MULTIPLEX PCR DIAGNOSIS FOR TAENIASIS AND CYSTICERCOSIS

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Abstract. Multiplex PCR was established for differential diagnosis of taeniasis and cysticercosis, including their causative agents. As a target gene, mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) was used. As regards identification of taeniid cestode parasites, multiplex PCR using taeniid species-specific and *Taenia solium* genotype specific-primers yielded differential products unique for *Taenia saginata*, *Taenia asiatica,* and Asian and American/African genotypes of *T. solium* with molecular sizes of 827, 269, 984, and 720 bp, respectively. The diagnostic results coincided with those based on the nucleotide sequences of *cox1* from all parasite materials examined. Moreover, multiplex PCR enabled to diagnose taeniasis by using copro-DNA extracted from fecal samples of *T. solium* or *T. saginata* carriers. From these results, it can be concluded that multiplex PCR is a useful tool not only for precise identification of taeniid cestode parasites, but also for detection of worm carriers for effective control of taeniasis and cysticercosis.

INTRODUCTION

Taenia solium, Taenia saginata and *Taenia asiatica* are causative agents of taeniasis in humans. *T. solium* also causes cysticercosis in humans, particularly, neurocysticercosis (NCC), which is characterized commonly by epileptic seizures, is the most serious (Flisser, 1998). In contrast, taeniasis is relatively innocuous or asymptomatic. However, gravid proglottids expelled from tapeworm carriers serve as new sources of infection for intermediate hosts. Particularly, in cases of *T. solium* carriers, early diagnosis and treatment are essential to preventing cysticercosis, due to autoinfection. To date, the diagnosis of taeniasis has been routinely performed based on the morphology of proglottids; however, the differentiation of *T. saginata* from *T. asiatica* is quite confusing due to morphological similarities. More recently, mitochondrial DNA analysis revealed the existence of two distinct genotypes of *T. solium*; however it is impossible to differentiate both genotypes morphologically (Nakao *et al*, 2002). To overcome the limitations of morphological identification, molecular approaches including DNA probes (Rishi and McManus, 1987, 1988; Flisser *et al*, 1988; Harrison *et al*, 1990; Chapman *et al*, 1995; Gonzalez *et al*, 2000), polymerase chain reaction (PCR), or PCR coupled with restriction fragment length polymorphism (Bowles and McManus, 1994; Mayta *et al*, 2000; Gonzalez *et al*, 2002; Rodriguez-Hidalgo *et al*, 2002; Yamasaki *et al*, 2002a) have been developed. Most recently, an advanced base excision sequence scanning thymine-base analysis using cytochrome *c* oxidase subunit 1 (*cox1*) and cytochrome *b* genes (*cob*) was established for comprehensive diagnosis of human taeniid cestodes (Yamasaki *et al*, 2002b) and then multiplex PCR using *cox1* was also introduced (Yamasaki *et al*, 2004). Recently, the differential diagnosis of cysticercosis/taeniasis has been reviewed (Ito and Craig, 2003). In the present study, multiplex PCR was improved and the diagnostic results have been introduced.

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MATERIALS AND METHODS

Parasite materials

A total of 57 taeniid parasite materials, including proglottids, cysticerci and eggs collected from geographically different localities were examined (Yamasaki *et al*, 2004); *T. saginata* (n=13), *T. asiatica* (n=13), taeniid eggs from China, *T. solium* from Latin America (n=7), Africa (n=4) and Asia (n=19). A taeniid cysticercus embedded in paraffin for histopathological examination was also tested.

Fecal samples

T. solium-infected fecal samples were obtained from the following sources (Yamasaki *et al*, 2004); 14 fecal samples collected in Guatemala in 1991 and 1994 had been diagnosed by copro-antigen detection test and

morphology of proglottids expelled after treatment (Allan *et al*, 1996). Four samples from Papua (formerly Irian Jaya), Indonesia, were available and had also been proven to be positive for *T. solium* by copro-antigen test kit (Virotech, Germany). Five stool samples from *T. saginata* carriers were also available. Seven fecal samples from non-infectious individuals were used as negative controls.

DNA extraction

Mitochondrial DNA samples (mtDNA) from parasite materials were prepared using DNeasy Tissue kit (Qiagen, Germany). However, some cysticerci developed in non-obese diabetic/Shi-severe combined immunodeficiency (NOD/Shi-*scid*) mice were lysed in 50 μl of 0.02 N NaOH and the resulting supernatants were used as template DNA. MtDNA from a small piece of formalin-fixed and paraffin-embedded specimen was prepared as follows; paraffin was melted at 70˚C and a tiny parasite tissue was separated, and then the parasite was lysed in 60 μl of 0.02N NaOH. Copro-DNAs from fecal samples of tapeworm carriers were extracted using QIAamp DNA Stool Mini kit (Qiagen, Germany).

Multiplex PCR

Multiplex PCR was performed according to Yamasaki *et al* (2004). In multiplex PCR-negative samples from Guatemala, nested PCR using species- and *T. solium* genotype-specific primers was followed by conventional PCR using Cox1/F and Cox 1/R primers (Nakao *et al*, 2002).

DNA sequencing and sequence analysis

The nucleotide sequences of PCR products were determined for further confirmation using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit. DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer and the nucleotide sequence data were analyzed using DNASTAR (version 3.75).

RESULTS

Identification of the causative agents of taeniasis and cysticercosis by multiplex PCR

Multiplex PCR using mtDNA prepared from parasite materials provided the most evident results. As shown in Fig 1-A, the diagnostic products with molecular sizes of 827, 269, 720, and 984 bp were amplified in *T. saginata* (lanes 1-10), *T. asiatica* (lanes 12-16), *T. solium* American/African (lanes 17-25) and Asian genotypes (lanes 26-32), respectively. The taeniid egg samples, as shown in lane 11, were identified as a mixture of *T. saginata* and *T. asiatica* because of the amplification of two products. In order to verify this, the oncospheres *in vitro* hatched derived

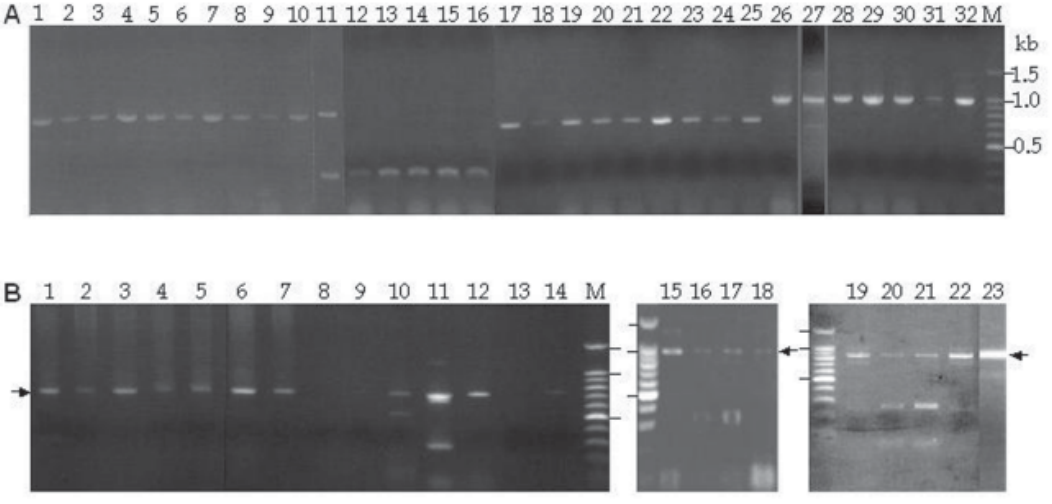

Fig 1- Differential diagnosis of taeniasis and cysticercosis including their causative agents by multiplex PCR. Panel A. Identification of taeniid cestode parasites using mtDNA prepared from taeniid parasites. The diagnostic *cox1* fragments with molecular sizes of 827, 269, 720, and 984 bp were amplified from *T. saginata* (lanes 1-10), *T. asiatica* (lanes 12-16), *T. solium* American/African genotype (lanes 17-25) and *T. solium* Asian genotype (lanes 26-32), respectively. A taeniid sample in lane 11 is a mixture of *T. saginata* and *T. asiatica.* Panel B. Differential diagnosis of tapeworm carriers. The carriers with *T. solium* American/African genotype from Guatemala were diagnosed by multiplex PCR (lanes 1-7) and nested PCR (lanes 9-12 and 14). The differential markers with molecular sizes of 984 and 827 bp were amplified from Asian genotype *T. solium* from Indonesia (lanes 15-18) and *T. saginata* (lanes 19-23), respectively. M indicates 100 bp ladder DNA markers.

from the eggs were injected into NOD/Shi-*scid* mice and allowed to develop in such mice. After 5 to 6 months, 28 cysticerci were recovered from two mice and it has been confirmed to be a mixture of *T. saginata* and *T. asiatica* by multiplex PCR using mtDNA prepared from an individual cysticercus (data not shown). Moreover, in the paraffin-embedded specimen, the 984bp-product was successfully amplified only using *T. solium* Asian genotype-specific primers (data not shown). DNA sequencing revealed

Table 1
Diagnostic results of tapeworm carriers by multiplex PCR.

Code	Year[a]	Status	Carriers from	Copro-antigen[b]	Multiplex PCR		Lane[e]
F 1	1994	*T. solium*	Guatemala	+	-		8
F 2	1994	*T. solium*	Guatemala	+	+	*T. solium*[d]	9
F 4	1994	*T. solium*	Guatemala	+	+	*T. solium*	1
F 6	1994	*T. solium*	Guatemala	+	+	*T. solium*[d]	10
F 7	1994	*T. solium*	Guatemala	+	+	*T. solium*[d]	11
F 8	1994	*T. solium*	Guatemala	+	+	*T. solium*[d]	12
F 9	1991	*T. solium*	Guatemala	+	-		13
F10	1994	*T. solium*	Guatemala	+	+	*T. solium*	2
F12	1994	*T. solium*	Guatemala	+	+	*T. solium*	3
F13	1994	*T. solium*	Guatemala	+	+	*T. solium*[d]	14
G 1	1994	*T. solium*	Guatemala	+	+	*T. solium*	4
G 6	1994	*T. solium*	Guatemala	+	+	*T. solium*	5
G 7	1994	*T. solium*	Guatemala	+	+	*T. solium*	6
G 8	1994	*T. solium*	Guatemala	+	+	*T. solium*	7
K03	2000	*T. solium*	Indonesia (Papua)	+	+	*T. solium*	15
K38	2000	*T. solium*	Indonesia (Papua)	+	+	*T. solium*	16
K44	2000	*T. solium*	Indonesia (Papua)	+	ND		
K50	2000	*T. solium*	Indonesia (Papua)	+	ND		
K54	2000	*T. solium*	Indonesia (Papua)	+	+	*T. solium*	17
K56	2000	*T. solium*	Indonesia (Papua)	+	ND		
K87	2000	*T. solium*	Indonesia (Papua)	+	+	*T. solium*	18
K90	2000	*T. solium*	Indonesia (Papua)	+	ND		
K10	2000	*T. solium*	Indonesia (Papua)	+	ND		
N 1	2002	*T. saginata*	United Kingdom	+	+	*T. saginata*	19
N 2	2002	*T. saginata*	Japan	-	+	*T. saginata*	20
N 3	2002	*T. saginata*	Brazil	+	+	*T. saginata*	21
N 4	2002	*T. saginata*	China	-	+	*T. saginata*	22
F 3	1995	*T. saginata*	Indonesia (Bali)	ND[c]	+	*T. saginata*	23
F 5	2002	noninfectious	United Kingdom	ND	-		
F11	2002	noninfectious	United Kingdom	ND	-		
G 3	2002	noninfectious	United Kingdom	ND	-		
G 4	2002	noninfectious	United Kingdom	ND	-		
G 9	2002	noninfectious	United Kingdom	ND	-		
G10	2002	noninfectious	United Kingdom	ND	-		
G12	2002	noninfectious	United Kingdom	ND	-		

[a] Year in which stool samples were collected.
[b] Copro-antigen data from Guatemala (Allan *et al*, 1996) and Indonesia.
[c] Not done.
[d] Detected by nested PCR using *T. solium* American genotype-specific primers.
[e] Corresponds to the lane numbers in Fig 1.

that the two nucleotides at positions 690 and 723 were guanine and cytosine, respectively, specific for *T. solium* Asian genotype, showing that the taeniid cysticercus is an Asian genotype of *T. solium* (data not shown).

Differential diagnosis of tapeworm carriers by multiplex PCR using copro-DNA

Multiplex PCR using copro-DNA extracted from worm carriers was able to diagnose worm carriers as taeniid species or *T. solium* genotype levels. As shown in Fig 1-B, the 720 bp-*cox1* fragments were detected from 7 of 14 *T. solium* carriers from Guatemala (lanes 1-7), however diagnostic products were not detected from the remaining 7 samples by multiplex PCR. However, target *cox1* was amplified using Cox1/F and Cox1/R primers; then, nested PCR using *T. solium* American/African genotype-specific primers was performed, resulting in 720-bp diagnostic products amplified from 5 of 7 (lanes 9-12, 14). It is noteworthy that the *cox1* fragment was detected from one worm carrier who expelled only egg-free immature proglottids (lane 14 in Fig 1B and code F13 in Table 1). The diagnostic bands were not detected by either multiplex PCR or nested PCR in the two samples (lanes 8 and 13). In the *T. solium* carriers from Indonesia, 984 bp products were detected in 4 samples (lanes 15-18). Similarly, 827 bp diagnostic markers were detected from all *T. saginata* carriers (lanes 19-23). No product was amplified from the negative control samples (data not shown).

DISCUSSION

Most molecular approaches have focused on differentiation of *T. saginata* from *T. solium* (Flisser *et al*, 1988; Harrison *et al*, 1990; Bowles and McManus, 1994; Chapman *et al*, 1995; Mayta *et al*, 2000; Gonzalez *et al*, 2000, 2002; Rodriguez-Hidalgo *et al*, 2002; Yamasaki *et al*, 2002a) and it appears useful in Europe, Latin America and Africa, where only *T. saginata* and *T. solium* (American/African genotype) occur. On the other hand, *T. asiatica*, beside *T. saginata* and *T. solium* (Asian genotype), is distributed in Asian regions, making the differential diagnosis of these taeniid cestodes more confusing. The taeniid sample (lane 11 in Fig 1-B) collected from different worm carriers in Yunnan Province, China was originally thought to be *T. saginata*, based on proglotiid morphology. However, it has been proved to be a mixture of *T. saginata* and *T. asiatica* by multiplex PCR. DNA sequencing of the *cox1* amplified by multiplex PCR revealed that the diagnostic results, based on the multiplex PCR, coincided with identification based on the nucleotide sequences of *cox 1* from all taeniid cestodes examined, indicating that multiplex PCR is a very precise tool.

In multiplex PCR using copro-DNA, worm carriers were detectable at taeniid species or *T. solium* genotype level. More importantly, a diagnostic product was detected from a worm carrier who had only immature proglottids (lane 14 in Fig 1-A, code F13 in Table 1), indicating that taeniid DNA from non-egg sources is present in feces of the carrier, and that prepatent carriers can be detectable. In cases of worm carriers with *T. solium* American genotype in Guatemala, 7 and 5 cases of 14 were diagnosed by multiplex PCR and nested PCR, respectively, with a sensitivity of 86% (12/14). In contrast, 4 and 5 worm carriers with *T. solium* Asian genotype from Indonesia and *T. saginata*, respectively, which were freshly collected and with which there were no problems with sample volume, were all positive. Indeed, the test sensitivity of the multiplex PCR seemed to increase depending on the storage conditions of the fecal sample. Thus, we recommend keeping fecal samples in >80% ethanol for DNA analysis after collection. In recent work on taeniasis in Indonesia, fecal samples have been stored in 80-99.5% ethanol after collection. Multiplex PCR using such fecal samples provided highly sensitive diagnostic results (Wandra *et al*, unpublished data). As summarized in Table 1, it appears that the sensitivity of multiplex PCR may be somewhat lower compared with that of the copro-antigen detection test. The copro-antigen assay has been used for detecting *T. solium* carriers, but the assay is genus-specific (Allan *et al*, 1990). In contrast, multiplex PCR enables differentiation of tapeworm carriers at species or *T. solium* genotype level. In particularly, the detection of *T. solium* carriers has an important implication that prevents cysticercosis due to autoinfection. Thus, multiplex PCR diagnosis will be useful for taeniasis/cysticercosis control that aims to detect tapeworm carriers and treat them in endemic areas of these infection. Even in nonendemic areas, the PCR-based diagnosis will be essential not only for routine identification of ethanol-fixed or paraffin-embedded samples, but also detection of worm carriers as immigrants or tourists who come from endemic regions in order to avoid locally-acquired taeniasis/cysticercosis.

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