

Bayesian modelling to estimate the test characteristics of coprology, coproantigen ELISA and a novel real-time PCR for the diagnosis of taeniasis

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Abstract

OBJECTIVE To estimate and compare the performances of coprology, copro-Ag ELISA and real-time polymerase chain reaction assay (copro-PCR) for detection of *Taenia solium* tapeworm carriers.

METHODS The three diagnostic tests were applied on 817 stool samples collected in two Zambian communities where taeniasis is endemic. A Bayesian approach was used to allow estimation of the test characteristics. Two (0.2%; 95% Confidence Interval (CI): 0–0.8), 67 (8.2%; 95% CI: 6.4–10.3) and 10 (1.2%; 95% CI: 0.5–2.2) samples were positive using coprology, copro-Ag ELISA and copro-PCR, respectively.

RESULTS Specificities of 99.9%, 92.0% and 99.0% were determined for coprology, copro-Ag ELISA and copro-PCR, respectively. Sensitivities of 52.5%, 84.5% and 82.7% were determined for coprology, copro-Ag ELISA and copro-PCR, respectively.

CONCLUSIONS We urge for additional studies exploring possible cross-reactions of the copro-Ag ELISA and for the use of more sensitive tests, such as copro-PCR, for the detection of tapeworm carriers, which is a key factor in controlling the parasite in endemic areas.

keywords taeniasis, *Taenia solium*, Bayesian methodology, diagnosis, sensitivity, specificity

Introduction

Even though taeniasis is a worldwide parasitic disease, detection of tapeworm carriers remains a public health issue. In humans, this zoonotic disease is caused by three *Taenia* species, that is, *Taenia saginata*, *T. solium* and *T. saginata asiatica* (Murrell 2005). While the life cycle differs from one species to another, clinical signs in infected individuals are quite similar (most carriers remain asymptomatic, but gastrointestinal dysfunctions, loss of appetite and weight loss may be observed). Unlike taeniasis whose symptoms are not of major clinical

importance, the pathology caused by the establishment of the larval stage of *T. solium* in the central nervous system of accidental human intermediate hosts (neurocysticercosis) may be responsible for a high disease burden in endemic areas (Murrell 2005). On the other hand, the presence of cysticerci of *Taenia* spp. in the specific intermediate hosts, that is, cattle for *T. saginata*, pigs for *T. solium* and *T. s. asiatica*, may be of economical importance due to carcass deterioration and/or condemnation in countries where carcass inspection at abattoir level is applied (Murrell 2005; Carabin *et al.* 2006; Praet *et al.* 2009).

Coprological examination of stool samples has remained the routine method for the diagnosis and identi-

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fication of *Taenia* spp. eggs to date. Although coprology shows a high specificity, its sensitivity is rather low (Allan *et al.* 1996; Flisser 2006; Somers *et al.* 2006), and the technique does not allow differentiating between *Taenia* species, which is essential for the clinical follow-up of patients living in areas where the parasites are sympatric.

As an alternative, an immunological technique, the coproantigen Enzyme-Linked Immunosorbent Assay (copro-Ag ELISA), has been developed (Allan *et al.* 1990). The method aims at detecting tapeworm antigens in faeces using polyclonal antibodies directed against the parasite. Some studies demonstrated a higher sensitivity of the assay than coprology (Allan *et al.* 1996) although this higher test performance has been questioned elsewhere (Somers *et al.* 2006). Moreover, like coprology, copro-Ag ELISA is only genus specific and cannot differentiate between *T. solium*, *T. saginata* and *T. s. asiatica* infections (Allan *et al.* 2003; Ito & Craig 2003). A modification of the copro-Ag ELISA described by Allan *et al.* (1990) has been reported to be *T. solium* species specific with a specificity of 100% (Guezala *et al.* 2009). This hybrid assay combined the use of polyclonal antibodies against *Taenia* adult tapeworm somatic extracts and an enzyme-conjugated rabbit IgG against *T. solium* adult excretory-secretory antigen. However, this test was only validated on a small number of samples and depends on the production of polyclonal antibodies, which may induce variation from one badge of antibodies to another and impairs test reproducibility.

The possibility of serological diagnosis has been described using *T. Solium*-specific antigens to detect antibodies against adult *T. solium* in serum by Western blot. The test is reported to have a sensitivity of 95% and a specificity of 100% (Wilkins *et al.* 1999). However, serological diagnosis of taeniasis has the disadvantage of residual antibodies from past exposure of individuals that might result in false positives (Wilkins *et al.* 1999; Allan *et al.* 2003; Ito & Craig 2003). The test is also currently only used for research purposes owing to the cost and accessibility of the assay as the purification process of the antigens requires expensive equipment (Wilkins *et al.* 1999; Allan *et al.* 2003; Ito & Craig 2003).

Molecular techniques have also been developed allowing species-specific tapeworm detection in faeces (Nunes *et al.* 2003, 2005; Yamasaki *et al.* 2004; Mayta *et al.* 2008; Nkouawa *et al.* 2009; Jeon *et al.* 2011). However, these methods have not yet been properly validated in the field.

This study aims at estimating and comparing the test characteristics (sensitivity and specificity) of coprology, copro-Ag ELISA and a newly developed real-time polymerase chain reaction assay (copro-PCR) for the

detection of *T. solium* in faeces. The three diagnostic tests have been applied on stool samples collected in two Zambian communities where taeniasis is endemic. Because none of the tests included in the study design is a gold-standard method and because no gold-standard test for the diagnosis of taeniasis exists, a Bayesian approach was used to allow estimation.

Materials and methods

Study design

The sampling protocol of this study has been described in Mwape *et al.* (2012). Briefly, community-based studies were conducted between August 2009 and October 2010 in the rural communities of Kakwiya in Petauke District and in Vulamkoko, Katete District of the Eastern province of Zambia. Faecal samples were collected from willing participants. The submitted samples were divided into two aliquots, one placed in 10% formalin (for coprology and copro-Ag ELISA) and the other in 70% ethanol (for copro-PCR), and were stored at 4 °C until use. All the samples were transported to Lusaka for analysis.

The collected faecal samples were tested using three diagnostic methods, namely the microscopic identification of *Taenia* spp. eggs in faeces (coprology; Ritchie 1948), the ELISA for the detection of *Taenia* spp. antigens in faeces (copro-Ag ELISA; Allan *et al.* 1990; with modifications by Mwape *et al.* 2012) and a newly developed real-time PCR assay for the detection of *T. solium* DNA in faeces (copro-PCR).

Novel real-time PCR assay for the detection of *T. solium* DNA in faeces (copro-PCR)

DNA isolation from the ethanol-stored samples for the copro-PCR test was carried out in Lusaka as described previously (Verweij *et al.* 2009). Briefly, approximately 200 µg faeces were suspended in 200 µl PBS containing 2% polyvinylpyrrolidone (PVPP; Sigma, Steinheim, Germany) and heated for 10 min at 100 °C. After sodium-dodecyl sulphate-proteinase K treatment (2 h at 55 °C), DNA was isolated with the QIAamp Tissue Kit spin columns (QIAGEN, Hilden, Germany). In each sample, 103 PFU/ml Phocin Herpes Virus 1 (PhHV-1) was added within the isolation lysis buffer, to serve as an internal control (Niesters 2002).

DNA amplification and detection was carried out at the Leiden University Medical Centre. *T. Solium*- and *T. saginata*-specific primers, and detection probes were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) from the Internal

Transcribed Spacer 1 (ITS1) sequences for *T. solium* and *T. saginata*, respectively (GenBank accession nos. EU747662 and AY392045). The *T. solium*-specific primers, TsoITS_145F and TsoITS_230R (Biolegio, The Netherlands) were chosen such that an 86-bp fragment inside the ITS1 sequence should be amplified. The TsoITS_169Tq_FAM double-labelled probe (Biolegio) was used to detect *T. solium*-specific amplification. *T. saginata*-specific PCR primers and a detection probe were chosen such that a 79-bp fragment within the ITS1 sequence should be amplified and detected for *T. saginata* specifically. The *T. saginata*-specific primers and probe set consisted of forward primer Tsag ITS_F529, reverse primer Tsag ITS_R607 and the *T. saginata*-specific double-labelled probe Tsag ITS_581Tq_Quasar705 (Biolegio). PhHV-1-specific primers and probe (Niesters 2002) set consisted of forward primer PhHV-267 s, reverse primer PhHV-337as and the double-labelled probe PhHV-305tq (Biolegio). National Center for Biotechnology Information (NCBI) BLAST search was used to test the theoretical specificity of the primers and probes.

To establish the PCR assays, genomic DNA was isolated using the QIAamp Tissue Kit (QIAGEN) from individual proglottids of *T. solium* and *T. saginata*. Serial 10-fold dilution series of DNA extracted from each cestode were tested with and without the presence of internal control DNA to estimate the influence of the internal control. Each dilution series was also tested with and without the other target to assess the ability to detect mixed infections. The PCR and the DNA isolation protocol used was further evaluated using DNA extracts of 23 stool samples from individual patients from a *T. solium* and *T. saginata* endemic area in Ecuador, in which microscopy revealed *Taenia* eggs. The specificity of

the PCR was tested against 150 DNA controls derived from a wide range of intestinal microorganisms (Ten Hove *et al.* 2008).

Amplification reactions were performed in white PCR plates in a volume of 25 µl with PCR buffer (HotstarTaq master mix; QIAGEN), 5 mM MgCl₂, 2.5 µg Bovine Serum Albumin (Roche Diagnostics Nederland B.V., Almere, the Netherlands), 1 pmol of each *T. solium*-specific primer, 2 pmol of each *T. saginata*-specific primer, 3.75 pmol of each PhHV-1-specific primer, 1.25 pmol of each specific detection probe and 5 µl of the DNA sample. Amplification consisted of 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 30 s at 60 °C. Negative and positive control samples were included in each amplification run. Amplification, detection and analysis were performed with the CFX real-time detection system (Bio-Rad Laboratories). The PCR output consists of a cycle-threshold (Ct) value, representing the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence and indicating the parasite-specific DNA load in the faecal sample tested. Any signal up to a Ct of 50 was considered as positive. All primers and detection probes are described in Table 1.

Bayesian analysis

Because none of the diagnostic tests included in this study is a gold standard, a Bayesian analysis was used to estimate the prevalence of *T. solium* taeniasis in this population and the characteristics of the tests. A multinomial Bayesian model adapted from Berkvens *et al.* (2006) was used. Prior information on the test characteristics was extracted from available literature or obtained through expert elicitation (experts of the Institute of Tropical Medicine of Antwerp (Belgium) and of the Leiden

Table 1 Oligonucleotide primers and detection probes for real-time PCR on different targets for the detection of *Taenia solium* and *Taenia saginata* DNA

Target organism	Oligonucleotide name	Oligonucleotide sequence
<i>Taenia solium</i>	Tsol ITS_145F	5'-ATGGATCAATCTGGGTGGAGTT-3'
	Tsol ITS_230R	5'-ATCGCAGGGTAAGAAAAGAAGGT-3'
	Tsol ITS_169Tq	FAM 5'-TGGTACTGCTGTGGCGGCGG-3'-BHQ 1
<i>Taenia saginata</i>	Tsag ITS_F529	5'-GCGTCGTCTTTGCGTTACAC-3'
	Tsag ITS_R607	5'-TGACACAACCGCGCTCTG-3'
	Tsag ITS_581Tq	Quasar705 5'-CCACAGCACCAGCGACAGCAGCAA-3'-BHQ2
Phocin herpes virus 1	PhHV-267s	5'-GGGCGAATCACAGATTGAATC-3'
	PhHV-337as	5'-GCGGTTCCAAACGTACCAA-3'
	PhHV-305tq	Cy5 5'-TTTTTATGTGTCCGCCACCATCTGGATC-3'-BHQ2

BHQ, black hole quencher; PCR, polymerase chain reaction.

Table 2 Prior information included in the Bayesian model (uniform distributions)*

Probability to have a positive result for copro-Ag ELISA if the individual is infected and positive for coprology	[0.9–1]
Probability to have a positive result for copro-Ag ELISA if the individual is infected and negative for coprology	[0.5–1]
Probability to have a positive result for copro-PCR if the individual is infected, positive for coprology and positive for copro-Ag ELISA	[0.95–1]
Probability to have a positive result for copro-PCR if the individual is infected, positive for coprology and negative for copro-Ag ELISA	[0.95–1]
Probability to have a positive result for copro-PCR if the individual is infected, negative for coprology and positive for copro-Ag ELISA	[0.5–1]
Probability to have a negative result for copro-PCR if the individual is not infected, negative for coprology and negative for copro-Ag ELISA	[0.95–1]
Probability to have a negative result for copro-PCR if the individual is not infected, negative for coprology and positive for copro-Ag ELISA	[0.95–1]
Probability to have a negative result for copro-PCR if the individual is not infected, positive for coprology and negative for copro-Ag ELISA	[0.95–1]
Probability to have a negative result for copro-PCR if the individual is not infected, positive for coprology and positive for copro-Ag ELISA	[0.95–1]

*The other probabilities are not constrained and left as uniform distributions [0–1].

Coprology, microscopic identification of *Taenia* spp. eggs in faeces; copro-Ag ELISA, enzyme-linked immunosorbent assay for the detection of *Taenia* spp. antigens in faeces; copro-PCR, polymerase chain reaction assay for the detection of *T. solium* DNA in faeces.

University Medical Centre, Leiden (The Netherlands)) and expressed as conditional probabilities (Table 2; Allan *et al.* 1990; Flisser 2006; Murrell 2005). The analysis was conducted in WinBUGS and R (Ihaka & Gentleman 1996; Lunn *et al.* 2000). The Markov chain Monte Carlo model was run for 25 000 iterations, and the first 5000 iterations were discarded as the burn-in phase. Criteria assessing the fit between prior information and test results were evaluated, that is, the Bayesian *P*-value (Bayesp), the Deviance Information Criterion (DIC) and the number of parameters effectively estimated by the model (pD) (Lunn *et al.* 2000; Spiegelhalter *et al.* 2002; Gelman *et al.* 2003; Berkvens *et al.* 2006).

Ethical clearance

The study protocol and consent forms were approved by the Institutional Ethics Committee of the University of

Zambia. Further approval was sought from the Ministry of Health, District Director of Health Offices in Petauke and Katete Districts and also from the community leaders before commencement of the study. Individuals of all ages were asked to participate in the study after obtaining informed consent. For individuals younger than 18, permission was sought from their parents or guardians.

The protocol for the development and validation of the novel real-time PCR assay based on the use of DNA extracts of 23 Ecuadorian patient stool samples was approved by the Ethical Committee of the Central University of Ecuador.

Results

Novel real-time PCR assay for the detection of *T. solium* DNA in faeces (copro-PCR)

In the NCBI BLAST search, primers and probes showed 100% specificity for *T. solium* and *T. saginata*. The Ct values obtained from testing the dilution series of *T. solium* and *T. saginata* DNA in both the individual assay and the multiplex assay were similar, and the same analytical sensitivity was achieved. The lowest amount of *T. solium* DNA and *T. saginata* amplified by PCR was estimated at 1 and 2.5 fg, respectively. The individual performance of the assays was not influenced by the presence of DNA from the other cestode or by the presence of DNA from the internal control. *T. solium*-specific amplification was detected in 15 of 23, *T. saginata*-specific amplification in 7 of 23, and amplification of *T. solium* and *T. saginata* both in 1 of 23 faecal samples from Ecuador in which *Taenia* eggs were detected by microscopy with Ct values of between 21.9 and 37.7 with a median Ct value of 27.8 cycles. No amplification of *T. solium* or *T. saginata* DNA was detected in any of the 150 control samples; only the amplification of the internal control was detected at the expected Ct value of ≈ 30 .

Bayesian analysis

A total of 944 faecal samples (718 from Petauke and 226 from Katete) were collected. Of these, 817 were sufficient in quantity for the three tests. Two (0.2%; 95% Confidence Interval (CI): 0–0.8), 67 (8.2%; 95% CI: 6.4–10.3) and 10 (1.2%; 95% CI: 0.5–2.2) of 817 samples tested positive using coprology, copro-Ag ELISA and copro-PCR, respectively. One sample tested positive for *T. saginata* taeniasis using copro-PCR. The comparative results and discrepancies for the three diagnostic methods are shown in Table 3.

Table 3 Results of the three diagnostic tests applied on 817 faecal samples of the same number of individuals

Coprology	Copro-Ag ELISA	Copro-PCR	No. of individuals
0	0	0	744
0	0	1	6
0	1	0	63
0	1	1	2
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	2

0 = negative test result; 1 = positive test result; No. of individuals, number of individuals for each result category. Coprology, microscopic identification of *Taenia* spp. eggs in faeces; copro-Ag ELISA, enzyme-linked immunosorbent assay for the detection of *Taenia* spp. antigens in faeces; copro-PCR, polymerase chain reaction assay for the detection of *T. solium* DNA in faeces.

Table 4 Estimates of the characteristics of the three diagnostic tests for the detection of *Taenia solium* taeniasis

Diagnostic test	Sensitivity (95% CI)	Specificity (95% CI)
Coprology	0.525 (0.111–0.965)	0.999 (0.995–1.000)
Coproantigen ELISA	0.845 (0.619–0.980)	0.920 (0.900–0.938)
Copro-PCR	0.827 (0.570–0.976)	0.990 (0.982–0.996)

CI, Credibility Interval; Coprology, microscopic identification of *Taenia* spp. eggs in faeces; copro-Ag ELISA, Enzyme-Linked Immunosorbent Assay for the detection of *Taenia* spp. antigens in faeces; copro-PCR, polymerase chain reaction assay for the detection of *T. solium* DNA in faeces.

Bayesian sensitivity and specificity estimates of the three diagnostic tests for the detection of *T. solium* taeniasis are presented in Table 4. There was no disagreement between the prior information and the test results as indicated by the Bayesp, the DIC and the pD.

Apparent prevalence as based on copro-PCR was estimated at 1.2% (95% CI: 0.5–2.2), while Bayesian analysis estimated a true prevalence of 0.6% (95% Credibility Interval: 0.1–1.7) in the tested population.

Discussion

This study gives an estimation of the characteristics of three diagnostic tests to detect *T. solium* taeniasis in individuals living in an endemic area. Bayesian analysis shows high specificities for the three tests (between 90% and 100%). However, specificity of the copro-Ag ELISA is lower than specificity estimates for the two other tests and lower than reported in the literature (Zamora *et al.*

2004). In the present study, 63 individuals are positive for copro-Ag ELISA, but negative for the other tests. Allan *et al.* (1990, 2003) demonstrated that antigen detection in faeces is genus specific with both *T. saginata* and *T. solium* reacting in the assay but with no cross-reactions with other parasite infections. This study reports for the first time the presence of *T. saginata* in this part of Zambia (one sample tested positive for *T. saginata* taeniasis using copro-PCR and was also copro-Ag positive). The sympatricity of *T. solium* and *T. saginata* may explain the difference in specificity between the tests. However, positive individuals in coprology were both confirmed being infected with *T. solium* using copro-PCR. Moreover, beef is rarely consumed in the area, and when consumed, it is boiled, suggesting a low *T. saginata* prevalence. Therefore, possible cross-reactions of the coproantigen ELISA with parasites other than *Taenia* spp. should be further investigated. The ability of the copro-Ag ELISA to detect immature tapeworm stages may also explain the higher number of copro-Ag ELISA positive cases compared with coprology (only detecting eggs and so adult, mature tapeworms). It is not clear to what extent free DNA originating from adult or juvenile tapeworms, besides DNA from helminth eggs, contributes to the performance of the copro-PCR. The latter requires further investigation.

On the other hand, knowing that coprology is genus specific and not species specific, the specificity of coprology to detect *T. solium* taeniasis as estimated here is particularly high. This can be explained by a low prevalence of *T. saginata* infections in the region even though reaching solid conclusions is impossible as apparent prevalence based on coprology is very low (only two individuals tested positive for coprology). Using such insensitive tests, a higher sample size should be envisaged to allow interpreting the estimated test characteristics.

Several authors have questioned sensitivity of coprology mainly because, even in high-prevalence *T. solium* pig and human cysticercosis areas, apparent prevalence of taeniasis as estimated using coprological detection is low. The discrepancy between high cysticercosis and low taeniasis prevalence remains a scientific issue (Flisser 2006). Even though the results of the present study support the hypothesis of false negative results using coprology, the true prevalence of taeniasis estimated here remains very low (0.6%) compared with a prevalence of active cysticercosis based on detection of circulating antigens of the metacestode of *T. solium* of 5.5% (Dorny *et al.* 2004; Mwape *et al.* 2012) and 12.2% (Mwape *et al.* 2012) estimated in the same communities. Moreover, some recent epidemiological studies indicate a high exposure to *T. solium* eggs, based on detection of

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antibodies directed against the metacestode of *T. solium* in endemic areas, suggesting a high level of environmental contamination with the eggs (Tsang *et al.* 1989; Garcia *et al.* 2001; Praet *et al.* 2010; Mwape *et al.* in preparation).

In conclusion, to our knowledge, this study compares for the first time the test characteristics of three tests for the diagnosis of *T. solium* taeniasis. We urge for additional studies exploring possible cross-reactions of the copro-Ag ELISA and for the use of more sensitive tests, such as copro-PCR, for the detection of tapeworm carriers, which is a key factor in controlling the parasite in endemic areas.

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