

Comparison of Kato–Katz, PCR and coproantigen for the diagnosis of *Taenia solium* taeniasis



Research Article

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Abstract

Four methods were compared for the diagnosis of human taeniasis caused by *Taenia solium*. Fecal samples from persons living in a *T. solium* endemic region of Madagascar were examined for taeniid eggs by the Kato–Katz method. Subsequently, samples positive ($n = 16$) and negative ($n = 200$) for *T. solium* eggs were examined by (i) amplification of the fragment of small subunit of the mitochondrial ribosomal RNA (*rrnS*) gene using conventional polymerase chain reaction (PCR) and (ii) a nested PCR of a fragment of the *T. solium* *Tso31* gene. Additionally, 12 egg-positive and all egg-negative samples were tested for coproantigen detection. A further 9 egg-positive fecal samples were examined using both PCRs. Of the 12 egg-positive samples tested by PCRs and coproantigen methods, 9 (75%) were positive by *rrnS* PCR, 3 (25%) using *Tso31*-nested PCR and 9 (75%) by coproantigen testing. None of the 200 egg-negative fecal samples was positive in either *rrnS* or *Tso31*-nested PCR. Twenty of the 25 egg-positive samples (80%) were positive in *rrnS* PCR, and DNA sequencing of PCR amplicons was obtained from 18 samples, all confirmed to be *T. solium*. Twelve of the 25 egg-positive samples (48%) were positive in the *Tso31*-nested PCR, all of which were also positive by *rrnS* PCR. It is suggested that species-specific diagnosis of *T. solium* taeniasis may be achieved by either coprological examination to detect eggs or coproantigen testing, followed by *rrnS* PCR and DNA sequencing to confirm the tapeworm species in egg-positive or coproantigen-positive samples.

Introduction

Taenia solium is the aetiological agent of neurocysticercosis in humans, one of a number of neglected tropical diseases recognized by the World Health Organization (2015). The parasite is transmitted in a cycle between humans, who harbour the adult tapeworm in the small intestine (taeniasis), and pigs where the larval stage (cysticercus) develops in the muscles and brain after ingesting feces or other items contaminated with *T. solium* eggs. Humans may also develop cysticercosis by ingesting eggs from the feces of a person harbouring the *T. solium* tapeworm. Infection in the brain and other nervous tissue of humans by *T. solium* cysts (neurocysticercosis) is a serious cause of morbidity in areas having poor sanitation and free-roaming pigs (Garcia *et al.*, 2020).

Efforts to prevent the transmission of *T. solium* and thereby reduce the incidence of neurocysticercosis rely on the treatment of patients with taeniasis, vaccination and medication of pigs, and improvements in sanitation and pig-rearing practices (Lightowlers, 2013).

Diagnostic tests for taeniasis are undertaken to determine the risk for transmission of cysticercosis in humans, identify endemic areas and to determine the outcomes of control programmes. *Taenia solium* taeniasis can be diagnosed by detection in the feces of eggs, tapeworm segments, parasite antigens or *T. solium* DNA in the feces (Praet *et al.*, 2013), or by serology with recombinant antigens (Levine *et al.*, 2007).

Human taeniasis is caused by 3 *Taenia* spp., including *T. solium*, *T. saginata* and *T. asiatica*. However, only *T. solium* causes neurocysticercosis and warrants a public health intervention. *Taenia saginata*, in particular, is widely distributed in areas where *T. solium* is prevalent; hence, diagnostic tests for *T. solium* taeniasis must differentiate *T. solium* from infection with other *Taenia* species. Egg morphology does not allow differentiation among *Taenia* species. Similarly, the coproantigen tests that have been described and well validated are unable to differentiate between *Taenia* spp. Recombinant antigens required to undertake species-specific

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serology (Levine *et al.*, 2007) are not readily available. Serological methods are also limited because they are unable to differentiate a current infection from past infection. At least 20 different DNA-based methods have been described for species-specific differentiation of *T. solium*, including at least 15 different polymerase chain reaction (PCR)-based methods; however, few have been validated carefully with parasitologically proven fecal samples from patients with *T. solium* taeniasis (Lightowlers *et al.*, 2016).

Here we compare 4 tests for the diagnosis of taeniasis and evaluate coproantigen and 2 PCR-based tests for species-specific diagnosis of *T. solium* taeniasis.

Methods

Human fecal samples

Human fecal samples were collected from people in a contiguous area of Betafo and Mandoto provinces of Madagascar as part of baseline evaluations for a *T. solium* control programme. The region was known to be endemic for *T. solium*. Two fecal samplings were undertaken. Initially, 960 samples were collected from randomly selected individuals. Samples were examined freshly for the presence of eggs and aliquots of approximately 2 g were suspended in a 10× volume of 90% ethanol (for DNA analyses) and stored at room temperature. Subsequently, a further 960 fecal samples were selected by purposive sampling, selecting random individuals proportional to the number of pigs present in the area, instead of proportional to the number of people as it was done in the first sampling. These samples were examined and stored in ethanol as above, and in addition, a 2 g sample was placed into a 10× volume of 10% formalin (for coproantigen testing) and stored at room temperature. A further 9 egg-positive fecal samples stored in ethanol were available from a previous study undertaken in Madagascar (Ramianrasoa *et al.*, 2020).

Egg detection

All fecal samples were examined using the Kato–Katz technique to identify taeniid eggs (Katz *et al.*, 1972). Most of the samples were evaluated using 1 slide for the Kato–Katz; however, during the second sampling, 78 samples were evaluated by using 2 slides from the same sample.

DNA isolation

Fecal DNA was isolated from all egg-positive fecal samples ($n = 25$) as well as from 200 randomly selected fecal samples which were found to be egg-negative. Approximately, 250 mg feces were placed in a 1.5 mL microtube and centrifuged at 1000 g for 1 min. The supernatant was discarded and the pellet was resuspended in 1 mL of distilled water by vortexing. Following re-centrifugation, the pelleted feces were processed for DNA isolation using the Qiagen QIAamp® PowerFecal® Pro DNA kit and Qiagen TissueLyser II homogenizer, according to the manufacturer's instructions with elution of fecal DNA in a volume of 50 µL. Purified DNA quantities were determined using the NanoDrop One instrument (ThermoFisher Scientific, Wilmington, DE, USA).

rrnS PCR

All egg-positive and 200 egg-negative fecal DNA samples were assessed by *rrnS* PCR. A 267 bp fragment of the small subunit of the mitochondrial ribosomal RNA was amplified, corresponding to positions 12 208–12 475 on the complete mitochondrial genome of *T. solium* (GenBank AB086256.1). The PCR was based on the generic *Taenia* spp. PCR described by Trachsel

et al. (2007) for the investigation of *Taenia* spp. infecting canines and also used by Ash *et al.* (2017) for human taeniasis. Modified primers (hCest3 5' TGA TTC TTT TTA GGG GAA GGT GTR GTG 3', hCest5 5' GCG GTG TGT ACA TGA GYT AAA C 3') were designed more specifically to suit amplification of the sequences from the 3 *Taenia* sp. infecting humans. Magnesium ion concentration and annealing temperature in PCR were optimized using purified *T. solium* genomic DNA (Gauci *et al.*, 2019). Fifty microlitre reaction volumes were prepared containing 3 mM MgCl₂, 50 µM deoxynucleotide triphosphate (dNTP) (Promega), 0.5 µM hCest3 and hCest5 primers, GoTaq Green Reaction Buffer (Promega), 1.25 U GoTaq Flexi DNA polymerase (Promega) and, unless otherwise noted, 2 µL isolated fecal DNA. Controls included 40 pg purified genomic DNA from *T. solium*, *T. saginata* and/or *T. asiatica* (Gauci *et al.*, 2019), and fecal DNA isolated from a volunteer known never to have been infected with *Taenia* sp. PCR conditions were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, followed by the final extension at 72°C for 5 min.

Agarose electrophoresis and DNA sequencing

PCR products were electrophoresed in 1.5% agarose, 0.5× Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer (0.05 M Tris, 0.05 M boric acid, 0.01 M EDTA), 1:10 000 Gel Red (Biotium). PCRs in which a 267 bp *rrnS* product could be detected in agarose electrophoresis were processed for DNA sequencing. Briefly, 10 µL of PCR amplicon were treated with 20 U exonuclease 1 (ThermoFisher) and 2 U shrimp alkaline phosphatase (ThermoFisher) at 37°C for 30 min and 85°C for 15 min. The DNA sequences of PCR amplicons were determined using the hCEST5 primer by Macrogen, North Korea, using Sanger sequencing. NCBI BLAST comparisons of *Taenia* spp. DNA sequences amplified from fecal DNA with the appropriate segment of *T. solium* (GenBank AB086256.1), *T. saginata* (GenBank AY684274.1) or *T. asiatica* (GenBank AP017670.1) mitochondrial DNA sequences readily allowed species differentiation.

Tso31-nested PCR

Egg-positive ($n = 25$) and 200 egg-negative fecal DNA samples were assessed by nested PCR amplifying the single copy of the *T. solium* genomic gene *Tso31* as previously described by Mayta *et al.* (2008). Initially, precisely the same commercial reagent suppliers and conditions described by Mayta *et al.* (2008) were used. The same nested PCR was undertaken using reagent sources as indicated above for the *rrnS* PCR and no improvement was found through the use of the particular reagent sources used by Mayta *et al.* (2008). For that reason, the following reagents and conditions were adopted for use in 50 µL *Tso31*-nested PCR reactions. Outer PCR: 3 mM MgCl₂, 50 µM dNTP (Promega), 0.5 µM F1 primers (5' ATG ACG GCG GTG CGG AAT TCT G 3') and R1 primer (5' TCG TGT ATT TGT CGT GCG GGT CTA C 3') and 4 µL fecal DNA. Incubations were 95°C for 3 min followed by 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min. The inner PCR used similar procedures with 2 µL of the outer PCR reaction, except for an MgCl₂ concentration of 2.5 mM and 40 cycles, 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'). PCR amplicons were analysed on an agarose gel as described above.

Coproantigen

The stool samples stored in 10% formalin were examined for coproantigens using the polyclonal antibody-based enzyme-

linked immunosorbent assay as described by Allan *et al.* (1990) with slight modifications (Mwape *et al.*, 2012). To determine the test result, the optical density of each stool sample was compared with the mean of a series of 8 reference *Taenia*-negative stool samples plus 3 standard deviations (cut-off).

Results

Egg detection

Four (0.4%) of the 960 fecal samples randomly selected from the study population were found to have *Taenia* eggs present using the Kato–Katz method. A further 12 fecal samples were found to have *Taenia* eggs present among the 960 samples obtained by purposive sampling (1.25%). All the egg-positive samples were identified as positive among the samples tested using a single slide, except for one, which was identified among the 78 samples tested by 2 slides, and was positive in both slides.

Species-specific diagnosis of *T. solium taeniasis*

A total of 25 fecal samples from different people, which were found to be positive for *Taenia* spp. eggs by Kato–Katz, were examined using *rrnS* and *Tso31*-nested PCRs, as well as 200 randomly selected egg-negative fecal samples. The concentration of DNA obtained from fecal samples using the PowerFecal® Pro DNA kit ranged from 0.1 to 120.1 ng µL⁻¹, depending on the organic content of the fecal sample. Twenty of the 25 egg-positive samples (80%) were positive in *rrnS* PCR. Of those 20 samples, the DNA sequence of the PCR product was successfully obtained from 18 samples, all of which were confirmed to be *T. solium* (GenBank accession no. OR098460). Twelve of the 25 egg-positive samples (48%) were positive in the *Tso31*-nested PCR, all of which were also positive by *rrnS* PCR.

Analytical sensitivity and specificity of the *Tso31* PCR

The analytical sensitivity of the *Tso31*-nested PCR was determined using purified *T. solium* genomic DNA (Fig. 1A) for comparison with the data published by Mayta *et al.* (1998). Positive reactions were seen using 500 fg DNA but not at 200 fg. Specificity of the assay is demonstrated in Fig. 1B. No reaction

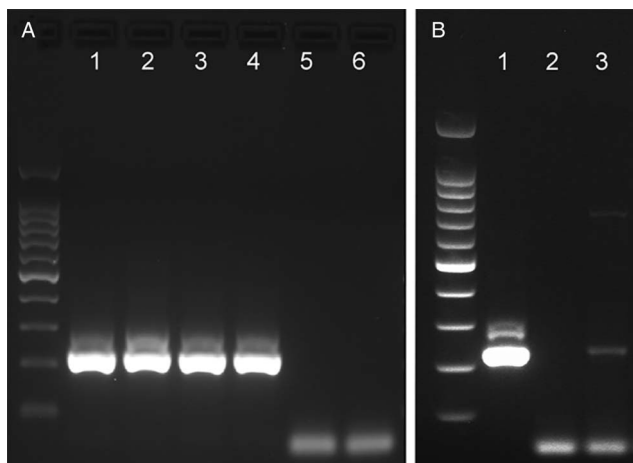


Figure 1. Agarose gel electrophoresis of PCR products showing analytical sensitivity and specificity of the *Tso31*-nested PCR. (A) Titration of *T. solium* DNA in PCR; 100 bp markers, lanes 1–6 PCRs containing 10 pg, 5 pg, 1 pg, 500 fg, 200 fg, 100 fg, DNA respectively. (B) PCR with different *Taenia* sp. DNA; 100 bp markers, lane 1 *T. solium*, lane 2 *T. saginata*, lane 3 *T. asiatica*.

products were found with *T. saginata* genomic DNA; however, a weak band was evident with genomic DNA from *T. asiatica*. This band was sequenced using the F589 primer which revealed a sequence (GenBank OQ476203) having 93% identity with the corresponding segment of the *Tso31*-nested gene.

Sensitivity comparison of PCRs with fecal DNA

The *rrnS* and *Tso31*-nested PCRs were compared using dilutions of DNA isolated from the feces of proven *T. solium* tapeworm carriers. Figure 2 shows the results of *rrnS* PCR (top panel) and *Tso31*-nested PCR (bottom panel) using the same DNA samples. DNA from egg-positive fecal samples remained detectably positive in *rrnS* PCR at 1:10 and 1:100 dilutions of the fecal samples; however, there was an absence of detectable product in some of the same dilution samples using the *Tso31*-nested PCR (Fig. 2).

Diagnostic sensitivity comparison of egg detection, PCR and coproantigen

The comparative diagnostic performance of PCR and coproantigen detection for the diagnosis of taeniasis is detailed in Table 1 for those samples for which *rrnS* PCR, *Tso31*-nested PCR and coproantigen testing were performed on each. Of the 12 egg-positive samples, 9 (75%) were positive by *rrnS* PCR, 3 (25%) were positive by *Tso31*-nested PCR and 9 (75%) were positive by coproantigen testing. Example results obtained in *rrnS* PCR and the *Tso31*-nested PCR are shown in Fig. 3. None of the 200 egg-negative fecal samples was positive in either *rrnS* or *Tso31*-nested PCR. One egg-positive sample was negative in all other tests and 2 egg-positive samples (8%) that were PCR-negative

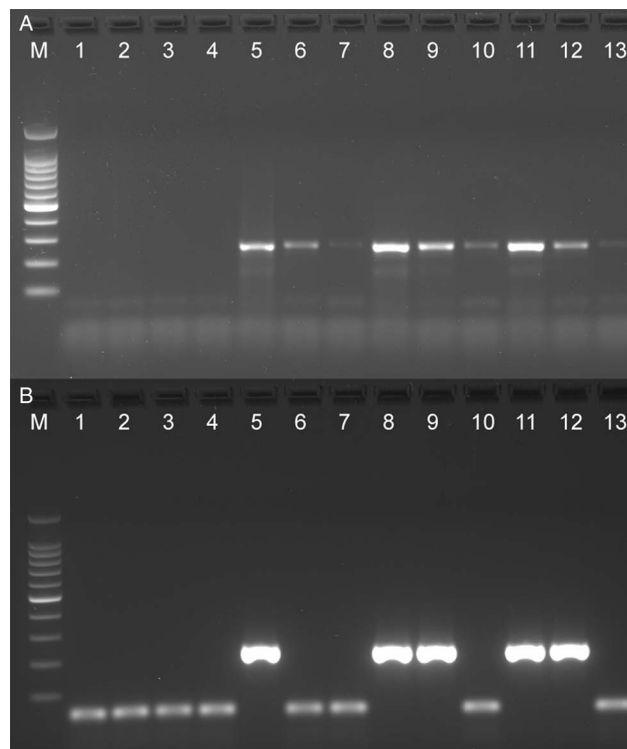


Figure 2. Comparative sensitivity of *rrnS* PCR (A) and *Tso31*-nested PCR (B) using dilutions of DNA isolated from fecal samples. 100 bp markers, lane 1 distilled water, lanes 2–4 fecal DNA from a known taeniasis-negative individual undiluted (2 µL), 1:10 dilution and 1:100 dilution, respectively; lanes 5–7, 8–10, 11–13 dilutions of fecal DNA from 3 fecal samples with proven *T. solium* infection, undiluted (2 µL), 1:10 dilution and 1:100 dilution, respectively.

Table 1. Comparison of the diagnostic performance of *rrnS* PCR, *Tso31*-nested PCR and coproantigen tests for the diagnosis of taeniasis in 12 fecal samples from different persons which were egg-positive for *Taenia* spp. by the Kato–Katz method. In addition, 200 egg-faecal samples which were negative for *Taenia* spp. eggs by Kato–Katz were also tested.

KK	<i>rrnS</i> PCR	<i>Tso31</i> -nested PCR	CoproAg	Number	% of KK positives
+	+	+	+	1	8
+	–	+	+	0	
+	+	–	+	6	50
+	+	+	–	2	16
+	+	–	–	0	
+	–	+	–	0	
+	–	–	+	2	16
+	–	–	–	1	8
–	–	–	+	2	
–	–	–	–	198	

KK, Kato–Katz test; CoproAg, coproantigen test; % of KK positives, percentage of all samples positive in Kato–Katz test.

were found to be positive by coproantigen testing. Two egg-negative samples were positive by coproantigen testing.

Discussion

Results of the detection of eggs in the feces by the Kato–Katz method corresponded with those of most egg-positive samples tested by *rrnS* PCR (Table 1). Of 12 fecal samples in which eggs were detected by Kato–Katz, 9 (75%) were positive in *rrnS* PCR. Of 9 fecal samples from proven cases of *T. solium* taeniasis (by egg detection and *rrnS* DNA sequencing), 7 (78%) were positive by coproantigen. Two of the 12 egg-positive samples were positive by coproantigen but not by PCR. Coproantigen testing identified 2 positive samples among the 200 egg-negative feces which were negative by PCR. It is unknown whether these samples were false positives in the coproantigen test or false negatives in Kato–Katz and PCR. Coproantigens can be detected in fecal samples collected prior to worm patency in both human (Tembo and Craig, 2015) and animal (Deplazes *et al.*, 1990; Allan *et al.*, 1992) cases of taeniasis, providing one possible explanation for these findings.

All taeniasis cases for which the tapeworm species was differentiated were found to be *T. solium*. A taeniasis survey undertaken in a different region of Madagascar by Rahantamalala *et al.* (2022) also identified only cases of *T. solium* infection. To date, there has been no confirmed case of *T. saginata* taeniasis described from Madagascar.

Analysis of the quantity of DNA obtained from the feces of 12 egg-positive samples revealed that the 4 samples with the smallest quantity of DNA were all negative in *rrnS* PCR utilizing 2 µL fecal DNA. Increasing the quantity of DNA to 20 µL led to 2 of these 4 samples being detected as PCR-positive. The relationship between the quantity of DNA from egg-positive samples used in *rrnS* PCR and the outcome of the test is shown in Fig. 4. One sample which was negative by both PCR and coproantigen tests was not among those with very low quantities of DNA.

We were unable to replicate the sensitivity of the *Tso31*-nested PCR described by Mayta *et al.* (2008) who recorded the test to have a sensitivity with purified *T. solium* genomic DNA as low as 200 fg, whereas in our case the test was positive at 500 fg but negative at 200 fg (Fig. 1). Variations in DNA quantitation methods are one possible reason to explain this difference. Direct comparison of the *rrnS* and *Tso31*-nested PCRs with dilutions of fecal

DNA from proven cases of *T. solium* taeniasis (proven by egg detection followed by *rrnS* PCR and DNA sequencing) found that the *rrnS* PCR was superior in sensitivity to *Tso31*-nested PCR in identifying cases of taeniasis (Fig. 2). As the quantity of fecal DNA decreased, the quantity of *rrnS* PCR-amplified DNA product declined but remained detectable. However, with the *Tso31*-nested PCR, DNA products were either abundant or completely absent, even when the *rrnS* PCR remained positive. While the *Tso31* PCR involved a pair of nested PCRs, the target (*Tso31*) is a single-copy gene in the genome (Mayta *et al.*, 2007). The *rrnS* PCR targets a segment of the mitochondrial genome. It is unclear just how many copies of the mitochondrial genome are there per cell in *T. solium*. Depending on cell type, human cells contain between 100 and 10 000 copies of the mitochondrial genome (Wai *et al.*, 2010). Ultrastructural investigations clearly identify multiple mitochondria in *T. solium* cells (Willms *et al.*, 2003) and those of other taeniids (Willms *et al.*, 2003; Jabbar *et al.*, 2010). The higher copy number of mitochondrial DNA targets for the *rrnS* PCR may explain the greater sensitivity of this technique with fecal DNA extracts from *T. solium* tapeworm carriers compared with the *Tso31*-nested PCR, despite the latter being a nested PCR which could have been expected to have greater analytical sensitivity. With a single sample of fecal DNA from a case of *T. asiatica* taeniasis, the *Tso31* PCR amplified a product of the same size as that obtained with *T. solium*, the sequence of which was similar to the sequence of the *Tso31* gene in *T. solium* (Fig. 1), suggesting that the *Tso31*-nested PCR may not be species-specific where *T. solium* and *T. asiatica* are sympatric.

Many PCR-based methods have been described for the diagnosis of *T. solium* taeniasis using fecal DNA; however, few have been validated using parasitologically proven cases of infection (Lightowers *et al.*, 2016). Of those that were well validated (e.g. Yamasaki *et al.*, 2004; Mayta *et al.*, 2008; Praet *et al.*, 2013; Rodriguez-Hidalgo *et al.*, 2015), they include procedures such as restriction fragment length polymorphism on the PCR amplicons (e.g. Rodriguez-Hidalgo *et al.*, 2015) or quantitative PCR (qPCR) (e.g. Praet *et al.*, 2013; Gordon *et al.*, 2015; Ng-Nguyen *et al.*, 2017). A comparison has not been made previously between the performance of different PCR-based methodologies using the same fecal DNA samples from confirmed cases of *T. solium* taeniasis. Rahantamalala *et al.* (2022) used an approach to species-specific diagnosis of taeniasis involving PCR with non-specific *cox1* primers followed by sequencing the DNA product

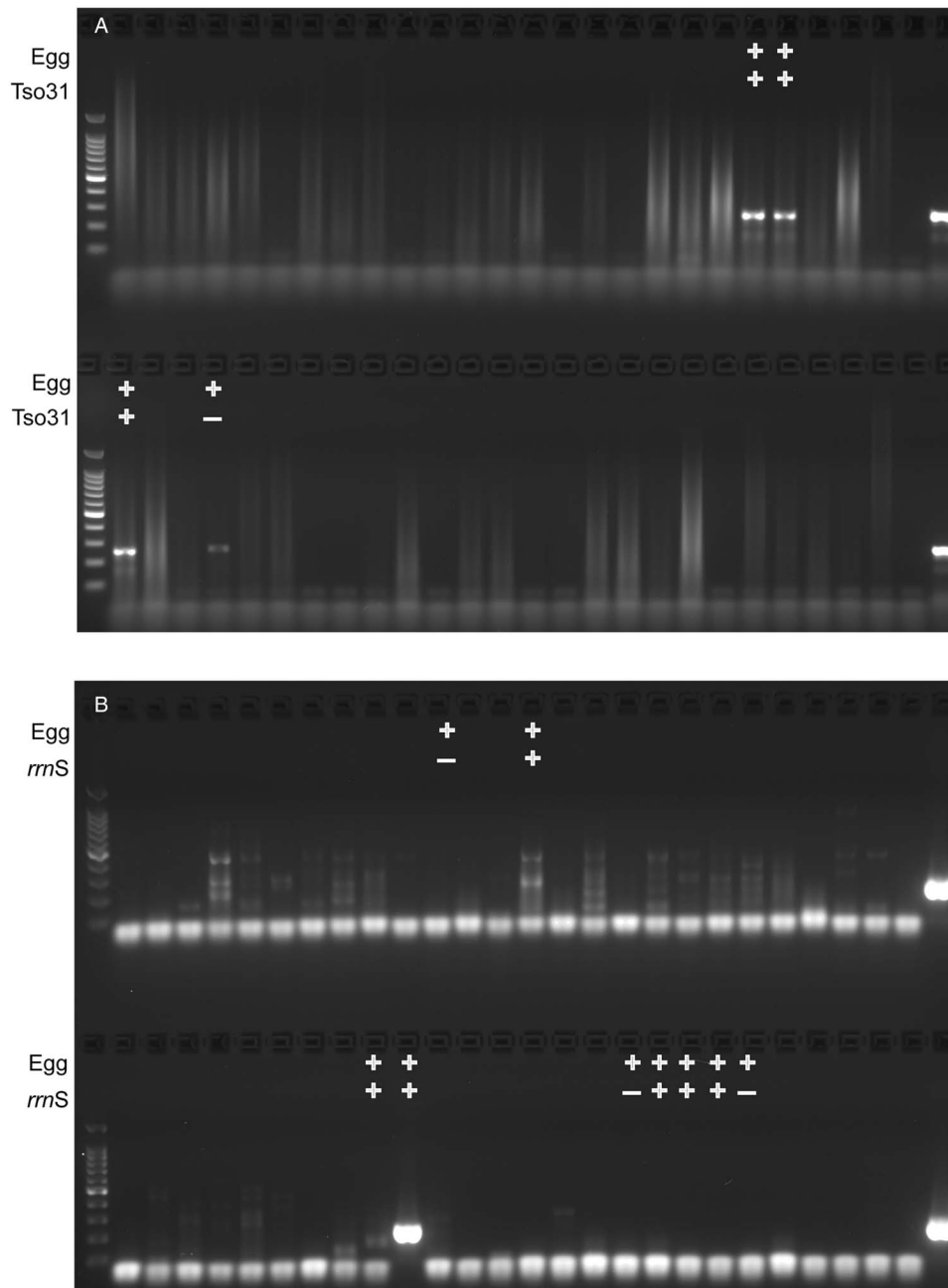


Figure 3. Agarose gel electrophoresis of (A) *rmS* PCR and (B) *Tso31*-nested PCR products obtained from DNA isolated from a subset of 52 human fecal samples that were tested by each method (different fecal samples shown in A and B). Egg+ indicates the fecal sample was positive for taeniid eggs by Kato–Katz. *Tso31*+ indicates that specific sample tested positive by *Tso31*-nested PCR, *rrnS*+ indicates that specific sample tested positive by *rmS* PCR. Left hand side lanes, 100 bp markers; right hand side lanes, *T. solium* DNA positive control.

in positive reactions, similar to the one we used. An advantage of the methodology used here, which targeted mitochondrial *rrnS*, is the small size of the target DNA sequence (267 bp) and that the 3 taeniid species infecting humans can be differentiated simply and unequivocally due to there being numerous sequence differences between the species across a region of only 30 bp. These differences can be determined from single-strand sequencing and without necessarily using sequence comparison software.

Full transmission of *T. solium* only occurs where pigs have access to materials contaminated with human feces. For this reason, transmission is limited to poor communities in developing countries. It is in these regions and countries where accurate diagnostic methods are needed for *T. solium* taeniasis. Techniques such as qPCR are poorly suited to these places.

Coproantigen testing could offer a simple, potentially inexpensive diagnostic method; however, the currently used methods are neither species-specific nor are the reagents available commercially. A publication has described a species-specific coproantigen test (Parkhouse *et al.*, 2020), which was evaluated with feces from 2 cases of *T. solium* taeniasis and 5 cases of *T. saginata* taeniasis, among other samples; however, there have been no further data published about this test in the ensuing 3 years since its description.

The data presented here suggest that a suitable method for species-specific diagnosis of *T. solium* taeniasis in fecal samples, which may be relatively simple and suitable for adoption in endemic countries, is an evaluation of fecal samples using a non-specific method such as egg detection or, where available, coproantigen testing, followed by a method where the positive

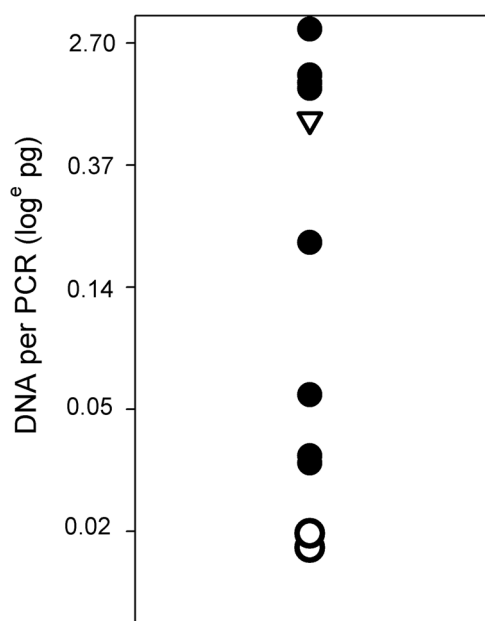


Figure 4. Relationship between the quantity of DNA isolated from 12 individual, egg-positive fecal samples and the outcome of *rrs* PCR for taeniasis. Closed symbols: PCR-positive samples; open symbols: PCR-negative samples; open circles: samples positive by coproantigen test; open triangle: samples negative by PCR and coproantigen tests.

samples are confirmed and speciated by *rrs* PCR and DNA sequencing.

Data availability. Data supporting results are provided within the article.

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Author's contribution. M. W. L. and M. D. conceived the study, obtained the funding and undertook data analyses. D. E. A. M., Mi. R., V. C. R. and Mo. R. coordinated the field activities. J. A. N. and N. S. R. organized and supervised field activities and coprological testing. H. A. R. advised on the study design. M. W. L. undertook the lab work for PCRs and DNA sequencing. C. G. G. and A. J. advised on PCR methods. K. E. M. undertook the coproantigen testing. M. W. L. drafted the manuscript and all authors contributed to the final manuscript.

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Competing interest. None.

Ethical standards. Ethics approval for the study was granted by the Madagascar Ministry for Health Ethics Committee for Biomedical Research No. 088-MSANP/SG/AMM/CERBM.

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