

Differentiating *Taenia solium* and *Taenia saginata* Infections by Simple Hematoxylin-Eosin Staining and PCR-Restriction Enzyme Analysis

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Species-specific identification of human tapeworm infections is important for public health purposes, because prompt identification of *Taenia solium* carriers may prevent further human cysticercosis infections (a major cause of acquired epilepsy). Two practical methods for the differentiation of cestode proglottids, (i) routine embedding, sectioning, and hematoxylin-eosin (HE) staining and (ii) PCR with restriction enzyme analysis (PCR-REA), were tested on samples from 40 individuals infected with *T. solium* ($n = 34$) or *Taenia saginata* ($n = 6$). Microscopic examination of HE staining of sections from 24 cases, in which conserved proglottids were recovered, clearly revealed differences in the number of uterine branches. Distinct restriction patterns for *T. solium* and *T. saginata* were observed when the PCR products containing the ribosomal 5.8S gene plus internal transcribed spacer regions were digested with either *AluI*, *DdeI*, or *MboI*. Both HE histology and PCR-REA are useful techniques for differentiating *T. solium* from *T. saginata*. Importantly, both techniques can be used in zones of endemicity. HE histology is inexpensive and is currently available in most regions of endemicity, and PCR-REA can be performed in most hospital centers already performing PCR without additional equipment or the use of radioactive material.

The life cycle of *Taenia solium* involves the pig as the normal intermediate host (harboring the larval vesicles, or cysticerci) and humans as the definitive host (harboring the adult tapeworm). Eggs are shed into the environment via human feces, and when ingested by pigs, they develop into tissue cysts, causing cysticercosis (5). Humans can also be infected with the larval stage after accidental ingestion of eggs excreted in their own feces or in the feces of another tapeworm carrier (7, 12). Human cysticercosis is endemic in most developing countries (7, 11). It often attacks the human central nervous system (CNS), causing a variety of neurologic symptoms, most commonly epilepsy (9, 11, 12).

Taenia saginata and *T. solium* are difficult to differentiate by parasitological examination because their eggs are indistinguishable (18). Correct identification is important because the consequences of human infection by these two parasites are very different. *T. saginata* is relatively innocuous, since only the intestinal tapeworm phase occurs in man, whereas infection with *T. solium* has major health effects due to extraintestinal infection by the larval or cyst phase in the CNS. Differentiation of the two human *Taenia* species is based on the number of uterine branches present in well-preserved gravid proglottids or on the absence or presence of hooks in the scolex of the tapeworm. Obtaining well-preserved and intact gravid proglottids or the scolex after treatment of the patient is often difficult due to the partial destruction of gravid proglottids or the recovery of only immature proglottids in the stool. In our exper-

ience with niclosamide and a ricine oil purgative, only immature proglottids were obtained for nearly half the patients. Other methods, such as biochemical analysis of total protein (6) or zymogram patterns (14, 15), have been explored but are difficult to interpret and inconsistent in their results.

Recently, DNA hybridization techniques have been used to differentiate between *T. solium* and *T. saginata* (13, 19, 20). However, hybridization is best performed with radioactive probes, which are expensive, are difficult to handle, and require special equipment. Simpler, more easily performed diagnostic assays for the diagnosis of these two cestodes are still needed, especially for use in developing countries. In this study we examined the utility of two methods for the differentiation of the two human taeniid species. The first is the use of hematoxylin-eosin (HE) staining of histological sections of whole gravid proglottids. The second is the use of PCR and restriction enzyme analysis (REA), which can be used to identify these tapeworms by using DNA from proglottids whether they are gravid or not, or from eggs. The PCR-REA we describe here is based on the amplification of ribosomal DNA (rDNA), which has been used to characterize the Asian taenia and to differentiate between strains of *Echinococcus granulosus* (2, 3, 23). For the differentiation of *T. solium* and *T. saginata*, we amplified the region spanning the 3' region of the 18S and the 5' region of the 28S ribosomal gene (including the 5.8S ribosomal gene) and then carried out REA of the PCR product. Typical restriction patterns were observed after electrophoresis and ethidium bromide staining.

MATERIALS AND METHODS

***Taenia* species proglottids and eggs.** Parasitological material was obtained from 40 individuals diagnosed by detection of *Taenia* species eggs upon micro-

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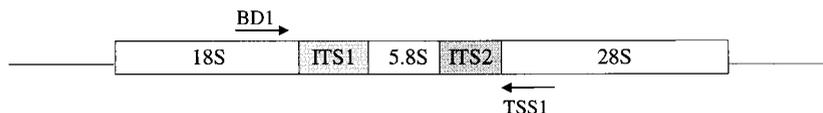


FIG. 1. Structure of a single rDNA repeat unit showing the primer annealing position for the PCR-REA.

scopic examination of stool or by a coproantigen detection enzyme-linked immunosorbent assay (1). *Taenia* proglottids were recovered from stool samples before or after taenicide treatment with 2 g of niclosamide followed by a purge with ricine oil (4). The proglottids were washed repeatedly with distilled water, followed by a final wash in 0.01 M Tris-HCl (pH 8.0) to remove all fecal material and debris. Samples obtained in the field were stored in 70% ethanol. A portion of the proglottids was stored at -70°C for later DNA isolation. The remaining tapeworm segments were stored at 4°C in 2.5% sodium dichromate. Eggs were obtained from the sediment of the sodium dichromate solution containing the stored tapeworm segments after centrifugation at $3,000 \times g$ at room temperature for 5 min.

Immature *T. solium* worms (not containing eggs) were recovered from hamsters, which had been orally infected with one to five *T. solium* cysticerci, as previously described (16). These tapeworms were used as a positive control for DNA analysis.

T. solium cysticerci were dissected from naturally infected cysticercotic pigs, washed with 0.01 M Tris-HCl (pH 8.0), and then cut to drain out the cyst fluid. The cyst tissue was again washed and stored at -70°C until needed.

Other tapeworms. *Diphyllobothrium latum* and *Hymenolepis nana* worms were recovered from infected patients treated with niclosamide and were processed as described above for *Taenia* species. *E. granulosus* scolices were obtained from hydatid cysts from naturally infected sheep.

Morphological identification. After trying, with poor results, to identify *Taenia* species by traditional methods in which a whole gravid proglottid is squashed between two glass plates and then injected with a carmine dye by using a 26-gauge needle (22), we developed a simpler and highly reliable method for *Taenia* identification. The method, which utilizes histological sections stained with HE to count the uterine branches, requires an intact gravid proglottid and follows the procedure used for processing biopsy samples with HE staining. The proglottid was fixed in neutral buffered 10% formalin, embedded in paraffin, and cut into longitudinal sections of 6 μm , which were stained and mounted; then uterine branches were counted under a light microscope at a magnification of $\times 40$. Proglottids were identified as *T. solium* when 10 or fewer branches arose to each side from the central uterus and as *T. saginata* when there were 12 or more branches (10).

We define a gravid proglottid as one that contains uterine branches filled with eggs. An immature proglottid is defined as one that does not have a fully mature reproductive system and is without eggs. A mature worm is defined as one with gravid proglottids, while an immature worm is one that does not have gravid proglottids.

DNA extraction. Frozen proglottids or cysts were homogenized manually in a glass tissue grinder in an ice bath. The homogenate was incubated for 1 h at 37°C with 10 volumes of lysis buffer (10 mM Tris-HCl, 100 mM EDTA, 0.5% sodium dodecyl sulfate [pH 8.0]) to which was added 200 μg of proteinase K (Gibco, Life Technologies)/ml. The sample was gently vortexed before incubation for 3 h at 50°C . The DNA was then extracted with phenol-chloroform-isoamyl alcohol (25/24/1 [wt/vol]) followed by chloroform-isoamyl alcohol (25/24/1 [wt/vol]) (21). The DNA was precipitated with cold ethanol and 3 M ammonium acetate at -20°C overnight and then pelleted by centrifugation at $12,000 \times g$. The pellet was then reconstituted in PCR water.

Oncosphere DNA was obtained from *Taenia* eggs by the following procedure. Embryonic plates were first dissolved by incubating eggs with 0.75% sodium hypochlorite (Mallinckrodt Baker, Phillipsburg, N.J.) in an ice bath for 15 min (17). After room temperature centrifugation at $3,000 \times g$ for 3 min, the sediment containing the oncospheres was washed twice with 0.01 M Tris-HCl (pH 8.0). The sediment was then subjected to three cycles of thawing and freezing in a dry-ice-ethanol mix in order to rupture the oncospheres; then DNA extraction was performed under the same conditions as those for proglottid DNA.

E. granulosus scolices were harvested from a fertile hydatid cyst. After centrifugation at room temperature at $3,000 \times g$ for 3 min, the sediment containing the scolices was washed three times with 0.01 M Tris-HCl (pH 8.0) and then subjected to DNA extraction as described above.

D. latum and *H. nana* were processed by the same technique as that for *Taenia* species.

Primers. rDNA is organized into units with very strongly conserved coding regions separated by relatively poorly conserved noncoding spacer regions (internal transcribed spacer 1 [ITS1] and ITS2). ITS regions have been widely used to differentiate between strains of *E. granulosus* (2, 3). Because ITS regions could be too variable for identification purposes, we included in the target DNA both ITS regions and the more conserved 5.8S gene.

Two primers were used. The first primer, BD1 (5' GTCGTAACAAGGTTT CCGTA 3') (2), was designed to hybridize with the 3' region of the 18S ribo-

somal gene. The second primer, TSS1 (5' ATATGCTTAAGTTCAGCGGGTA ATC 3'), was designed to hybridize with the 5' region of the 28S ribosomal gene (as shown in Fig. 1).

PCR and REA. The PCR was performed on a Perkin-Elmer Cetus thermocycler system 2400, in a total volume of 50 μl by using 100 ng of total DNA. The amplification was performed in $1 \times$ PCR buffer (Gibco, Life Technologies) containing 2.5 mM MgCl_2 , 0.2 mM (each) dATP, dGTP, dCTP, and dTTP, 0.5 μM each primer, and 1 U of *Taq* polymerase (Perkin-Elmer Cetus). Cycles for PCR consisted of 5 min at 94°C followed by 30 cycles consisting of 94°C for 1 min (denaturation), 56°C for 1 min (annealing), and 72°C for 2 min (elongation). Ten microliters of PCR product was separated by electrophoresis on a 1.0% agarose gel containing 0.5 μg of ethidium bromide/ml to confirm the presence of amplification products. In initial experiments the band was further purified with the Qiaex kit (Qiagen Inc., Chatsworth Calif.), but since this did not give superior results compared with direct digestion of the PCR product, the latter method was used throughout the remaining experiments. Seventeen microliters of the PCR product was digested in $1 \times$ enzyme buffer, 1 U of enzyme (*AluI*, *DdeI*, or *MboI*). Tubes containing the reaction mixture were incubated for 3 h at 37°C . Fifteen microliters of the reaction mixture was separated by horizontal electrophoresis in a 2.5% agarose gel stained with ethidium bromide.

Investigators were blinded to the identities of both PCR and histology samples.

RESULTS

Over a 1-year period we obtained specimens of *Taenia* species from 40 patients through area hospitals or field studies. In 60% (24 of 40) of the samples, well-preserved proglottids were recovered and identified by histology. Of the remaining 40%, no intact gravid proglottid was available for histology. All 40 samples were identifiable by PCR-REA.

Using HE-stained histological sections of gravid proglottids, we easily differentiated between *T. solium* and *T. saginata* by counting the number of uterine branches present. Of the 24 samples examined histologically, 18 were identified as *T. solium* and 6 as *T. saginata* (Fig. 2). Results were similar whether the proglottid was preserved in ethanol, formalin, or sodium dichromate.

PCR-REA differentiation of cestode species. Specimens that were preserved in ethanol or sodium dichromate were useful for PCR amplification, but those fixed in neutral buffered formalin were not. PCR amplification with primers BD1 and TSS1 resulted in the detection of a single band of approximately 1,300 bp for all cestodes studied, including *E. granulosus*, *H. nana*, and *D. latum* (Fig. 3). Furthermore, PCR amplification gave the same product regardless of the stage of the cestode tested (i.e., DNA from *T. solium* eggs, cysts, or immature worms or from *T. saginata* eggs, as well as from mature worms of both species).

Three restriction enzymes, *AluI*, *DdeI*, and *MboI*, proved useful for differentiating cestode species, since each enzyme gave a unique identification pattern for each cestode. There were 34 isolates which gave a restriction pattern consistent with the *T. solium* pattern (similar to that obtained with *T. solium* cysticerci from naturally infected pigs or with immature worms collected from hamsters), whereas 6 produced a restriction pattern consistent with that of *T. saginata* isolates. Results of electrophoretic analysis of *T. solium* amplified products digested with the three different enzymes are shown in Fig. 3. The patterns of *D. latum* and *E. granulosus* samples were clearly different both from each other and from that of *T. solium* or *T. saginata*.

All of the 18 specimens histologically identified as *T. solium*

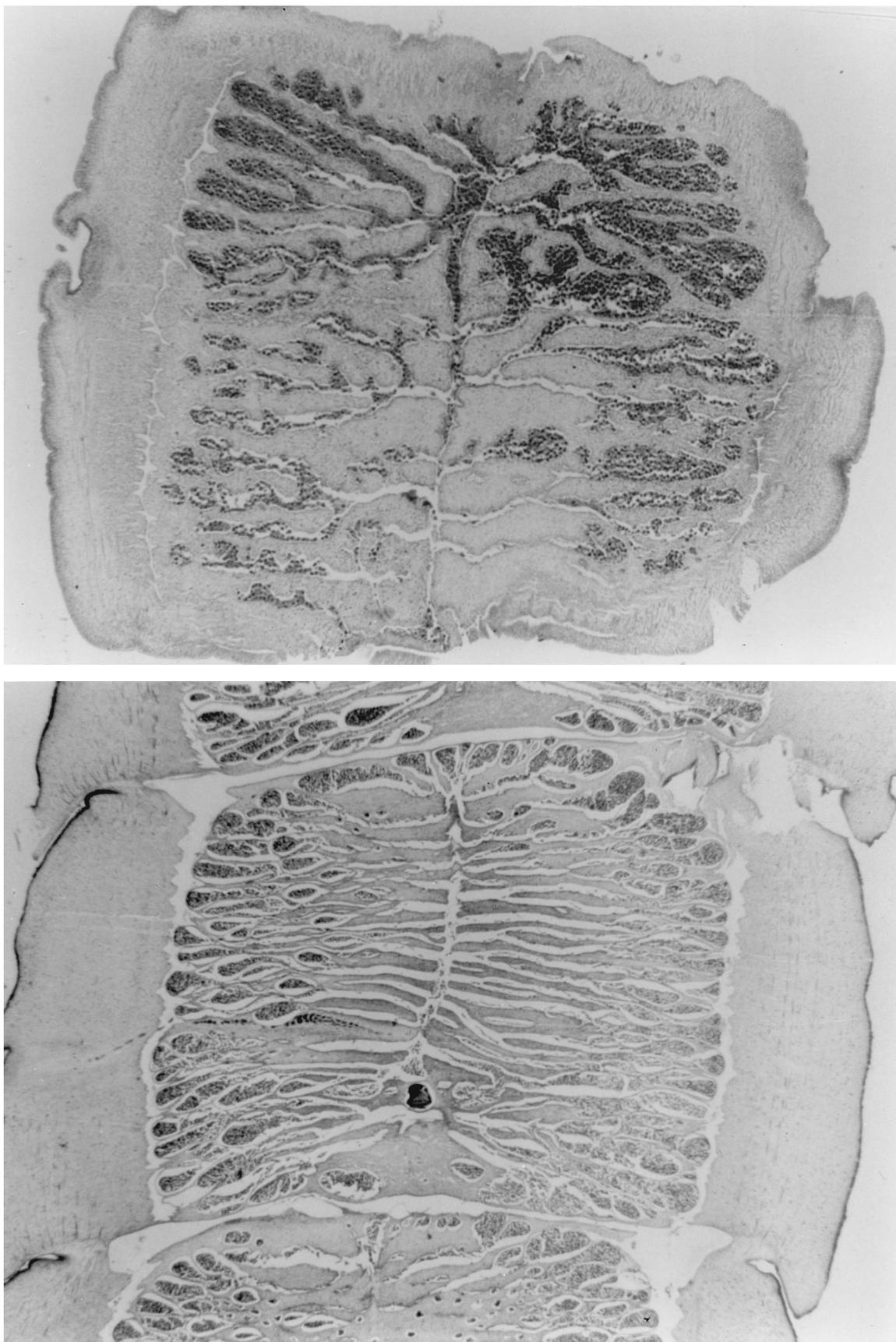


FIG. 2. Sections of HE-stained gravid proglottids of *T. solium*, with fewer than 10 uterine branches (top), and *T. saginata*, with more than 12 uterine branches (bottom). Magnification, $\times 40$.

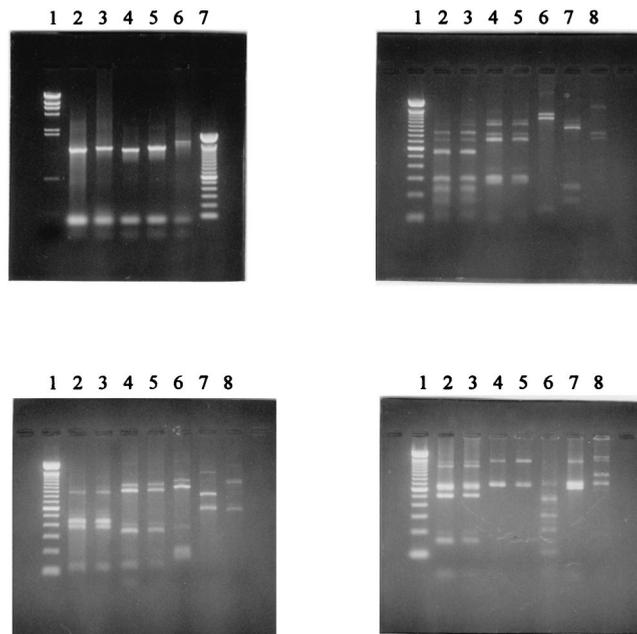


FIG. 3. PCR-amplified fragments and enzyme restriction patterns. (Upper left) PCR-amplified fragments obtained by using DNA templates from *T. solium* (lane 2), *T. saginata* (lane 3), *D. latum* (lane 4), *H. nana* (lane 5), or *E. granulosus* (lane 6). Lane 1, λ DNA/*Hind*III fragments Lane 7, 100-bp ladder. (Upper right) Restriction patterns obtained when PCR products were digested with *Alu*I. PCR was carried out with DNA templates from *T. solium* harvested from a hamster (lane 2), a *T. solium* cyst (lane 3), *T. saginata* recovered from two patients (lane 4 and 5), *D. latum* (lane 6), *H. nana* (lane 7), and *E. granulosus* (lane 8). Lane 1, 100-bp ladder. (Bottom) Restriction patterns obtained by digestion of PCR products with *Dde*I (left) or *Mbo*I (right). Lanes are as described for *Alu*I digestion.

were also identified as *T. solium* by PCR-REA. Similarly, the six specimens identified histologically as *T. saginata* were also identified as *T. saginata* by PCR-REA.

Eggs directly obtained in the concentrated sediment from gravid proglottids of *T. solium* or *T. saginata* could be identified by PCR-REA. We were not successful in amplifying the DNA of *T. solium* or *T. saginata* eggs obtained from stool samples of infected hosts.

DISCUSSION

The differentiation of *Taenia* species is important because of their very different clinical and epidemiological consequences. Patients with *T. solium* proglottids have a risk of developing cysticercosis, while those with *T. saginata* are not at risk for this disease. Proglottids obtained from stool samples, after treatment, could easily be identified by simple HE staining of histological sections of gravid proglottids and/or by a practical molecular technique (PCR-REA). The need for these tools became apparent when we were not able to obtain scolices for identification and when carmine staining of preparations of squashed proglottids gave equivocal or uncertain results. Frequently the proglottids were partially torn, or even after staining, not all the branches could be clearly noted. Both histology and PCR-REA gave clear and definitive identification of cestode species. PCR-REA was able to identify *Taenia* species even when examination by histology could not be performed, because this method does not rely on the availability of intact gravid proglottids.

Histologic identification is simple, useful, and inexpensive

and can be performed in any pathology or histology laboratory. It does not require any extra procedures or equipment. A careful search of journals and textbooks concerning tropical medicine or parasitology failed to find any reference to the use of this method for identification. Moreover, the proglottids can be stored and transported fixed in either sodium dichromate, alcohol, or formalin. However, this method is practical only when gravid proglottids are available. For 16 (40%) of our patients, proglottids could not be analyzed by this method because they were damaged or immature.

When PCR is available and no gravid proglottid is available, specimen identification can be confirmed by DNA analysis. The DNA-based identification techniques described by Rishi and McManus (19), Flisser et al. (8), Harrison et al. (13), and Chapman et al. (6a) are all hybridization methods not easily performed in developing countries. Compared to previously described methodologies, the PCR-REA method described here has many advantages. It avoids the use of scarce and expensive radioactive reagents and special equipment. It enables the distinction between *T. solium* and *T. saginata* to be made in only two steps and only requires the use of agarose gels stained with ethidium bromide for the visualization of bands. Fresh worms or those fixed in either dichromate or ethanol are suitable for this assay; however, formalin-fixed material did not amplify (data not shown). In addition, although the PCR-REA method was able to amplify eggs from proglottids, it was not successful in amplifying eggs obtained from fixed or fresh stool specimens.

Although ITS regions are normally used to demonstrate intraspecific variations, in our studies only one pattern was observed for each cestode species. Whether there may be differences between isolates of *T. solium* or *T. saginata* obtained from different geographical regions is still uncertain. Because species identification of *T. solium* and *T. saginata* is important for clinical and epidemiological purposes, further studies are now under way to refine these techniques and permit the detection and distinction of taenia eggs directly from clinical stool samples.

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