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DNA Differential Diagnosis of Taeniasis and Cysticercosis by Multiplex PCR

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Multiplex PCR was established for differential diagnosis of taeniasis and cysticercosis, including their causative agents. For identification of the parasites, multiplex PCR with cytochrome *c* oxidase subunit 1 gene yielded evident differential products unique for *Taenia saginata* and *Taenia asiatica* and for American/African and Asian genotypes of *Taenia solium* with molecular sizes of 827, 269, 720, and 984 bp, respectively. In the PCR-based detection of tapeworm carriers using fecal samples, the diagnostic markers were detected from 7 of 14 and 4 of 9 *T. solium* carriers from Guatemala and Indonesia, respectively. Test sensitivity may have been reduced by the length of time (up to 12 years) that samples were stored and/or small sample volumes (ca. 30 to 50 mg). However, the diagnostic markers were detected by nested PCR in five worm carriers from Guatemalan cases that were found to be negative by multiplex PCR. It was noteworthy that a 720 bp-diagnostic marker was detected from a *T. solium* carrier who was egg-free, implying that it is possible to detect worm carriers and treat before mature gravid proglottids are discharged. In contrast to *T. solium* carriers, 827-bp markers were detected by multiplex PCR in all *T. saginata* carriers. The application of the multiplex PCR would be useful not only for surveillance of taeniasis and cysticercosis control but also for the molecular epidemiological survey of these cestode infections.

Taenia solium, *Taenia saginata*, and *Taenia asiatica* are known as causative agents of taeniasis in humans. *T. solium* also causes cysticercosis in humans. In particular, neurocysticercosis caused by larval *T. solium* cysticerci developed in the central nervous system is the most serious disease characterized by diverse neurologic symptoms, most commonly epileptic seizure (5, 20). In contrast to cysticercosis, taeniasis is relatively innocuous, with the adult stages of these cestodes infecting the small intestine of humans and causing a few specific symptoms, such as abdominal pain and nausea (15). However, gravid proglottids filled with eggs expelled from tapeworm carriers serve as a new source of infection for intermediate hosts, particularly in developing countries where sanitary conditions are poor. Therefore, early detection and adequate treatment of taeniasis is important for the prevention of cysticercosis infections. Furthermore, reliable epidemiological information for use in the effective control of taeniasis or cysticercosis, including accurate tools for parasite identification, is needed. To date, proglottids and scolices expelled from tapeworm carriers or cysticerci collected from intermediate hosts have been identified morphologically. In Asian regions, however, *T. saginata* and *T. asiatica* are frequently confused due to their morphological similarities. Moreover, a recent study demonstrates that two distinct genotypes of *T. solium* exist, i.e., Asian and American/African genotypes (14).

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DNA differential diagnosis is considered very useful for the accurate identification of human taeniid cestode samples. In order to overcome the limitations of identification of taeniid cestodes based on the morphology, various molecular approaches have been developed, including the use of DNA probes (4, 6, 7, 9, 16, 17), PCR, or PCR coupled to restriction fragment length polymorphism (3, 8, 13, 18, 23). Such molecular approaches have been recently reviewed (11). However, most of these studies have focused on the differentiation of *T. solium* from *T. saginata* and, to date, no copro-PCR test for human taeniasis has been developed. Most recently, a new method based on the thymine bases of mitochondrial genes has been developed for comprehensive differential diagnosis of *T. saginata*, *T. asiatica*, and two genotypes of *T. solium* (22, 23). That method, although it provides precise diagnostic results, is somewhat complicated. In the present study, a more simple and reliable multiplex PCR has been established for differential diagnosis of causative agents of taeniasis and cysticercosis. In addition, the method was also assessed for specific detection of *Taenia* spp. DNA in fecal samples from tapeworm carriers.

MATERIALS AND METHODS

Parasite materials. For molecular identification of taeniid cestode parasites, a total of 57 taeniid parasite materials, including proglottids, cysticerci, and eggs, were examined (Table 1). Proglottids were obtained from tapeworm carriers. Of 26 cysticerci examined, 20 were collected from naturally infected intermediate hosts, and the remaining 6 were from nonobese diabetic/Shi-severe combined immunodeficiency (NOD/Shi-*scid*) mice as animal models for cysticercosis (10). A taeniid egg sample from Yunnan province, China was also analyzed as same as other materials. The viable eggs were prepared from gravid proglottids collected from different tapeworm carriers, and then oncospheres hatched in vitro were

TABLE 1. Taeniid cestode samples examined in the present study

Species (*n*)	Developmental stage (no. of samples examined)	Locality	Accession no.[c] for cox1	Lane no.[d]	Comment
T. saginata (13)	Cysticercus (1)	Brazil (Mato Grosso do Sul)	AB107246	1	
	Proglotiid (1)	Brazil	AB107237	2	
	Proglottid (1)	Ecuador	AB107238	3	
	Proglottid (1)	Ethiopia	AB107241	4	Expelled after treatment in Japan
	Cysticercus (1)	Belgium	AB107242	5	Developed in NOD/Shi-*scid* mice
	Proglottid (1)	Indonesia (Bali)	AB107240	6	
	Proglottid (3)	Thailand (Bangkok)	AB107244 and AB107245	7	
	Proglottid (2)	Nepal (Katumandu)	AB107243	8	
	Cysticercus (1)	China (Xinjiang)	AB066495	9	
	Proglottid (1)	China (Yunnan)	AB107247	10	
T. saginata and *T. asiatica* eggs[a]		China (Yunnan)	AB107247 and AB107235	11	
T. asiatica (13)	Cysticercus (1)	Taiwan	AB066494	12	Developed in NOD/Shi-*scid* mice
	Proglottid (1)	Taiwan	AB107234		
	Cysticercus (2)	South Korea	AB107236	13	Developed in NOD/Shi-*scid* mice
	Cysticercus (1)[b]	China (Yunnan)	AB107235	14	Developed in NOD/Shi-*scid* mice
	Proglottid (7)	China (Yunnan)	AB107234	15	
	Cysticercus (1)[b]	Indonesia (Bali)	AB107236	16	Developed in NOD/Shi-*scid* mice
T. solium (30)	Cysticercus (1)	Brazil	AB066492	17	
	Cysticercus (1)	Bolivia (Santa Cruz)	AB066491	18	
	Proglottid (1)	Peru	AB066490	19	
	Cysticercus (2)	Ecuador	AB066491	20	
	Cysticercus (1)	Mexico	AB066490	21	
	Proglottid (1)	Mexico	AB066490	22	
	Cysticercus (2)	Mozambique	AB066493	23	
	Cysticercus (1)	Tanzania	AB066493	24	
	Cysticercus (1)	Cameroon	AB066490	25	
	Cysticercus (1)	Thailand	AB066487	26	
	Cysticercus (1)[b]	Indonesia (Irian Jaya)	AB066488	27	Developed in NOD/Shi-*scid* mice
	Proglottid (1)	Indonesia (Irian Jaya)	AB066488	28	
	Cysticercus (4)	India (Panjab)	AB066489	29	
	Cysticercus (2)	India (Vellore)	AB066489		
	Proglottid (7)	China (Yunnan)	AB066486	30	
	Proglottid (1)	China (Yunnan)	AB066486	31	
	Cysticercus (2)	China (Jiling)	AB066485	32	

[a] The eggs were injected into immunodeficient mice for DNA analysis of developed cysticerci.
[b] The materials were lysed in 0.02 N NaOH at 90°C for 10 min.
[c] The numbers were deposited in DDBJ/EMBL/GenBank databases.
[d] These numbers correspond to the lanes in Fig. 1.

injected into the peritoneal cavity of NOD/Shi-*scid* mice. After 5 to 6 months, cysticerci developed in such mice were recovered and identified individually by multiplex PCR. All parasite materials were kept in absolute ethanol at −30°C for DNA preparation after collection.

Fecal samples. *T. solium*-infected fecal samples were obtained from the following sources: 14 from Guatemala were collected in 1991 and 1994 and 9 from Papua (formerly Irian Jaya), Indonesia, were collected in 2000 (Table 2). In the cases from Guatemala, all samples were from worm carriers diagnosed by copro-antigen detection where *T. solium* was identified morphologically after treatment and stored at −20 to −30°C prior to testing (2). In one sample from Bali, Indonesia, a *T. solium* carrier was suspected. One sample was from a carrier who expelled only immature (nongravid) tapeworms, including four scolices and strobillae from at least seven worms (code F13 in Table 2). The fecal samples from Papua, Indonesia, where *T. saginata* has not been detected, had been proven to be positive for *T. solium* by using copro-antigens based on diagnosis by using a commercial kit (Ag-ELISA) that utilized anti-*T. solium* antibodies (Virotech, Rüsselshein, Germany). In addition, stool samples from four volunteers infected with *T. saginata* in 2002 were also examined. The fecal samples from seven non-infected volunteers from the United Kingdom collected in 2002 were used as negative controls. These fecal samples were also stored at −20 to −30°C until used. Fecal samples from Guatemala and the United Kingdom were tested as a blind test.

DNA extraction. Mitochondrial DNA (mtDNA) from parasite materials was prepared by using DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. However, three cysticerci obtained from NOD/Shi-*scid* mice were lysed in 50 μl of 0.02 N sodium hydroxide at 90°C for 10 min, and the resulting supernatants were used directly as template DNA (Table 1). Copro-DNAs from fecal samples of tapeworm carriers were extracted by using the QIAamp DNA Stool Minikit, which requires at least 0.2 g of feces (Qiagen). Only small amounts (ca. 30 to 50 mg) in five of nine fecal samples from Papua, Indonesia, were available, and these were probably insufficient to extract copro-DNA. In addition, to evaluate the detection limit of taeniid DNA in fecal samples, a given number of *T. saginata* viable eggs (1, 2, 4, 10, 20, 200, and 2,000) were experimentally mixed with 0.2 g of stool samples from a noninfected volunteer under the microscope, and DNA derived from the eggs was extracted by using the same kit.

Multiplex PCR. Cytochrome *c* oxidase subunit 1 gene (*cox1*) was used as a target gene. Based on the nucleotide sequences of *cox1* from human taeniid cestodes, the following forward primers were designed to be amplified different sizes of products; 5′-TTGATTCCTTCGATGGCTTTTCTTTTG-3′, specific for *T. saginata* (Tsag, positions 322 to 348 from AB066495); 5′-ACGGTTGGATT AGATGTTAAGACTA-3′, specific for *T. asiatica* (Tasia, positions 880 to 904 from AB066494); 5′-GGTAGATTTTTTAATGTTTTCTTTA-3′, specific for the *T. solium* American/African genotype (Tsol/Amer, positions 429 to 453 from AB066485 to AB006489); and 5′-TTGTTATAAATTTTTGATTACTAAC-3′, specific for the *T. solium* Asian genotype (Tsol/Asia, positions 165 to 189 from AB066490 to AB066492). Species- or genotype-specific nucleotides were introduced at the 3′ end of each forward primer. Reverse primer (Rev, 5′-GACAT AACATAATGAAAATG-3′, positions 1148 to 1129) were common to all species. A PCR cocktail contained mixed primers and 0.5 U of the *Ex Taq*DNA polymerase Hot Start (TaKaRa, Tokyo, Japan) in 25 μl of a reaction mixture. Standard multiplex PCR protocols consisted of 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 60°C), and extension (90 s at 72°C), plus one cycle of 5 min at 72°C. Subsequently, PCR-amplified products were electrophoresed on 0.9 to 1.0% agarose gels. In the multiplex PCR with copro-DNA samples for

TABLE 2. Summary of multiplex PCR diagnoses for detection of tapeworm carriers

Code	Yr[a]	Status	Origin of carrier	Copro-antigen[c]	Multiplex PCR finding
F1	1994	*T. solium*	Guatemala	+[b]	–[e]
F2	1994	*T. solium*	Guatemala	+	*T. solium*[d]
F4	1994	*T. solium*	Guatemala	+	*T. solium*
F6	1994	*T. solium*	Guatemala	+	*T. solium*[d]
F7	1994	*T. solium*	Guatemala	+	*T. solium*[d]
F8	1994	*T. solium*	Guatemala	+	*T. solium*[d]
F9	1991	*T. solium*	Guatemala	+	–
F10	1994	*T. solium*	Guatemala	+	*T. solium*
F12	1994	*T. solium*	Guatemala	+	*T. solium*
F13	1994	*T. solium*	Guatemala	+	*T. solium*[d]
G1	1994	*T. solium*	Guatemala	+	*T. solium*
G6	1994	*T. solium*	Guatemala	+	*T. solium*
G7	1994	*T. solium*	Guatemala	+	*T. solium*
G8	1994	*T. solium*	Guatemala	+	*T. solium*
K03	2000	*T. solium*	Indonesia (Papua)	+	*T. solium*
K38	2000	*T. solium*	Indonesia (Papua)	+	*T. solium*
K44	2000	*T. solium*	Indonesia (Papua)	+	–
K50	2000	*T. solium*	Indonesia (Papua)	+	–
K54	2000	*T. solium*	Indonesia (Papua)	+	*T. solium*
K56	2000	*T. solium*	Indonesia (Papua)	+	–
K87	2000	*T. solium*	Indonesia (Papua)	+	*T. solium*
K90	2000	*T. solium*	Indonesia (Papua)	+	–
K10	2000	*T. solium*	Indonesia (Papua)	+	–
F3	1995	*T. solium*	Indonesia (Bali)	ND[c]	*T. saginata*
N1	2002	*T. saginata*	United Kingdom	+	*T. saginata*
N2	2002	*T. saginata*	Japan	–	*T. saginata*
N3	2002	*T. saginata*	Brazil	+	*T. saginata*
N4	2002	*T. saginata*	China	–	*T. saginata*
F5	2002	Noninfectious	United Kingdom	ND	–
F11	2002	Noninfectious	United Kingdom	ND	–
G3	2002	Noninfectious	United Kingdom	ND	–
G4	2002	Noninfectious	United Kingdom	ND	–
G9	2002	Noninfectious	United Kingdom	ND	–
G10	2002	Noninfectious	United Kingdom	ND	–
G12	2002	Noninfectious	United Kingdom	ND	–

[a] Year in which the stool samples were collected.
[b] Data from Guatemala are based on the report by Allan et al. (2).
[c] ND, not done. +, positive; –, negative.
[d] Detected by nested PCR with genotype-specific primers.
[e] –, negative.

detection of worm carriers, a volume of PCR cocktail was 50 μl to minimize the effect of inhibitory substances contained in the copro-DNA samples. Annealing was performed at 56°C. In multiplex PCR-negative samples from Guatemala, nested PCR with species- and genotype-specific primers was followed by conventional PCR with Cox1/F and Cox 1/R primers (14).

DNA sequencing and sequence analysis. *T. solium* examined in the present study had been identified previously based on the nucleotide sequences of *cox1* and cytochrome *b* gene (14, 22). In the present study, in order to confirm whether diagnostic results obtained by multiplex PCR were reliable or not, the complete nucleotide sequences of *cox1* from *T. saginata* (12 of 13 samples) and *T. asiatica* (12 of 13 samples) were determined. For this purpose, *cox1* (~1.8-kb fragment) was amplified with Cox I/F and Cox I/R primers, and samples for DNA sequencing were prepared by using an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster, Calif.) if necessary. In addition, the products amplified by multiplex and nested PCR with copro-DNA samples were also directly sequenced. In some cases, the PCR fragments were subcloned into pT7Blue T-vector (Novagen, Darmstadt, Germany) if necessary. DNA sequencing was performed on an ABI Prism 310 genetic analyzer, and the nucleotide sequence data were analyzed by using DNASTAR (version 3.75).

Nucleotide sequences. The nucleotide sequences determined in the present study have been deposited in DDBJ/EMBL/GenBank databases under accession numbers AB107237 to AB107247 (*T. saginata*) and AB107234 to AB107236 (*T. asiatica*).

RESULTS

Differentiation of the causative agents of taeniasis and cysticercosis by multiplex PCR. The amplification of diagnostic *cox1* fragments, particularly for the Asian genotype of *T. solium*, was dependent on the nucleotide sequences of the primers used. Although three forward primers (positions 165 to 189, 666 to 690, and 701 to 723) for *T. solium* Asian genotype were tested, the use of one primer (positions 165 to 189) provided the best amplification of target gene fragments (data not shown). The successful amplification of diagnostic products was also dependent on the ratio of the forward and reverse primers and the optimal ratio was 1:2:4 for Tsag and Tsol/Amer (0.2 pmol), Tasia and Tsol/Asia (0.4 pmol), and a reverse primer (0.8 pmol), respectively. Under this condition, multiplex PCR with mtDNA prepared from parasite materials yielded the most evident results. As shown in Fig. 1A, the diagnostic products with molecular sizes of 827, 269, 720, and 984 bp were amplified in *T. saginata* (lanes 1 to 10) and *T. asiatica* (lanes 12 to 16) and in the American/African (lanes 17 to 25) and Asian (lanes 26 to 32) genotypes of *T. solium*, respectively. Two diagnostic bands (827 and 269 bp) were detected when a taeniid egg sample from Yunnan province, China, was tested (Fig. 1A, lane 11). This suggested that *T. saginata* and *T. asiatica* were mixed in the original proglottid samples. In order to verify this, therefore, the taeniid oncospheres hatched in vitro were injected into NOD/Shi-*scid* mice and allowed to develop

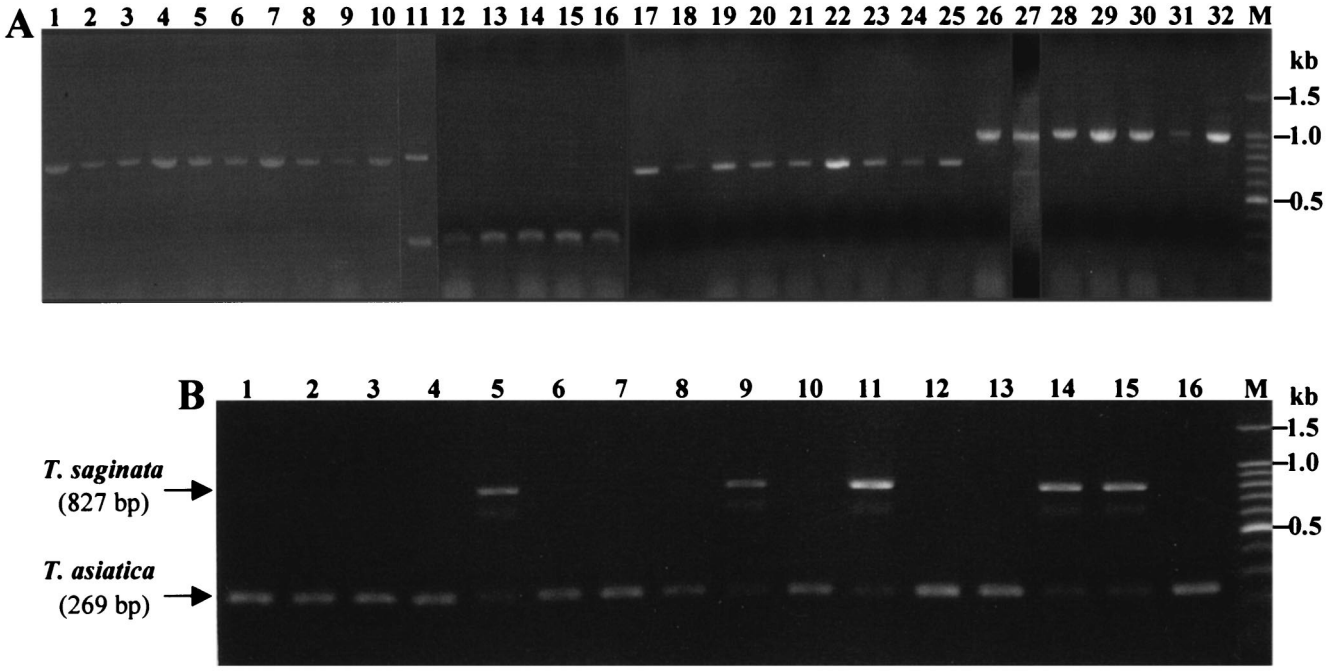

FIG. 1. (A) Multiplex PCR with mtDNA prepared from causative agents of taeniasis or cysticercosis. Lanes 1 to 10, *T. saginata*; lane 11, a mixture of *T. saginata* and *T. asiatica*; lanes 12 to 16, *T. asiatica*; lanes 17 to 25, American/African genotype of *T. solium*; lanes 26 to 32, Asian genotype of *T. solium*. The lane numbers correspond to the lanes shown in Table 1. (B) Confirmation by multiplex PCR of cysticerci developed in immunodeficiency mice. The cysticerci originated from the eggs in lane 11 of panel A. Lanes 1 to 4, 6 to 8, 10, 12, 13, and 16, *T. asiatica*; lanes 5, 9, 11, 14, and 15, *T. saginata*. M, DNA size markers (100-bp ladder [Promega]).

in such mice. As shown in Fig. 1B, multiplex PCR with individual mtDNA prepared from a total 28 cysticerci recovered from two mice has been proven that *T. saginata* and *T. asiatica* were included in the original sample. Of 28 cysticerci, 12 and 16 were determined to be *T. saginata* (Fig. 1B, lanes 5, 9, 11, 14, and 15) and *T. asiatica* (Fig. 1B, lanes 1 to 4, 6 to 8, 10, 12, 13, and 16), respectively. The representative data are shown (Fig. 1B). These diagnostic results obtained by multiplex PCR were also supported by those based on the nucleotide sequence analysis of *cox1* from individual 28 cysticerci.

Differential diagnosis of tapeworm carriers by multiplex PCR with copro-DNA. In order to evaluate the detection limit of taeniid DNA in fecal samples of tapeworm carriers, multiplex PCR was performed with DNA samples prepared from a given number of *T. saginata* eggs. As shown in Fig. 2, 827-bp products unique for *T. saginata* were amplified in a dose-dependent fashion. The target band was detectable if at least five eggs were contained in 1 g of feces (lane 2); however, multiplex PCR provided more reliable diagnostic results if more than 50 eggs were present in 1 g of fecal sample (lane 5). Figure 3 shows the results obtained by multiplex PCR with copro-DNA extracted from fecal samples of tapeworm carriers. These results are also summarized in Table 2. The diagnostic products were detected from 7 of 14 *T. solium* American genotype carriers from Guatemala (lanes 1 to 7) by multiplex PCR. However, diagnostic products were detected from five of the remaining seven samples found to be negative by multiplex PCR when nested PCR with Tsol/Amer and Rev primers was performed (lanes 9 to 12 and lane 14, also very faint in the lane 9). Any products from these negative seven cases were not amplified when nested PCR with either Tsag or Tsol/Asia primer sets was performed (data not shown). It was noted that a faint *cox1* fragment was detected from a Guatemalan worm carrier who expelled only immature (non gravid) tapeworms (Fig. 3, lane 14, and Table 2, code F13). In *T. solium* Asian genotype carriers from Papua, Indonesia, 984-bp products were detected in four of nine fecal samples (lanes 15 to 18), but five of nine stool samples were not detected (data not shown). In contrast to the *T. solium* carriers, the 827-bp diagnostic markers were detected in all *T. saginata* carriers (lanes 19 to 23). One sample from Bali, Indonesia, was considered to be from a *T. solium* carrier; however, it was diagnosed as *T. saginata* by multiplex PCR (lane 23). In cases of negative controls from the United Kingdom, the product was not amplified by either multiplex PCR (lanes 24 to 30) or nested PCR using any of the primer sets (data not shown).

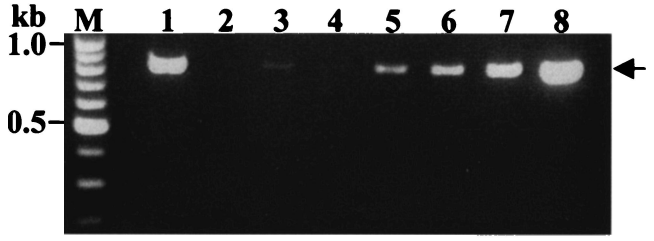

FIG. 2. Detection limit of target gene by multiplex PCR with DNA samples prepared from taeniid eggs. Target *cox1* fragments were amplified by a dose-dependent fashion (indicated by an arrow). Lane 1, positive control with mtDNA from a *T. saginata* proglottid, lanes 2 to 8, 5, 10, 20, 50, 10^2, 10^3, and 10^4 eggs/g of feces, respectively.

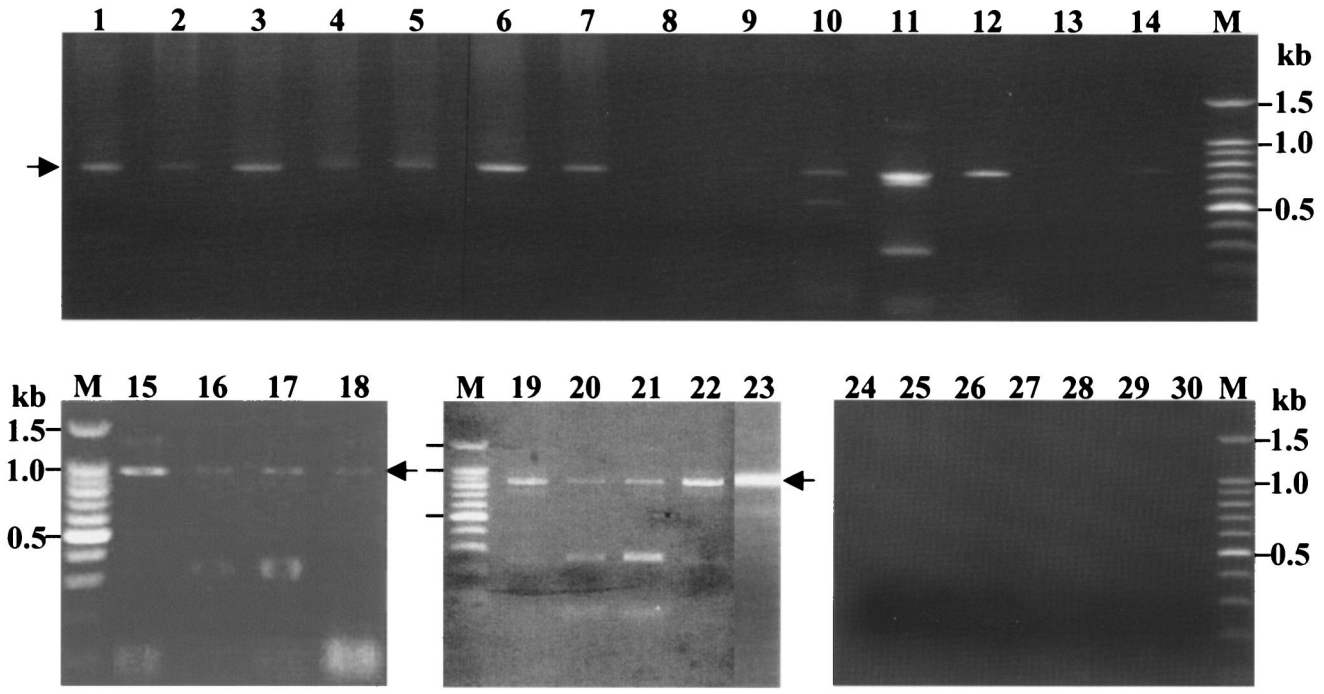

FIG. 3. Differential diagnosis of tapeworm carriers by multiplex and nested PCR with copro-DNA. Diagnostic markers with molecular sizes of 720, 984, and 827 bp were detected from tapeworm carriers with the *T. solium* American/African genotype from Guatemala (lanes 1 to 7), and nested PCR results with Tsol/Amer and Rev primers products are shown in lanes 8 to 14. The worm carriers with the Asian genotype of *T. solium* from Indonesia and *T. saginata* are shown in lanes 15 to 18 and lanes 19 to 23, respectively. Lanes 24 to 30 are from negative controls. M, DNA size markers.

DISCUSSION

Differentiation of *T. solium* and *T. saginata* based on the DNA analysis has been possible for several years (3, 4, 6–9, 13, 17–18, 22). Identification of Asian *Taenia* spp. and Asian versus American/African genotypes of *T. solium*, as well as application for stool samples has not previously been reported. Multiplex PCR for the differential diagnosis of *T. saginata* and *T. solium* was described by González et al. (8). The method coupled with restriction fragment length polymorphism has been further applied for differentiation of geographical isolates of *T. saginata* and *T. solium* (8), and it appears useful for the diagnosis of taeniasis and cysticercosis in Europe, America, and Africa, where *T. saginata* and *T. solium* (American/African genotype) are distributed. In Asian regions, on the other hand, since *T. saginata*, *T. asiatica*, and *T. solium* (Asian genotype) are distributed sympatrically, methods capable of differentiating these taeniid cestodes are necessary. Indeed, taeniid proglottids collected from different worm carriers in Yunnan province, China, where *T. solium* is also endemic, were originally thought to be only *T. saginata* on the basis of proglottid morphology; however, it has subsequently been proven to be a mixture of *T. saginata* and *T. asiatica* by multiplex PCR. The diagnostic results obtained by multiplex PCR were same as those based on the nucleotide sequences of *cox1* from taeniid cestode samples examined, indicating that the multiplex PCR can be used with a high degree of accuracy.

In DNA differential diagnosis of human taeniid cestodes, it is interesting to determine whether distinct genotypes or geographical variations exist in *T. saginata* and *T. asiatica*. The DNA sequencing of *cox1* revealed that *T. saginata* showed slight polymorphism (0.2 to 0.6%) among the 10 different geographical isolates tested (Table 1, AB06695 and AB107237 to AB107247); however, distinct phylogenetic genotypes such as those seen in *T. solium* were not detected. The *cox1* genes from*T. asiatica* samples collected from four different localities were almost identical (with a similarity of 99.8%, Table 1, AB066494 and AB107234 to AB107236). It has previously been reported that geographically different strains or isolates have been detected in *T. saginata* (7, 21), however such variations observed in *T. saginata* and *T. asiatica* did not interfere DNA differential diagnosis by multiplex PCR used here.

Target gene fragments were detectable if at least 5 eggs of *T. saginata* were present in 1 g of fecal sample. It has been reported that even one *T. solium* egg could be detected by PCR, however 50 or more eggs were needed for more reliable diagnostic (4). In the current study, neither eggs of *T. solium* nor *T. asiatica* were available, however a similar sensitivity might be expected in cases of *T. solium* or *T. asiatica* eggs. Interestingly, as indicated in lane 14 in Fig. 3, taeniid DNA was detected from egg-free fecal samples (same as code F13 in Table 2). Given the turnover of taeniid parasite material into the feces, there is very good chance that taeniid DNA is present in feces where taeniid eggs are not. A similar situation has been observed in a volunteer *T. saginata* carrier from the United Kingdom, where it was possible to detect DNA in feces by PCR prior to patency (unpublished data). This suggests that DNA from non-egg sources is present in feces from tapeworm carriers. No diagnostic marker was amplified from five of nine carriers with the *T. solium* Asian genotype from Indonesia; however, this was considered due to small amounts of fecal

samples. In contrast, 720-bp diagnostic markers were detected from 7 of 14 carriers with the *T. solium* American genotype from Guatemala. Two samples (Fig. 3, lanes 8 and 13, and Table 2, codes F1 and F9) from Guatemala were both determined to be negative by multiplex PCR and by nested PCR. In contrast, *T. solium* carriers, all four *T. saginata* samples from human volunteer infections, which were freshly collected and with which there were no problems with sample volume, were positive. Indeed, the sensitivity of the multiplex PCR technique seems to be dependent on the conditions of sample storage and the volumes of the fecal samples. In the present study, fecal samples from Guatemala stored at −20 to −30°C for 12 years and small volumes of stool samples from Indonesia had to be used. In recent research on taeniasis in Indonesia, fecal samples stored in 80 to 99.5% ethanol after collection were used and provided more reliable diagnostic results (T. Wandra, unpublished data). Thus, PCR diagnosis using copro-DNA for the detection of taeniasis can be tested under different circumstances if the stool samples are preserved in ethanol.

As summarized in Table 2, it appears that the sensitivity of multiplex/nested PCR may be somewhat lower in comparison with that of copro-antigen detection test; however, PCR diagnosis has an advantage in its ability to differentiate human taeniid cestode species. The copro-antigen assay is genus specific, not species specific, detecting both *T. solium* and *T. saginata* worm carriers (1). In PCR-based diagnosis, the ability to differentially diagnose tapeworm carriers, particularly *T. solium* carriers, has important implications: greater clinical relevance (given the greater clinical risks associated with *T. solium* cysticercosis due to the autoinfection) and epidemiological relevance (given the epidemiological importance of the ability to differentiate taeniasis to the species level). The main risk factor for acquiring cysticercosis in humans and pigs is the presence of *T. solium* tapeworm carriers within the household (19) but not *T. saginata* and *T. asiatica*. In the present study, the diagnostic markers were detected from one case of *T. solium* carriers who was egg-free, implying that it is possible to detect worm carriers prior to patency. In areas where taeniasis is endemic, therefore, multiplex PCR diagnosis will be useful for taeniasis control that aims to detect tapeworm carriers and treat them. The use of praziquantel at a single low dose is strongly recommended for the treatment of taeniasis due to either *T. saginata* or *T. solium* (12). PCR-based diagnosis is also applicable, even in areas where this organism is not endemic, for diagnosing tapeworm carriers among immigrants or tourists who have returned from such regions in order to avoid locally acquired taeniasis or cysticercosis.

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REFERENCES

1. **Allan, J. C., G. Avila, J. Garcia-Noval, A. Flisser, and P. S. Craig.** 1990. Immunodiagnosis of taeniasis by coproantigen detection. Parasitology **101:**473–477.
2. **Allan, J. C., M. Velasquez-Tohom, J. Garcia-Noval, A. R. Torres, P. Yurrita, C. Fletes, F. de Mata, H. de Soto de Alfaso, and P. S. Craig.** 1996. Epidemiology of intestinal taeniasis in four rural Guatemalan communities. Ann. Trop. Med. Parasitol. **90:**157–165.
3. **Bowles, J., and D. P. McManus.** 1994. Genetic characterization of the Asian *Taenia*, a newly described taeniid cestode of humans. Am. J. Trop. Med. Hyg. **50:**33–44.
4. **Chapman, A., V. Vallejo, K. G. Mossie, D. Ortiz, N. Agabian, and A. Flisser.** 1995. Isolation and characterization of species-specific DNA probes from *Taenia solium* and *Taenia saginata* and their use in an egg detection assay. J. Clin. Microbiol. **33:**1283–1288.
5. **Flisser, A.** 1998. Neurocysticercosis in Mexico. Parasitol. Today **4:**131–137.
6. **Flisser, A., A. Reid, Z. E. Garcia, and D. P. McManus.** 1988. Specific detection of *Taenia saginata* eggs by DNA hybridization. Lancet **ii:**1429–1430.
7. **González, L. M., E. Montero, L. J. S. Harrison, R. M. E. Parkhouse, and T. Gárate.** 2000. Differential diagnosis of *Taenia saginata* and *Taenia solium* infection by PCR. J. Clin. Microbiol. **38:**737–744.
8. **González, L. M., E. Montero, S. Puente, R. López-Velez, M. Hernández, E. Sciutto, L. J. S. Harrison, R. M. E. Parkhouse, and T. Gárate.** 2002. PCR tools for the differential diagnosis of *Taenia saginata* and *Taenia solium* taeniasis/cysticercosis from different geographical locations. Diagn. Microbiol. Infect. Dis. **42:**243–249.
9. **Harrison, L. J. S., J. Delgado, and R. M. E. Parkhouse.** 1990. Differential diagnosis of *Taenia saginata* and *Taenia solium* with DNA probes. Parasitology **100:**459–461.
10. **Ito, A., K. Nakaya, Y. Sako, M. Nakao, and M. Ito.** 2001. NOD-*scid* mouse as an experimental animal model for cysticercosis. Southeast Asian J. Trop. Med. Public Health **32**(Suppl. 2)**:**83–89.
11. **Ito, A., and P. S. Craig.** 2003. Immunodiagnostic and molecular approaches for the detection of taeniid cestode infections. Trends Parasitol. **19:**377–381.
12. **Ito, A., C. Urbani, JM. Qiu, D. A. Vuitton, DG. Qiu, D. D. Heath, P. S. Craig, F. Zheng, and P. M. Schantz.** 2003. Control of echinococcosis and cysticercosis: a public health challenge to international cooperation in China. Acta Trop. **86:**3–17.
13. **Mayta, H., A. Talley, R. H. Gilman, J. Jimenez, M. Verastegui, M., Ruiz, H. H. Garcia, and A. E. González.** 2000. Differentiating *Taenia solium* and *Taenia saginata* infections by simple hematoxylin-eosin staining and PCR-restriction enzyme analysis. J. Clin. Microbiol. **38:**133–137.
14. **Nakao, M., M. Okamoto, Y. Sako, H. Yamasaki, K. Nakaya, and A. Ito.** 2002. A phylogenetic hypothesis for the distribution of two genotypes of the pig tapeworm *Taenia solium* worldwide. Parasitology **124:**657–662.
15. **Pawlowski, Z. S., and M. G. Schultz.** 1972. Taeniasis and cysticercosis (*Taenia saginata*). Adv. Parasitol. **10:**269–343.
16. **Rishi, A. K., and D. P. McManus.** 1987. DNA probes which unambiguously distinguish *Taenia solium* from *T. saginata.* Lancet **i:**1275–1276.
17. **Rishi, A. K., and D. P. McManus.** 1988. Molecular cloning of *Taenia solium* genomic DNA and characterization of taeniid cestodes by DNA analysis. Parasitology **97:**161–176.
18. **Rodriguez-Hidalgo, R., D. Geysen, W. Benitez-Ortiz, S. Geerts, and J. Brandt.** 2002. Comparison of conventional techniques to differentiate between *Taenia solium* and *Taenia saginata* and an improved polymerase chain reaction-restriction fragment length polymorphism assay using a mitochondrial 12S rDNA fragment. J. Parasitol. **88:**1007–1011.
19. **Sarti, E., P. M. Schantz, A. Plancarte, M. Wilson, O. Gutierrez, A. S. Lopez, J. Roberts, and A. Flisser.** 1992. Prevalence and risk factors for *Taenia solium* taeniasis and cysticercosis in humans and pigs in a village in Morelos, Mexico. Am. J. Trop. Med. Hyg. **46:**677–685.
20. **Simanjuntak, G. M., S. S. Margono, M. Okamoto, and A. Ito.** 1997. Taeniasis/cysticercosis in Indonesia as an emerging disease. Parasitol. Today **13:** 321–323.
21. **Wouters, G., J. Brandt, and S. Geerts.** 1987. Observations on possible strain differences in *Taenia saginata*, p. 76–80. *In* S. Geerts, V. Kumar, and J. Brandt (ed.), Helminth zoonoses. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
22. **Yamasaki, H., M. Nakao, Y. Sako, K. Nakaya, M. O. Sato, W. Mamuti, M. Okamoto, and A. Ito.** 2002. DNA differential diagnosis of human cestodes by base excision sequence scanning thymine-base reader analysis with mitochondrial genes. J. Clin. Microbiol. **40:**3818–3821.
23. **Yamasaki, H., M. Nakao, Y. Sako, K. Nakaya, M. O. Sato, W. Mamuti, N. Xiao, M. Okamoto, and A. Ito.** 2002. DNA Differential diagnosis of human taeniid cestodes using restriction fragment length polymorphism and base excision sequence scanning T-base system, p. 189–193. *In* Proceedings of the 10th International Congress of Parasitology. Monduzzi Editore, Boronia, Italy.