A SIMPLE AND EFFECTIVE MULTIPLEX PCR TECHNIQUE FOR DETECTING HUMAN PATHOGENIC *TAENIA* EGGS IN HOUSEFLIES

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Abstract. *Taenia solium*, *T. saginata*, and *T. asiatica* are cestode pathogens causing taeniasis in humans. Houseflies can transfer *Taenia* eggs to food. However, houseflies are thought to carry only small numbers of *Taenia* eggs, sometimes fewer than 10. Although several PCR-based methods have been developed to detect *Taenia* DNA, these require more than 10 eggs for adequate detection. We developed a multiplex PCR method with high specificity for the discrimination among the eggs of the three *Taenia* species, *T. solium*, *T. saginata*, and *T. asiatica*, using 18S ribosomal DNA (rDNA) as a genetic marker. This technique was found to be highly sensitive, capable of identifying the *Taenia* species from only one egg. This multiplex PCR technique using 18S rDNA specific primers should be suitable to diagnose *Taenia* eggs.

Keywords: houseflies, *Taenia solium*, *T. saginata*, *T. asiatica,* 18S ribosomal RNA, multiplex PCR

INTRODUCTION

Taeniasis is a disease caused by an infection with *Taenia solium*, *T. saginata*, or *T. asiatica* adult worms (Eom, 2006). Among these species, only the accidental ingestion of *T. solium* eggs can cause neurocysticercosis (Ito *et al*, 2006). Humans are the definitive hosts of *T. saginata* and *T. solium* following the consumption of inadequately cooked beef and pork containing metacestodes, respectively (Galán-Puchades and Fuentes, 2013). *Taenia asiatica* has hepatic tropism in pigs and wild boar and causes cysticercosis (Galán-Puchades and Fuentes, 2000, 2013). The distribution of *T. solium* and *T. saginata* is world-wide, while *T. asiatica* has only been reported in Asia (Galán-Puchades and Fuentes, 2013).

All three species of *Taenia* have been reported to be found in Thailand (Anantaphruti *et al*, 2007; Anantaphruti, 2013), which is why the Thai Ministry of Public Health is concerned (Anantaphruti *et al*, 2007). About 1-2% of the population in northern and northeastern Thailand are estimated to have taeniasis (Anantaphruti *et al*, 2007). Cases of cysticercosis have been reported from Thailand (Waikagul *et al*, 2006; Anantaphruti *et al*, 2007; Anantaphruti, 2013).

In taeniasis endemic areas, houseflies

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have been reported to carry eggs from *Taenia* and other helminthes (Maipanich *et al*, 2008, 2010, 2011, 2012, 2014). The eggs adhere to the fly's body when it feeds on human feces contaminated with helminths. In Thailand, *Taenia* eggs have been observed on the common housefly (*Musca domestica* Linnaeus, 1758) and the blowfly (*Chrysomya megacephala* Fabricius, 1794) and frequently co-exist with other helminth eggs, such as *Trichuris trichiura*, hookworm, *Opisthorchis* sp, and *Echinostoma* sp (Maipanich *et al*, 2008, 2010, 2011, 2014). However, eggs of different *Taenia* species appear similar to each other and it is difficult to distinguish among *Taenia* species (Jimenez *et al*, 2010). In addition, only small numbers of *Taenia* eggs are usually found on the houseflies collected (Maipanich *et al*, 2014) making them difficult to identify.

Previously, PCR-based techniques have been developed to distinguish among *Taenia* spp using mature proglottids (González *et al*, 2000, 2010). Multiplex PCR using the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) as the genetic marker has been used to discriminate among *T. solium*, *T. saginata*, and *T. asiatica* eggs. However, this technique is not reliable for analyzing small numbers of eggs (<50) per gram of stool (Yamasaki *et al*, 2004). We found this technique is sufficiently sensitive to amplify DNA from one *Taenia* egg (unpublished data).

A method with a high sensitivity and specificity, such as multiplex real-time PCR, can be used to detect small quantities of DNA but requires high-throughput equipment and relatively expensive reagents compared to conventional PCR (Gordon *et al*, 2015). We aimed to develop a simple, relatively inexpensive multiplex PCR method with a high sensitivity and specificity using the nuclear 18S ribosomal RNA (rRNA) gene as a specific genetic marker. We used this method to discriminate among eggs of *T. solium, T. saginata*, and *T. asiatica* and to detect *Taenia* eggs on houseflies collected from taenia endemic areas.

MATERIALS AND METHODS

Housefly samples

Houseflies were caught in Ban Nong Bua, Tha Song Yang District, Tak Province, Thailand in February, 2013 in buckets used as fly traps containing rotten shrimp as bait. The traps were left near food shops in the village for about 8 hours during the day. Flies were collected from the traps and stored on ice. Groups of 30 flies were separated and kept in a capped bottle containing 15 ml formalin-detergent (FD) solution (10 ml formalin and 50 ml detergent dissolved in 440 ml water) as a preservative and transported to our department for examination.

Housefly examination

The preserved houseflies, *C. megacephala*, were washed in an ultrasonic cleaner with FD solution (Elma Transsonic Digital D-7700, Singen, Germany at level 5 for 15 minutes at room temperature. After washing, the flies were dried and identified. Sediment in the washing solution was examined under a stereomicroscope (SZ30 Binocular Stereo Zoom Microscope; Olympus, Tokyo, Japan) to determine helminthic objects. *Taenia* eggs were selected based on their appearance, which is usually 30-40 μm in size with a thick striated outer shell and three pairs of hooklets. All *Taenia* eggs were then preserved in absolute ethanol and stored at -20ºC until examined.

DNA sample preparation

Tissue from each worm was extracted from mature proglottids obtained from specimens at the Department of Helmin-

thology, using a genomic DNA mini kit for tissue (Geneaid, Taipei, Taiwan) following the manufacturer's instructions. The DNA concentration was measured using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies, Wilmington, DE). The genomic DNA was then diluted with sterile double distilled water as required for the PCR. Shells from the egg(s) were broken using a sterile coverslip on a sterile glass slide and then washed with 10 µl sterile double distilled water. The broken eggs used for each preparation were then heated at 96°C for 5 minutes using a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The genetic material was then immediately used to make the DNA template for the PCR.

Serial dilutions of genomic DNA were made each for *T. solium*, *T. saginata*, and *T. asiatica* from 10^{-5} ng to 1 ng and used to test primer sensitivity along with one and five *T. solium* eggs. Genomic DNA from *Ascaris lumbricoides*, *A. suum*, *T. trichiura*, hookworm, *Strongyloides stercoralis*, and *Opisthorchis viverrini* were also prepared from adult worm specimens preserved in 70% ethanol at the Department of Helminthology. Genomic DNA was extracted and measured following the *Taenia* tissue extraction method described above, to test primer specificity.

Primer design

The 18S rRNA gene small subunit (SSU) was used as a PCR target because it shows sequence conservation within a species but has sufficient sequence variability between genera to enable the phylogenetic discrimination of genera and species (Yan *et al*, 2013). PCR primers for discriminating *Taenia* species were created based on the nucleotide sequences of the 18S rDNA sequences from taeniid cestodes, which are available in GenBank. The following forward primers were designed to amplify different PCR amplicons: Tasia18S: 5′-CCACGAGGACGTGGCAGC-3′, primer specific for *T. asiatica* (positions 1912-1929 of AB731617 and GQ260088; product size, 519 bp); Tsag18S: 5′-TTCAGCGCCTCATCTAGCTG-3′, primer specific for *T. saginata* (positions 2024-2043 of JQ609338 and DQ768166; product size, 407 bp); and Tsol18S: 5′-GATGGAGTCCGGCGTTTG-3′, primer specific for *T. solium* (positions 2156-2173 from DQ157224 and GQ260091; product size, 275 bp). Each species-specific forward primer was used with a genera-specific reverse primer which amplifies the DNA from all three *Taenia* species: Trev18S: 5′-CGTGAACGAGGAATTTCCCCTAGT-3′ (positions 2411-2431 from all the above 18S rDNA sequences used). All primers were synthesized by *AITbiotech*, Singapore.

PCR conditions

The singleplex PCR for the three *Taenia* species used different forward primers but the same reverse primer. PCR reaction mixture was composed of 1X TopTaq Master Mix Kit (1.25 U TopTaq DNA polymerase, 200 µM of each dNTP, and 1.5 mM $MgCl_2$) (Qiagen, Hilden, Germany), 2 µM of each species-specific forward primer, the reverse primer and 5 ng/µl of genomic DNA. The products were run on a T100™ Thermal Cycler (Bio-Rad Laboratories). Before selecting the optimal conditions, we followed those of Yamasaki *et al* (2004), but were unable to amplify genomic DNA. Final PCR conditions were as follows: 95°C for 3 minutes; followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds; with a final extension at 72°C for 5 minutes. PCR amplicons were checked for size by running on 2.0% agarose gel with 1 µl ethidium bromide and

Table 1
Genomic DNA and primer concentrations used.

Species	Genomic DNA		Forward primer		Reverse primer	
	Concentration (ng)	Volume (µl)	Concentration (µM)	Volume (µl)	Concentration (µM)	Volume (µl)
Duplex PCR						
T. solium / *T. saginata*	10^{-2}/1	1/1	2/4	1/1	5	2
T. solium / *T. asiatica*	10^{-2}/1	1/1	2/4	1/1	5	2
T. saginata / *T. asiatica*	2/5	1/1	5	1/1	10	2
Multiplex PCR						
T. solium / *T. saginata* / *T. asiatica*	10^{-2}/2/5	1/1/1	2/4/6	1/1/1	10	3

visualized under a UV transilluminator. The PCR product for each *Taenia* species was sequenced by a ABI 3730XL DNA analyzer (*AITbiotech*) using BigDye Terminator chemistry with user-supplied primers. Primer specificity was checked by BLAST sequence alignment.

Duplex and multiplex PCR conditions involved an initial denaturation of 95°C for 3 minutes; followed by four cycles of 95°C for 30 seconds, 58°C for 45 seconds, and 72°C for 90 seconds, then 29 cycles of 95°C for 30 seconds, 58°C for 45 seconds, and 72°C for 1 minute; with a final extension at 72°C for 10 minutes. Genomic DNA and primer concentrations were different and specific for each sample of mixed genomic DNA (Table 1). The Mg^{2+} concentration was increased to 3 µM with the aim of increasing product yield.

Molecular identification of *Taenia* eggs on houseflies

To determine the specificity and sensitivity of the PCR reactions, 14 *Taenia* eggs collected from house flies were divided into five samples to prepare the genetic material for the methods described above. All sets of genetic material were then amplified by multiplex PCR.

Comparison of sensitivity with previously developed techniques

To compare the sensitivity of our results with previous methods, DNA was extracted from viable *T. solium* eggs (one or five) using the TopTaq Master Mix kit. PCR amplification of *cox1* was performed according to the method previously described (Yamasaki *et al*, 2004).

RESULTS

Sensitivity

We first determined the detection limit of 18S rRNA gene PCR amplifica-

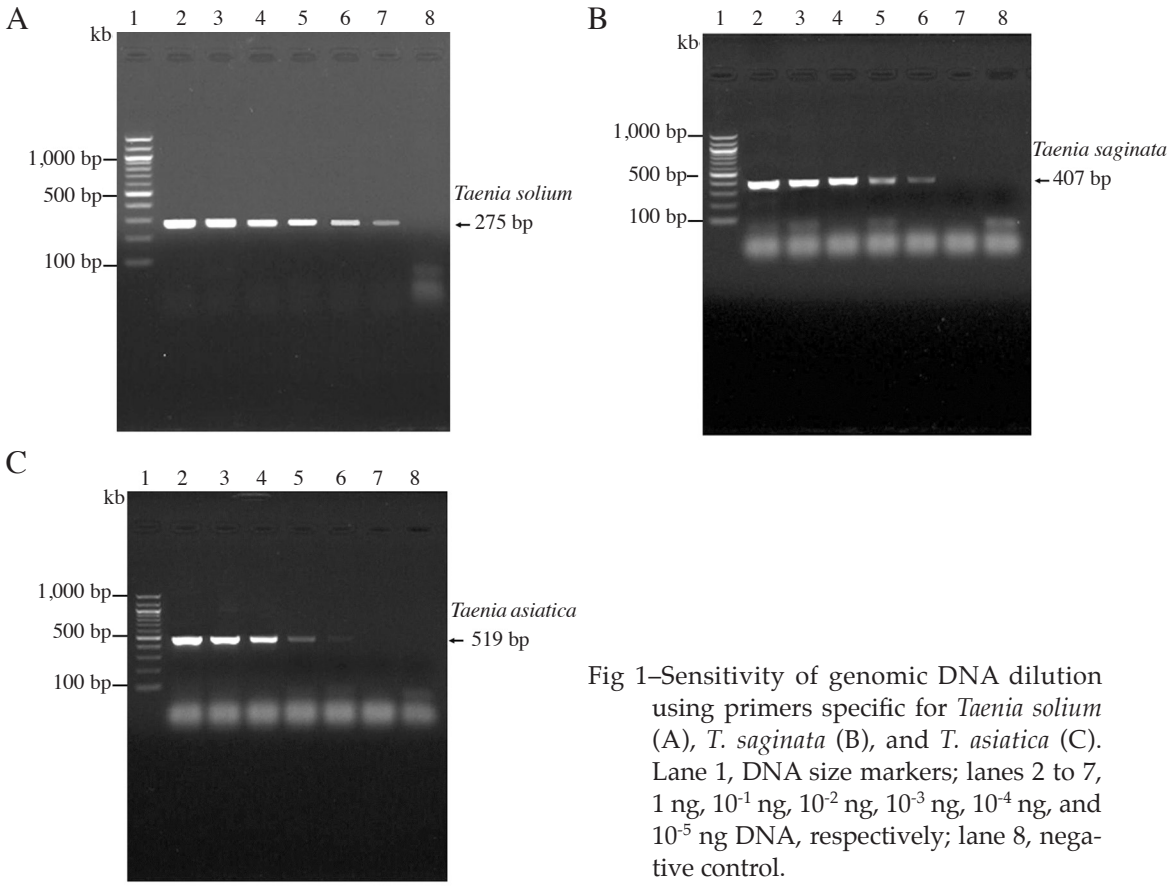

Fig 1–Sensitivity of genomic DNA dilution using primers specific for *Taenia solium* (A), *T. saginata* (B), and *T. asiatica* (C). Lane 1, DNA size markers; lanes 2 to 7, 1 ng, 10^{-1} ng, 10^{-2} ng, 10^{-3} ng, 10^{-4} ng, and 10^{-5} ng DNA, respectively; lane 8, negative control.

tion of the DNA samples prepared from adult worms.

The lower limit of detection for the genomic DNA was 10^{-5} ng for *T. solium* (Fig 1A), 10^{-4} ng for *T. saginata* (Fig 1B), and 10^{-4} ng for *T. asiatica* (Fig 1C). PCR amplification of the SSU 18S rRNA gene was shown to detect *Taenia* eggs with higher sensitivity than that of *cox1* because the positive PCR products for the 18S rRNA were obtained from reactions using only one *T. solium* egg. In contrast, no PCR product was detected on gel electrophoresis following the amplification of *cox1* (Fig 2). The detection limit of taeniid DNA was performed using DNA samples prepared from a given number of *T. solium* eggs.

Specificity

Primer specificity. Singleplex PCR for the 18S rDNA used the same conditions as those employed for sensitivity, but a different genomic DNA template was used (Fig 3). The singleplex PCR results show a single amplicon of 275, 407, and 519 bp for *T. solium* (lane 1), *T. saginata* (lane 2), and *T. asiatica* (lane 3), respectively. Fig 3 also shows the results of PCR amplification using *T. solium*-specific primers to evaluate primer specificity from other parasites (lane 8: *A. lumbricoides*; lane 9: *A. suum*, lane 10: *T. trichiura*; lane 11: hookworm; lane 12: *S. stercoralis*; lane 13: *O. viverrini*). No PCR product was detected when DNA from other parasites was used

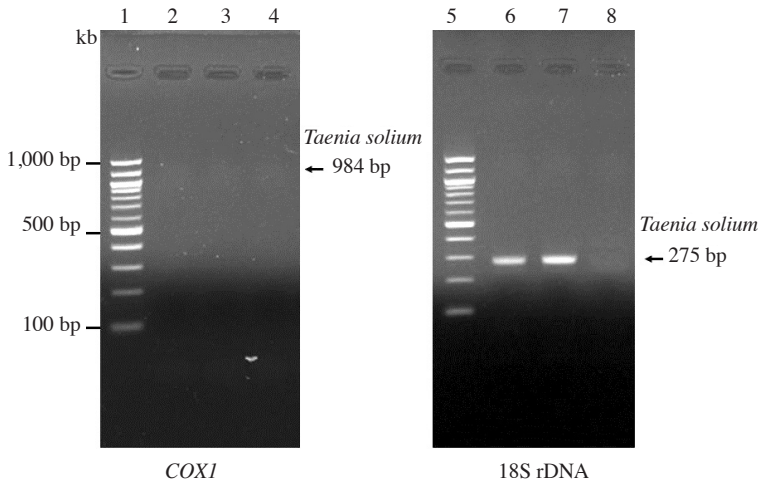

Fig 2–Detection of different numbers of *Taenia solium* eggs comparing *cox1* (lanes 2-4) and 18S rDNA markers (lanes 6-8). Lanes 1 and 5, DNA size markers; lanes 2 and 6, one egg; lanes 3 and 7, five eggs; lanes 4 and 8, negative controls.

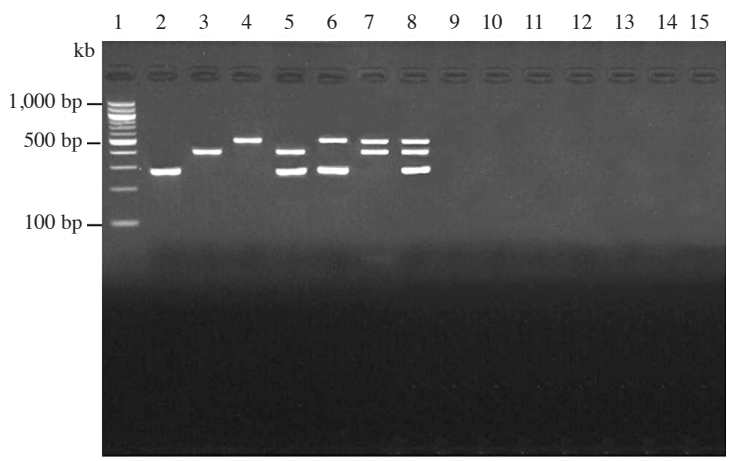

Fig 3–Multiplex PCR amplification of diagnostic fragments from the mature proglottids of *Taenia solium*, *T. saginata*, and *T. asiatica* using 18S rDNA primers. Lane 1, DNA size markers; lanes 2–4, singleplex PCR of *T. solium*, *T. saginata*, and *T. asiatica*, respectively; lanes 5-7, duplex PCR of *T. solium* + *T. saginata*, *T. solium* + *T. asiatica*, and *T. saginata* + *T. asiatica*, respectively; lane 8, multiplex PCR of all three *Taenia* species; lanes 9-14, primer specificity with the parasites *Ascaris lumbricoides*, *A. suum*, *Trichuris trichiura*, hookworm, *Strongyloides stercoralis*, and *Opisthorchis viverrini*, respectively; lane 15, negative control.

in the singleplex assay.

Differentiation among *T. solium*, *T. saginata*, and *T. asiatica* by multiplex PCR amplification of 18S rRNA

The successful amplification of diagnostic products was also dependent on the concentration of genomic DNA shown in Table 1. Multiplex PCR reactions containing specific primers for *T. solium*, *T. saginata*, and *T. asiatica* were evaluated for specificity using duplex and multiplex PCR of genomic DNA. As shown in Fig 3, two bands (275 and 407 bp) were amplified from *T. solium* and *T. saginata* DNA templates (lane 5), two (275 and 519 bp) from *T. solium* and *T. asiatica* DNA templates (lane 6), and two (407 and 519 bp) from *T. saginata* and *T. asiatica* DNA templates (lane 7). The multiplex PCR products contained three DNA bands (275, 407, and 519 bp) from the amplification of mixed *T. solium*, *T. saginata*, and *T. asiatica* DNA templates (lane 8). Therefore, the specificity for *T. solium*, *T. saginata*, and *T. asiatica* was 100%.

Identification of *Taenia* eggs collected from houseflies by multiplex PCR

Fourteen *Taenia* eggs collected from houseflies were identified at the species level using adjusted multiplex PCR conditions. The eggs were separated into three

groups of five, five, and four eggs. The PCR product amplified from all three groups of eggs were 275 bp, which is the specific band size for *T. solium*.

DISCUSSION

Houseflies are potential transmitters of helminth infections (Maipanich *et al*, 2008, 2010, 2011, 2012, 2014), with soil-transmitted helminth larvae and eggs, including those of *Taenia* spp, frequently detected adhering to houseflies in small numbers (Maipanich *et al*, 2008, 2010, 2011, 2012, 2014). The morphological characteristics of the different species of *Taenia* eggs are similar making it difficult to distinguish among species (Jimenez *et al*, 2010). A highly sensitive and specific technique is needed to identify small numbers of *Taenia* eggs on houseflies. In this study we developed a multiplex PCR method with a high sensitivity and specificity but low cost to discriminate among *T. solium*, *T. saginata*, and *T. asiatica* eggs using the SSU 18S rRNA gene as the target marker.

This technique was successful at amplifying DNA from a single *Taenia* egg, while the previous target markers, *cox1* and NADH dehydrogenase subunit 2 (*NADH2*), could not (Jeon *et al*, 2009). The method was also successfully able to amplify small amounts of genomic DNA (~10^{-4} to 10^{-5} ng). This may be possible because rRNA is an essential housekeeping gene, so tandemly repeated copies are present in large clusters on the chromosome (Ide *et al*, 2010). The 18S rRNA marker was specific for detecting the three *Taenia* species without cross reaction with DNA from other parasites (*A. lumbricoides*, *A. suum*, *T. trichiura*, hookworm, *S. stercoralis*, and *O. viverrini*).

In the past decade, many efforts have been made to develop sensitive and specific techniques to discriminate eggs of *Taenia* species, particularly from stool samples (Yamasaki *et al*, 2004; Mayta *et al*, 2008; Gordon *et al*, 2015). These investigations used the mitochondrial genes *cox1* and *NADH2* as DNA markers, and demonstrated equal levels of sensitivity and specificity (Yamasaki *et al*, 2004; Jeon *et al*, 2009). However, they were limited to amplification of genetic material from ≥50 eggs to obtain a reliable diagnosis following copro-PCR (Yamasaki *et al*, 2004).

In the present study, *cox1* primers from a previous study were used to amplify a single *Taenia* egg, but the PCR amplification was not successful. Therefore, that primer is unable to detect very small numbers of *Taenia* eggs. We compared PCR amplification of eggs (without feces) using two markers: 18S rDNA and *cox1* genes. The 18S rDNA primer was able to detect the DNA from only one egg but the *cox1* primer was not. We had no false positive amplification products using species-specific primers. We also had no false positives using freshly purified/sterile water, tubes, and reagents, and kept our PCR work area separate from other areas.

18S rDNA appears to be more conserved and numerous in the genome than mitochondrial genes (Hwang and Kim, 1999). The 18S rRNA gene has also been used previously in the molecular diagnosis of *Taenia* spp (Foronda *et al*, 2005). PCR products amplified with 18S rRNA-specific primer pairs contained a highly conserved region and another hypervariable region, both in sequence and length. Nucleotide sequences of this gene were used to discriminate among *T. solium*, *T. saginata* and *T. asiatica*.

Although multiplex real-time PCR techniques, including the present one,

have been developed to obtain increased sensitivity in the detection of intestinal helminths from fecal samples, they come at a cost. These include the use of specific fluorogenic probes, which require complex standard curves to be performed to achieve ≥ 90% PCR efficiency with control DNA, and a real-time PCR machine (Gordon *et al*, 2015). The loop-mediated isothermal amplification method was also developed for use in the field without a PCR machine, but this requires at least five eggs for adequate detection, and is limited to only *T. saginata* and *T. asiatica* (Nkouawa *et al*, 2009). Because of the limitations of other methods, simple multiplex PCR has been suggested as the most suitable technique for the detection of *Taenia* eggs.

In the present study, houseflies carrying *Taenia* eggs were collected from an endemic area for taeniasis and cysticercosis (Maipanich *et al*, 2011). All *Taenia* eggs were identified as those of *T. solium*. This housefly species (*C. megacephala)* has been reported to be the potential transmitter of helminth eggs and larvae (Förster *et al*, 2009), so it is important to attempt to eliminate them from endemic areas of cysticercosis to avoid contamination of food and drinking water.

In conclusion, this simple multiplex PCR method, using 18S rDNA as the target marker, is a relatively inexpensive, sensitive and specific technique for detecting and discriminating among *T. solium*, *T. saginata*, and *T. asiatica* using very small numbers of eggs, such as those carried on a housefly. Of note, this method is for use with purified eggs, not fecal material. We propose the 18S rDNA-specific primers are a suitable alternative DNA marker for the routine diagnosis of *Taenia* eggs, particularly in endemic areas.

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