection of *Taenia* species. Compared to PCR, LAMP has the advantages of reaction simplicity and cost-effectiveness. LAMP does not need sophisticated and expensive equipments, and a simple water bath or a heat block is sufficient to furnish a constant temperature for reactions requiring only 1 to 2 h. Another useful feature of LAMP is that a white precipitate of magnesium pyrophosphate leading to turbidity of reaction mixtures as a byproduct of gene amplification makes it easy to distinguish positive samples from negative samples. In fact, we could discriminate between positive and negative samples by a naked eye in all cases (data not shown). Although limited numbers of clinical specimens were analyzed in the present study, it has been shown that the LAMP method has the potential for use in the differential diagnoses of infections with *Taenia* species that could be easily applicable in countries where taeniasis/cysticercosis are endemic for better control and prevention of these diseases.

ACKNOWLEDGMENTS

Fecal and/or parasite samples used for this study were obtained through joint projects on epidemiological studies of taeniasis/cysticercosis in Indonesia, Thailand, and China (3, 11, 24). We sincerely thank T. Wandra, J. Waikagul, and T. Y. Li for their great help with these samplings.

This work was supported by the leadership in science and technology in Asia from the Ministry of Education, Culture, Sports, Science and Technology, Japan; International Collaboration Research Fund from the Japan Society for the Promotion of Science (JSPS) (17256002); and JSPS-Asia/Africa Science Platform Fund to A.I.

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JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2009, p. 252–254
0095-1137/09/$08.00+0 doi:10.1128/JCM.01476-08

Vol. 47, No. 1

Development of an Immunochromatographic Test To Detect Antibodies against Recombinant Em18 for Diagnosis of Alveolar Echinococcosis[▽]

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Received 1 August 2008/Returned for modification 17 September 2008/Accepted 29 October 2008

An immunochromatographic test (ICT) for the rapid detection of antibodies to *Echinococcus multilocularis* was developed. The ICT showed a sensitivity of 94% and a specificity of 95.4%. High degrees of agreement were observed between the ICT and an enzyme-linked immunosorbent assay (κ = 0.93) and between the ICT and immunoblot analysis (κ = 0.97). It is expected that the ICT developed in this study will be useful for the serodiagnosis of alveolar echinococcosis.

Alveolar echinococcosis (AE), caused by the larval stage of *Echinococcus multilocularis*, is a serious parasitic disease of humans in countries of the higher latitudes of Northern Hemisphere. In the previous decade, a lot of new data have been published on prevalence of *E. multilocularis* in final and intermediate hosts in areas where it had previously not been recorded (5). Humans are accidentally infected with *E. multilocularis* by ingestion of eggs excreted with the feces of carnivores harboring adult tapeworm of this species. It is thought that humans become exposed to *E. multilocularis* by handling of infected definitive hosts or by ingestion of food contaminated with eggs. Oncospheres hatched from eggs in the small intestine of humans migrate via the portal system into various organs, mainly the liver, and differentiate and develop into the metacestode stage. The metacestodes propagate asexually like a tumor, leading to organ dysfunction. Since clinical symptoms usually do not become evident until 10 or more years after initial parasite infection, early diagnosis and treatment especially during asymptomatic period are important for reduction of morbidity and mortality (14). About a third of patients have cholestatic jaundice, and about a third of patients have epigastric pain. In the remaining patients, *E. multilocularis* infections are incidentally detected during medical examination for symptoms such as fatigue, weight loss, and hepatomegaly (15). At present, diagnosis of AE is primarily based on imaging techniques including echography, computed tomography, magnetic resonance imaging, and positron emission tomography with [^{18}F]fluoro-deoxyglucose (3). However, these imaging techniques are sometime limited by the small size of visualized lesions and atypical images, which are difficult to distinguish from abscesses or neoplasms. Moreover, these imaging techniques are unsuitable for diagnosis in isolated communities. Therefore, immunological tests have been considered important methods to confirm clinical findings, to give diagnostic help by providing information on the parasite in case of unclear images, or to survey in areas of endemicity where imaging techniques are not readily available (4, 9, 11). Previously, we have reported an enzyme-linked immunosorbent assay (ELISA) and an immunoblot analysis (IB) by using recombinant *E. multilocularis* 18-kDa antigen (Em18), the breakdown product of ezrin-radixin-moesin-like protein (2) that is also known as EM10 (8), EM II/3 (7), or EM4 (10) by the cysteine peptidase, and demonstrated that these two tests have a high potential for differentially diagnosing AE (1, 12, 16, 18). However, these two methods are time-consuming and require special materials and equipments, which make them not suitable for clinical applications. In contrast, an immunochromatographic test (ICT) is a simple, rapid, and reliable method for detection of specific antibodies to infectious agents. In the present study, we developed an ICT with rEm18 antigen for diagnosis of AE and compared ICT with ELISA and IB.

The rEm18 was expressed in a bacteria system as described previously (16) with some modifications. Briefly, a DNA fragment encoding the Em18 was amplified by PCR with the primers 5′-GGGAATTCAAGGAGTCTGACTTAGCGGA T-3′ and 5′-TTGGATCCTAGGGCTTCACTTTCATCATCC TG-3′. The PCR products were digested with EcoRI and BamHI and cloned into bacterial expression vector pTWIN-1 (New England Biolabs, Beverly, MA) for producing a fusion protein with chitin binding domain/mini-inteins. The cloned plasmid was transfected into *Escherichia coli* ER2566 strain and expression of the recombinant protein was induced by the addition of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to the culture. The expressed rEm18 was purified by using a chitin column (New England Biolabs) according to the manufacturer's instructions. The purified rEm18 did not have the fusion partner, because rEm18 was released by intein activity of the fusion partner itself during purifications (6). The purified rEm18 (1 mg/ml) and anti-goat immunoglobulin G (IgG) antibody (1 mg/ml) were sprayed onto a nitrocellulose membrane in a 1-mm-wide line as test and control lines, respectively. The nitrocellulose membrane with rEm18 and anti-goat IgG antibody, absorbent pad, and substrate reservoir pad were assembled on a laminated membrane card, and the assembled sheet was cut into strips 5 mm in width. The strip was placed into a plastic assay device (Mitsubishi Chemical Medience, Tokyo, Japan) with windows for applying a serum sample and

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▽ Published ahead of print on 5 November 2008.

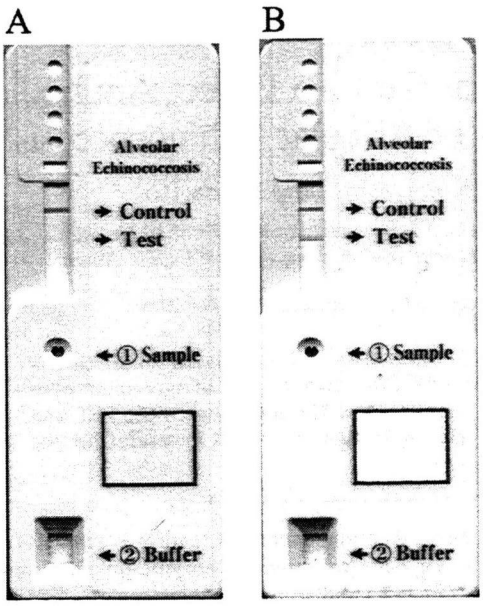

FIG. 1. Examples of ICT tests with negative and positive sera. (A) Result with a negative serum (one blue band at control line in the result window); (B) result with a positive serum (two blue bands at control and test lines in the result window). The inscriptions "Sample" and "Buffer" represents the positions for loading of sample and of substrate solution, respectively.

a substrate solution. For assay, first, 10 μl of serum sample was mixed 20 μl of a serum dilution buffer containing 0.1 mg of alkaline phosphatase-conjugated anti-human IgG antibody (Dako, Tokyo, Japan)/ml in a tube, and the mixed serum sample was applied into the sample window of the plastic device. Soon after application of the serum sample (within 30 s), 200 μl of the substrate solution was loaded onto the substrate reservoir pad, and the result was evaluated after 20 min. BCIP (5-bromo-4-chloro-3-indolylphosphate) was used for color development. As shown in Fig. 1, a sample was considered positive if two color lines corresponding to rEm18 and anti-goat IgG antibody appeared in the result window, and a sample was considered as negative if one color line corresponding to anti-goat IgG antibody appeared in the result window. In cases where there was no appearance of a colored anti-goat IgG antibody line, the assay was invalid even if a colored rEm18 line appeared. ELISA and IB were performed as described previously (16), except using the rEm18 prepared in the present study.

A total of 94 serum samples, including 50 serum samples from AE patients, 24 serum samples from cystic echinococcosis (CE) patients, and 20 serum samples from healthy persons, were examined by ICT, ELISA, and IB. Each diagnosis of AE and CE had been carried out by imaging techniques, clinical findings, histological observations (if feasible), and/or serology of IB with recombinant Em18 (16) or EmAgB8/1 (13). As shown in Table 1, 47 AE and 2 CE patient sera were determined to be positive by ICT, and none of sera from healthy persons showed positive reactions; thus, the sensitivity and specificity of ICT were 94.0 and 95.4%, respectively. There were no significant differences in sensitivity and specificity among ICT, ELISA, and IB ($P > 0.1$, Pearson chi-square test).

TABLE 1. Results of ICT, ELISA, and IB with sera from AE patients, CE patients, and healthy persons

Serum sample source	Total no. of samples examined	Samples examined by:					
		ICT		ELISA		IB	
		No. positive	%	No. positive	%	No. positive	%
AE patient	50	47	94.0	47	94.0	47	94.0
CE patient	24	2	8.3	1	4.2	3	12.5
Healthy subject	20	0	0	0	0	0	0

Two CE patient sera, determined to be positive by ELISA and/or IB, were also positive by ICT. This is not an incomprehensible result, because it is known that a few CE patient sera react to rEm18 even though rEm18 is highly specific antigen for AE (11, 12, 16, 18). These results indicated that the ICT is a sensitive and specific method for the diagnosis of *E. multilocularis* infection.

The results obtained by ICT were compared to those of previously established ELISA and IB with rEm18 (Table 2). All ELISA-positive samples, except one from AE patient, were ICT positive. Two ELISA-negative samples with the optical density values 0.068 and 0.079 close to the cutoff optical density value of 0.093 at 405 nm were ICT positive, and both were also positive by IB (data not shown). All IB-positive samples, except for one from a CE patient, were ICT positive, and none of the IB-negative samples was ICT positive. The degrees of agreement between ICT and ELISA and between ICT and IB were estimated by kappa analysis (17). A kappa statistics value of >0.75, 0.40 to 0.75, or <0.4 represents excellent agreement, good to fair agreement, and poor agreement, respectively. High degrees of agreement were observed between ICT and ELISA ($\kappa = 0.93$) and between ICT and IB ($\kappa = 0.97$), which indicated that ICT is reliable.

In conclusion, we developed a rapid, simple, sensitive, and specific ICT with rEm18 for detection of specific antibodies to *E. multilocularis* infection. Although ICT, ELISA and IB with rEm18 show similarities to each other with regard to both sensitivity and specificity, ICT has the following advantages: (i) expertise, experience, and special equipment are not required; (ii) 20-min incubation is enough to detect specific antibodies; and (iii) it is more economical than ELISA and IB. These advantages suggest a high diagnostic potential for the ICT in clinical practice in providing immediate and proper treatments and in mass-screening programs in areas of endemicity as a

TABLE 2. Comparison of ICT with ELISA and IB[a]

Samples examined by ICT	Samples examined by:					
	ELISA			IB		
	No. positive	No. negative	Total	No. positive	No. negative	Total
Positive	47	2	49	49	0	49
Negative	1	44	45	1	44	45
Total	48	46	94	50	44	94

[a] Results with a total 94 sera shown in Table 1 were used for comparisons.

primary screening tool. Further analysis on stability of ICT and a large-scale evaluation might be necessary.

This study was financially supported in part by Hokkaido Translational Research Project from the Ministry of Education of Japan on the development of the rapid test for echinococcosis (from 2007 onward) to A.I.

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