Nested PCR for the Specific Diagnosis of *Taenia solium* Taeniasis

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**Word Count:** Abstract = 174; text = 2823

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Running title: TAENIASIS Tso31-NESTED-PCR
ABSTRACT

Taeniasis due to *Taenia solium* is a disease with important public health consequences since the larval stage is not exclusive to the animal intermediate, the pig, but also infects humans causing neurocysticercosis. An early diagnosis and treatment of *T. solium* tapeworm carriers is important to prevent human cysticercosis. Current diagnosis based on microscopic observation of eggs lacks both sensitivity and specificity. In the present study a nested PCR assay targeting the *Tso31* gene, was developed for the specific diagnosis of taeniasis due to *T. solium*. Initial specificity and sensitivity testing was performed using stored known *T. solium* positive and negative samples. The assay was further analyzed under field conditions by conducting a case-control study of pre-treatment stool samples collected from an endemic population. Using the archived samples the assay showed 97% (31/32) sensitivity and 100% (123/123) specificity. Under field conditions the assay had a 100% sensitivity and specificity using microscopy/ELISA coproantigen as the gold standards. The *Tso31* nested PCR described here might be a useful tool for the early diagnosis and prevention of taeniasis/cysticercosis.

**Keywords:** Taenia, Taeniasis, Nested-PCR, Neurocysticercosis.
INTRODUCTION

Human Taeniasis/cysticercosis is an important public health problem, particularly in developing countries. Taeniasis is a parasitic disease of the human intestinal tract caused by the adult stage of the closely related tapeworms: *Taenia solium*, *Taenia saginata* and *Taenia saginata asiatica*. The disease is a consequence of ingesting cysts present in raw or undercooked infected meat. After ingestion, the cyst reaches the human intestine where it develops into an adult tapeworm releasing proglottids filled with eggs that are passed in the stools (7). Ingestion of these eggs by the intermediate hosts results in the development of cysts in soft tissues, a disease known as cysticercosis. Cysticercosis affects swine (*T. solium*) and cattle (*T. saginata*) causing economic loss (1, 12). Unlike *T. saginata* eggs, *T. solium* eggs are capable not only of infecting the animal intermediate host, but also humans, causing human cysticercosis (11, 10). In humans the larval stage often localizes in the central nervous system causing a clinical disorder known as neurocysticercosis (10, 9) a major cause of seizures and epilepsy in most of the developing world. Human cysticercosis is highly endemic in Latin America, Asia and Africa, especially in those countries where domestic pig husbandry is practiced. It is also increasing in industrialized countries due to immigration of tapeworm carriers (9, 30).

The human *T. solium* carrier is the sole source of cysticercosis infections for pigs and neurocysticercosis in humans. The early identification of taeniasis due to *T. solium* is of great importance due to its epidemiological implications. Currently the diagnosis of taeniasis is based on the detection of eggs by microscopic
observation of fecal samples. This technique lacks both sensitivity and specificity, since eggs of most members of the family Taenidae are morphologically indistinguishable and are shed intermittently. Detection of *T. solium* coproantigen by the ELISA technique (2, 3, 5) is also used. The method is more sensitive than microscopy but cross reacts with *T. saginata*. Differentiation of *T. solium* and *T. saginata* is based on the morphological characteristics of the scolex or gravid proglottids (21). Recovery of scolices after treatment is unusual for *T. solium* and in many cases both the scolex and proglottids can be recovered only after special treatment (18).

DNA based differentiation of *T. solium* and *T. saginata* has been described and includes the use of probes (4, 6, 15), the polymerase chain reaction (PCR) with species specific primers (13, 14, 31), and PCR followed by restriction enzyme analysis (21, 26, 32). However, these methods require pure parasite DNA, which means that the DNA has to be extracted from a single proglottid and adequately cleaned because these primers may amplify any eukaryotic DNA causing cross amplification. A few reports have described the use of DNA based techniques to differentiate *T. solium* from *T. saginata* from fecal samples (23, 24, 31) but they still lack sensitivity.

The goal of this study was to develop a rapid and sensitive PCR based technique for the specific detection and diagnosis of taeniasis due to *T. solium* directly from fecal samples.
**MATERIALS AND METHODS**

**Parasitic material from infected animals.**

*T. solium* cysticerci were dissected from naturally infected pigs, washed twice with 0.01M Tris-HCl (pH 8.0), and stored at –70°C until needed. Immature tapeworms were obtained from hamsters that were infected under laboratory conditions with one to five *T. solium* cysticerci (20). *Echinococcus granulosus* scolices were obtained from hydatid cysts excised from naturally infected animals.

DNA from *Entamoeba hystolitica* was kindly provided by Dr. WA Jr. Petri (University of Virginia). DNA from *Fasciola hepatica* was provided by Dr. P. Herrera (Universidad Peruana Cayetano Heredia).

**Tapeworms from infected individuals.**

Taenia tapeworms were obtained from naturally infected patients after informed consent and treatment. After recovery, proglottids were identified as *T. solium* or *T. saginata* by PCR-REA as described previously (21). Recovered parasitic material was identified by examination of scolex (when recovered), uterine lateral branch counting (histology) and PCR-REA. A total of 25 *T. solium* proglottids and 17 *T. saginata* proglottids obtained from different patients were used to test the specificity of the PCR assay.

Proglottids of *Diphyllobothrium sp* (*n*=2), *Hymenolepis diminuta* (*n*=1) and *Hymenolepis nana* (*n*=1) tapeworms were recovered from infected patients after informed consent and treatment. The specimens were identified by PCR-REA as described (21).
Positive stool samples.

All known positive samples were obtained from previous field studies (18). Taeniasis positive patients identified upon microscopic examination were given standard medical treatment as indicated (18). Informed consent was approved by the ethical committee of the John Hopkins School of Public Health and the Universidad Peruana Cayetano Heredia. Stool samples were collected following treatment and preserved by diluting one volume of fecal sample with two volumes of 2.5% (w/v) of potassium dichromate. Samples diluted in potassium dichromate were kept at room temperature or 4°C until used. A total of 35 fecal samples collected from patient with taeniasis were analyzed in the present study, 32 patients were identified as *T. solium* carriers and 3 as *T. saginata* carriers.

Stool samples positive for *H. nana* (n=10), *Diphyllobothrium sp.* (n=5), *Strongyloides stercoralis* (n=3), and hookworm sp. (n=2) were also tested by Tso31 nested PCR. All stool samples were positive for their respective parasite by microscopy and were preserved in potassium dichromate.

Testing of all previously identified positive samples were performed randomly in a blinded fashion along with 100 stool samples that were negative for taeniasis by microscopy. Those negative samples were obtained in previous studies from shantytown in Lima, Peru which is non-endemic for taeniasis. These included samples positive to parasites such as *Ascaris lumbricoides* (n=1), *H. nana* (n=4), *Giardia lamblia* (n=33), *Cryptosporidium sp.* (n=1), *Cyclospora cayetanensis* (n=2), *Blastocystis hominis* (n=1), *Chilomastix mesnili* (n=12), *Iodamoeba butschlii* (n=2), *Entamoeba coli* (n=29) and *Endolimax nana* (n=22).
DNA extraction from stool samples.

One milliliter of potassium dichromate preserved stool samples was washed three times with reagent grade water by centrifugation at 18300xg (14000rpm) for 10 min. The pellet was then extracted following the FastDNA® SPIN® Kit for Soil DNA extraction protocol (25).

**Primers**

Primers were designed based on the recently published gene sequence that encodes the *T. solium* oncosphere specific protein Tso31 (Gene bank accession DQ861410). The outer PCR was performed using the primer pair F1: 5’ ATG ACG GCG GTG CGG AAT TCT G 3’ and R1: 5’ TCG TGT ATT TGT CGT GCG GGT CTA C 3’ and was predicted to amplify a 691bp segment. The second PCR (nested PCR) was performed using primers: F589: 5’ GGT GTC CAA CTC ATT ATA CGC TGT G 3’ and R294: 5’ GCA CTA ATG CTA GGC GTC CAG AG 3’ and were predicted to amplify a 234 bp DNA fragment.

**PCR conditions.**

The master mix for the outer PCR was composed of 1X PCR buffer (Applied Biosystems, Foster city, CA), 3 mM of MgCl₂ (Applied Biosystems), 200uM of each dNTP (Invitrogen, Carlsbad, CA), 0.2 µg/µl of BSA, 0.8µM of each primer, 0.125U of Taq Polymerase (Invitrogen) and 2.0 µl of extracted DNA in a total volume of 25 µl. The nested PCR was carried out in a PCR mix similar to that used for the outer PCR except that the final concentration of MgCl₂ was 2.5 mM and one micro-liter of
the first PCR amplification was used as a DNA template.

PCR amplification was carried out in a PTC200-MJ research cycler (Bio-Rad, Hercules, CA). The outer PCR amplification consisted of an initial denaturation step at 95°C for 3 min, followed by 20 amplification cycles each consisting of a denaturation step at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. The second PCR was carried out similar to the outer PCR except that 40 cycles were performed with an annealing step at 60°C for 30 sec. Amplification products were electrophoresed using a 2% agarosa gel. The gels were stained with ethidium bromide (10 μg/ml) for 10 min. Stained gels were then visualized under the UV light and documented.

Analytical sensitivity of the Tso31 Nested PCR.

A fecal sample, determined to be negative both by microscopy and coproantigen test, was used to assess the detection limit of the Tso31 nested PCR. The sample was distributed in 250 mg aliquots and each aliquot was contaminated in duplicate with 1; 2; 5; 10; 40; 50 or 100 *T. solium* eggs. The aliquots containing eggs were processed for DNA extraction as describe above for stool samples.

To determine the pure DNA detection limit of the Tso31 nested PCR, a DNA extracted from *T. solium* cysts was quantitated using a spectrophotometer and diluted in PCR grade water in tenfold dilutions. Diluted DNA was then amplified as described above.
Field testing

To further test the Tso31 nested PCR assay and confirm the results of the PCR on archived specimens, we conducted a case control study using samples obtained before treatment from an endemic population in Puno, Peru. Out of 982 stool samples collected 11 (1.1%) were positive by both ELISA-coproantigen (2) and microscopy. Tapeworm infected patients were given standard treatment and post-treatment stools were collected for confirmation. Those 11 positive samples were considered as cases. Four negative controls per case were randomly selected among the samples that tested negative for taeniasis both by ELISA-coproantigen and microscopy (44 out of 971). Specimens were coded so that the PCR was performed in a blinded fashion. All samples had DNA extracted and were tested by the Tso31 nested PCR as described below.
RESULTS

PCR from DNA extracted from parasites.-

All DNA samples extracted from *T. solium* proglottids, cysts and tapeworms from hamsters after Tso31 nested PCR gave a single amplification product of 234bp (Fig 1). All the 25 DNA samples extracted from *T. solium* proglottids were positive by Tso31 nested PCR. None of the 17 DNA samples extracted from *T. saginata* proglottids amplified by Tso31 nested PCR. The Tso31 nested PCR produced no amplification when DNA obtained from *E. granulosus* scolices, proglottids of *H. nana* (n=1), *H. diminuta* (n=1) or *Diphyllobothrium* sp (n=2) was used as target DNA. *Entamoeba hystolitica* and *Fasciola hepatica* DNA did not produce any amplification when assayed by the Tso31 nested PCR.

Tso31 nested PCR with defined samples in laboratory conditions.-

A total of 31 fecal samples out of the 32 known to be positive to *T. solium* were amplified using the Tso31 nested PCR described above. The only stool sample negative by the Tso31 nested PCR did not amplify even when dilutions were made to discard presence of inhibitors or when DNA was extracted again from the original sample. However, the DNA extracted from the tapeworm recovered from this patient gave a positive amplification. There was no amplification using DNA samples extracted from the three *T. saginata* carriers. No amplification products were observed when 100 taeniasis negative stool samples obtained from subjects living in Lima, a non endemic
region, were tested by Tso31 nested PCR. Similarly none of the stool samples were positive by Tso31 nested PCR for *T. solium* when tested with specimens positive for *Diphyllobothrium sp.* (n=10), *H. nana* (n=5), *Strongyloides stercoralis* (n=3) or hookworm (n=2). Also, no amplification was observed when using DNA extracted from stool samples positive for *Giardia lamblia*, *Cryptosporidium sp.*, *Cyclospora cayetanensis*, *Blastocystis hominis*, *Chilomastix mesnili*, *Iodamoeba butschlii*, *Entamoeba coli*, or *Endolimax nana*. Using *T. solium* positive samples that were previously identified after treatment (archived stool samples), the sensitivity and specificity of the Tso31 nested PCR was 97% and 100% respectively (Table 1).

The lower detection limit of the Tso31 nested PCR in a fecal sample was 10 eggs per 250 mg of stool sample (40 eggs/g stool sample) using the FastDNA® SPIN® Kit for Soil DNA extraction (Fig 1). While the detection limit using purified DNA was 100 femtograms (Fig 2).

**Tso31 nested PCR with samples from a field epidemiological study.**

Out of the 55 samples (11 cases and 44 controls) analyzed in the case-control study, ten of the initial 11 cases gave a positive amplification when tested by the Tso31 nested PCR assay. Tapeworms recovered after treatment of the eleven cases were identified at the species level by PCR-REA. One tapeworm recovered from a taeniasis positive patient by microscopy and ELISA-coproantigen (2, 3) was further identified as *T. saginata* by PCR-REA. The stool sample obtained from this patient consistently did not give any amplification product in the Tso31 nested PCR. Ten of the recovered tapeworms were identified as *T. solium* all these
samples were positive when tested by the Tso31 nested PCR assay. Thus, under field conditions, both the specificity and sensitivity of the Tso31 nested PCR was 100%. Although samples tested in the case control study were positive by microscopy to other parasites and multiple infections were common (29 were infected with more than two parasites) the Tso31 nested PCR had no cross-reactions with any of these parasites. Among the control samples four were infected with *Ascaris lumbricoides*, two with *Enterobius vermicularis*, and four with *Hymenolepis nana*. 
DISCUSSION

Specific identification of *Taenia solium* is important because of the clinical and epidemiological consequences to public health. Patients with *T. solium* tapeworms have a risk of developing cysticercosis, while those with *T. saginata* do not. Several DNA-based assays have recently been developed to differentiate *T. solium* from *T. saginata*; however most of them rely on the use of pure parasite DNA. Parasite DNA, however, can only be obtained from proglottids recovered after treatment of tapeworm carriers. Elimination of proglottids after treatment is often erratic.

Other researchers have developed and used PCR based assays for the detection of *T. solium*. The PCR–RFLP described by Nunes et al., 2005 (24) has a detection limit of 17 eggs/g of stool sample but it is not highly sensitive (66.6%). The multiplex PCR based on the HDP2 sequence (13, 24) has been reported to have a detection of DNA equivalent to 2 eggs but has not been tested with fecal specimens, furthermore when fecal samples were artificially inoculated with known number of eggs the detection limit was of 4375 *T. saginata* eggs (35,000 pg of DNA) (23). The multiplex PCR targeting the mitochondrial *cox1* gene provides reliable results with more than 50 eggs/g feces, but it was only 48% sensitive when tested with 23 positive samples (31). Although nested PCR is a two-step procedure, it seems to be the best way to overcome the challenge of amplifying DNA extracted from stool samples. We have tested a battery of positive samples using the primers of the Tso31 nested PCR in a simple PCR format. The results of these tests were unreliable and weak amplifications and poor sensitivity was observed.
Reports on the specific detection or differentiation of T. solium from T. saginata directly from stool samples are scarce, probably because of the difficulties encountered in extracting DNA from parasite eggs and the presence of PCR inhibitors which affects the sensitivity of PCR assays. Release of oncospheres from eggs is a difficult process, which requires the use of extreme and often detrimental procedures such as treatment with bleach. The use of such procedures usually results in damage to the DNA and absence of PCR amplification. The high sensitivity achieved by this study suggest that the FastDNA SPIN® Kit for Soil is a method that overcomes with both the difficulty of obtaining DNA from Taenia eggs and the presence of inhibitors in stool samples. It is also probable that the Fast DNA extraction not only extracts DNA from eggs but also from the tapeworm tegument cells since we were able to amplify DNA extracted from stool samples from hamsters that had infertile T. solium tapeworms (data not shown).

We have tested a large collection of samples both from tapeworm carriers and non-carriers that were stored with no special storage conditions other than the suspension of specimens in 2% potassium dichromate. Some samples have been stored for up to 3 years under room temperature conditions without apparent adverse effect on the performance of the Tso31 nested PCR. Potassium dichromate is widely used for preservation and viability studies of different parasites present in fecal specimens (17, 27, 28, 29) it has been also reported for Cryptosporidium cysts and bacteria present in human fecal samples (19, 22). Extraction of DNA from Cryptosporidium cysts presents similar challenges to those encountered with DNA extraction from Taenia eggs. The high sensitivity achieved by this study suggest that the FastDNA SPIN® Kit for Soil is a method that overcomes with both the difficulty of obtaining DNA from Taenia eggs and the presence of inhibitors in stool samples. It is also probable that the Fast DNA extraction not only extracts DNA from eggs but also from the tapeworm tegument cells since we were able to amplify DNA extracted from stool samples from hamsters that had infertile T. solium tapeworms (data not shown).
DNA recovery from cryptosporidium (16).

The Tso31 nested PCR test amplifies only DNA from *T. solium* and it does not cross-react with DNA extracted from other parasites or from stool samples positive to other parasites. However, the assay has not been performed using DNA obtained from *Taenia saginata asiatica* or samples positive to this parasite. It is probable that no cross-reaction will occur since this parasite is a *T. saginata* subspecies.

The Tso31 nested PCR developed here for the detection of *T. solium* in stool samples is highly sensitive and specific. The test is reliable under conditions endemic for taeniasis and it constitutes a powerful tool for the early diagnosis of taeniasis and is a potential tool for the control of human taeniasis/cysticercosis.
ACKNOWLEDGMENTS

This study was supported by the Bill and Melinda Gates Foundation grant # 23981, the NIH grant # P01 AI51976, the Global Research Training grant # D43 TW006581, and the NIH BRAVO/MIRT grant # 5 T37 MD001427.

We would like to express our gratitude to Cesar Jeri for his contribution in the present study. We also appreciate the technical assistance of M-C Camila, J. B. Phu; and D. Sara.
**Table 1.** - Results of the Tso31 nested PCR assay on archived stool samples from *Taenia solium* positive and negative patients.

<table>
<thead>
<tr>
<th>Tso31-nested-PCR</th>
<th><em>T. solium</em> taeniasis</th>
<th>% Predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative*</td>
</tr>
<tr>
<td>Positive</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>123</td>
</tr>
</tbody>
</table>

%Sensitivity-specificity: 96.9% 100.0%

*Negative samples included 100 samples from a nonendemic population, 3 samples positive to *T. saginata*, 10 positive to *H. nana*, 5 positive to *Diphyllobothrium sp.*, 3 positive to *Strongyloides stercoralis* and 2 positive to hookworms.
Table 2. - Performance of the Tso31 nested PCR in a case-control study conducted using stool samples obtained from a taeniasis endemic population before treatment.

<table>
<thead>
<tr>
<th>Tso31-nested- PCR</th>
<th>Cases&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>T. solium</em></td>
<td><em>T. solium</em> negative</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cases were taeniasis positive stool samples positive by ELISA-coproantigen and/or by microscopy.

<sup>b</sup> One sample was considered initially as a case, but the tapeworm recovered after treatment was identified as *T. saginata* by PCR-REA and by morphology of the scolex.
**FIGURE LEGENDS**

**Figure 1.** - Tso31 nested PCR amplification using DNA extracted from different sources.

Electrophoresis was performed using 5 µl of amplification product. Lanes 1 & 13: 100 bp ladder; lanes 2 - 8: DNA from a contaminated sample with 100, 50, 20, 10, 5, 2, and 1 *T. solium* eggs respectively; lane 9: DNA from a *T. solium* proglottid; lane 10: DNA form *T. saginata* proglottid; lane 11: DNA from a *T. solium* positive stool sample; lane 12: DNA from a *T. saginata* positive stool sample.

**Figure 2.** – Analytical sensitivity of the Tso31 nested PCR

Lanes 1 & 10: 100 bp ladder. DNA extracted from *T. solium* cysts was spectrophotometrically quantitated and diluted. PCR was carried out using different DNA concentrations as follows; lanes 2 - 9: 10 ng; 1 ng, 100 pg; 10 pg; 1 pg; 100 fg; 10 fg; 10 fg; and 1 fg respectively.
REFERENCES


