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Development of a Species-Specific Coproantigen ELISA for Human *Taenia solium* Taeniasis

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Abstract. *Taenia solium* causes human neurocysticercosis and is endemic in underdeveloped countries where backyard pig keeping is common. Microscopic fecal diagnostic methods for human *T. solium* taeniasis are not very sensitive, and *Taenia saginata* and *Taenia solium* eggs are indistinguishable under the light microscope. Coproantigen (CoAg) ELISA methods are very sensitive, but currently only genus (*Taenia*) specific. This paper describes the development of a highly species-specific coproantigen ELISA test to detect *T. solium* intestinal taeniasis. Sensitivity was maintained using a capture antibody of rabbit IgG against *T. solium* adult whole worm somatic extract, whereas species specificity was achieved by utilization of an enzyme-conjugated rabbit IgG against *T. solium* adult excretory-secretory (ES) antigen. A known panel of positive and negative human fecal samples was tested with this hybrid sandwich ELISA. The ELISA test gave 100% specificity and 96.4% sensitivity for *T. solium* tapeworm carriers ($N = 28$), with a J index of 0.96. This simple ELISA incorporating anti-adult somatic and anti-adult ES antibodies provides the first potentially species-specific coproantigen test for human *T. solium* taeniasis.

INTRODUCTION

Human taeniasis caused by *Taenia solium* (the pork tapeworm) is a potentially dangerous zoonosis that increases the risk of the tapeworm carrier and its contacts to acquire cysticercosis or neurocysticercosis. Humans are the only host of the adult stage of the tapeworm. When humans, however, become accidentally infected with *T. solium* eggs, usually because of a lack of good hygiene, development of the intermediate larval stage (cysticercus) of the parasite may occur. *T. solium* cysticercosis is a common parasitosis of the central nervous system, usually causing neurocysticercosis. This serious illness can cause severe neurologic damage and in some cases be fatal.¹ *T. solium* is endemic in areas of developing countries with poor sanitation and reliance on free-range porcine husbandry. Backyard free-ranging pigs gain access to infected human feces, allowing the completion of the parasite life cycle.

Diagnostic methods for human taeniasis traditionally depend on microscopic examination of the stool sample to search for eggs, but this copro-parasitologic approach is not very sensitive, and the eggs of *T. saginata* (human beef tapeworm) and *T. solium* are indistinguishable under the light microscope.² Histologic identification by uterine branch staining of proglottids requires intact mature proglottids, which may not always be available.³ Development of polymerase chain reaction (PCR) for amplification of *Taenia* spp. DNA is a species-specific method but requires sophisticated equipment and is not practical for mass-screening surveys.⁴

The detection of parasite-specific antigens released in the feces of *Taenia* spp. carriers (coproantigens) has improved diagnostic potential and is currently the most reliable approach for surveillance of human taeniasis.⁵ The ELISA test for coproantigen detection (ELISA-CoAg) developed by Allan and others^{6,7} has proven very useful for diagnosis of human taeniasis, including *T. solium* taeniasis, but the test is genus specific rather than species specific, giving cross-reactions

with *T. saginata* and *T. asiatica* infections.^{8–10} This ELISA test used hyperimmune rabbit IgG (polyclonal antibodies) raised against *Taenia* adult tapeworm derived crude somatic extract (known as WWE for whole worm extract), but nevertheless, was able to detect very small amounts of coproantigen present in human fecal samples.^{7,11} Epidemiologic studies showed that this genus-specific ELISA test could detect up to 2.6 times more human tapeworm carriers than microscopy alone.^{12,13} Coproantigens are also produced by both mature and immature tapeworms, allowing their identification at early stages^{7,14} and from the first week after infection in an experimental *T. solium*–hamster model.¹⁵ Once the tapeworm carrier has been successfully treated, the patient becomes negative for coproantigens at ~5 days after treatment.^{7,13} Lack of species specificity for the ELISA-CoAg, however, remains a problem in areas where *T. saginata* (and/or *T. asiatica*) is co-endemic.^{7,10,16} This seems to be caused by crude WWE antigens being shared by members of the *Taenia* genus. There is some evidence, however, that ES antigens of canid *Taenia* species were potentially more specific than somatic antigens.^{17,18} The high sensitivity (> 95%) of the ELISA-CoAg test based on anti-*T. solium* WWE antibodies makes it a very useful mass screening test,⁵ but the combined use of anti-*T. solium* WWE antibodies and anti-*T. solium* ES antibodies could provide greater specificity for the coproantigen ELISA.

Therefore, in this study, an ELISA for *T. solium* coproantigen detection was developed based on rabbit IgG raised against *T. solium* WWE as a capture antibody in combination with an enzyme-conjugated rabbit IgG raised against *T. solium* adult ES antigens. The sensitivity and specificity values were calculated, and the test performance was evaluated using a defined panel of human fecal samples. Also, the capacity of potential coproantigen detection for the new test was assessed using serial dilutions of antigen preparations in human feces.

MATERIALS AND METHODS

Fecal samples. For the initial assay standardization process, positive and negative pools of fecal supernatants were used. The positive pools were produced by mixing positive fecal samples

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of confirmed *T. solium* carriers. The original samples were all positive to the following: standard WWE-ELISA-CoAg test and microscopy (*Taenia* egg positive), and the patients had confirmed release of *T. solium* proglottids and/or scolices. The pools were prepared by mixing the positive fecal samples with 10% phosphate-buffered saline (PBS)-formaldehyde at a rate of 1:5. The pools used were kindly provided by the Parasitic Diseases of the Nervous System Research Unit, INCN/UPCH (Lima, Peru).

For the sensitivity and specificity calculation, a panel of 94 human fecal samples was tested. The panel comprised 28 confirmed *T. solium* positive samples, 33 confirmed *T. saginata* samples, 6 *Taenia asiatica* samples, and 27 confirmed *Taenia* spp.-negative samples including 7 non-endemic negative controls, as well as 20 samples positive only to other parasite infections including *Ancylostoma duodenale*, *Ascaris lumbricoides*, *Blastocystis hominis*, *Chilomastix mesnili*, *Endolimax nana*, *Entamoeba coli*, *Enterobius vermicularis*, *Giardia lamblia*, *Hymenolepis nana*, *Iodamoeba butschlii*, *Strongyloides stercoralis*, and *Trichuris trichiura*. The *T. solium*- and *T. saginata*-positive fecal samples were obtained from endemic regions of Peru. Species were confirmed by PCR^{3,9} and/or staining and counting of uterine branches of proglottids eliminated by the patients.^{3,19} The *T. asiatica* fecal samples were obtained from China and Indonesia and were confirmed by PCR.²⁰ Fecal samples from patients with other parasites were obtained from different parts of Peru considered of low risk for *T. solium*, including Lima. The negative controls used were from fecal samples obtained from persons residing in an area of Peru (Iquitos) non-endemic for cysticercosis.

Individual fecal samples were processed by mixing the fecal material in a 1:5 proportion with PBS-formalin 5%. The samples were mixed using a vortex to form a slurry and centrifuged at 3,000g for 30 minutes at 25°C. The supernatant was recovered, aliquoted in 1.5-mL vials, and stored at 4°C until used. On the day of use, vials containing fecal supernatants were vortex mixed and re-centrifuged at 3,000g for 15 minutes before use.

***Taenia solium* extracts/antigens.** Antigen preparations were obtained by *in vitro* maintenance of immature adult *T. solium* tapeworms obtained by experimentally infecting golden hamsters (*Mesocricetus aureatus*).^{7,14} Hamsters were each infected with four *T. solium* cysts obtained from naturally infected pigs and killed 32 days later. Immature tapeworms were harvested from hamster intestines, washed in sterile PBS, and maintained *in vitro* in protein-free culture media supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 0.007% NaHCO₃, 1% glucose, and antibiotics^{21,22} for collection of ES antigens, and the proglottids were also processed to obtain WWE.^{6,7} The *T. solium* antigen extracts were also used to prepare dilution curves to determine the antigen detection capabilities of the ELISA. The starting concentration of both extracts (WWE and ES) was 1.25 µg/mL, which were serially diluted to obtain concentration curves. A pool of negative human feces was used to dilute the antigen preparations. This pool came from *Taenia* negative persons from an endemic area of Peru (Tumbes).

Anti-*T. solium* antibodies. Hyperimmune rabbit IgG was raised against *T. solium* adult WWE and separately against *T. solium* adult ES antigen.⁷ In brief, rabbits were immunized with *T. solium* WWE obtained from specimens grown in immunosuppressed golden hamsters according to Allan and

others.⁷ with 0.5 mL antigen plus an equal volume of Freund complete adjuvant. Boosts were given at 10- and 14-day intervals with the same amount of antigen in incomplete adjuvant. The IgG of hyperimmune rabbit serum fraction was purified using Protein A-coupled sepharose.⁷ Protein A-purified IgG was dialyzed in excess PBS (pH 7.2) at 4°C and re-concentrated by vacuum dialysis. The hyperimmune IgG was divided into two equal portions, one of which was conjugated to horseradish peroxidase following the method of Wilson and Nakane.²³ Pure IgG or peroxidase-conjugated IgG was aliquoted and stored at -80°C until further use. The protein concentration of antigen extracts (WWE, ES) in the polyclonal antibody preparations was estimated by a micro-Bradford protein detection assay.²⁴ The values obtained were of 1.78 and 1.76 mg/mL for WWE and ES antibodies, respectively.

***Taenia solium* coproantigen ELISA.** Two coproantigen ELISAs were assessed. The first was the already established genus (*Taenia*)-specific ELISA of Allan and others,^{7,13} which used capture and conjugated polyclonal antibodies against adult WWE of *T. solium* (WWE-ELISA). The second was a novel "hybrid" coproantigen ELISA that incorporated anti-*T. solium* WWE capture antibodies and anti-*T. solium* adult ES conjugated antibodies (WWE-ES ELISA). For the hybrid WWE-ES ELISA, the test protocol was as follows: Immulon 4 HBX microtiter plate (Dynex) coated with a 1:4,000 dilution (0.445 µg/mL) of capture antibody (anti-WWE) at 100 µL/well, diluted in bicarbonate coating buffer (pH 9.6) (Sigma, U.K.). Sealed plates were incubated at 4°C overnight. Plates were washed three times with 200 µL of washing buffer composed of PBS (pH 7.3 ± 0.2; 0.15 mol/L; OXOID) plus 0.1% C58 H114 O26 polyoxyethylene 20 sorbitan monolaurate (Tween-20; Mallinckrodt), leaving wash buffer on for 30- to 60-second intervals each time. A blocking buffer composed of PBS plus 0.3% Tween-20 was added at 100 µL/well to all wells including blanks, re-sealed, and left 1 hour at room temperature on an orbital shaker. Heat-inactivated fetal bovine serum (HIFBS; Gibco) was added (60 µL/well) to all microtiter wells including blanks, followed by 40 µL of fecal supernatant sample to wells in duplicate. For the blank wells, 40 µL of the sample diluent (PBS-formalin 5%) was added. Plates were re-sealed and incubated for 1 hour at room temperature on an orbital shaker. Plates were washed as above. Peroxidase-conjugated anti-*T. solium* ES antibodies were added at a 1:4,000 dilution (0.44 µg/mL) in blocking buffer plus 15% HIFBS (100 µL/well) to all wells except the blank. Plates were re-sealed and incubated for 1 hour at room temperature on an orbital shaker. Plate contents were discarded, and the plate was washed as above. The substrate TMB (3,3',5,5' tetramethylbenzidine, Sureblue reserve; KPL) was added at room temperature to all wells including blanks and the plates foil wrapped and incubated on the shaker for 5 minutes to develop. The OD values of well reactants were read after 5 minutes at a wavelength of 650 nm.

Positive-negative cut-off determination. For comparison purposes, two cut-off values were determined for the test. One was calculated as the average of the OD values of the negative fecal samples plus 3 SD, and the other cut-off value was calculated using the Youden (*J*) index as an indicator of the test performance and optimal cut-off.²⁵ The Youden index, or *J* index, is a statistical tool to assess the performance of a given test, reducing it to a single number value. It is especially useful when introducing modifications into a diagnostic test. The best

cut-off point was established by choosing the optimal *J* index that indicated the best sensitivity and specificity combination. The results were processed using Microsoft Excel software. The average results of the OD values were sorted along with its real status of infection (positive or negative to *T. solium*) and the sensitivity, specificity, and *J* index were calculated for each OD value as if it were used as a cut-off value.

Sensitivity was calculated by dividing the number of true infected samples that gave a positive result in the test by the total number of true infected samples. Specificity was calculated by dividing the number of true negative samples that gave negative test results by the total number of true negative samples. The *J* index was calculated by the following formula:

$$J = (S + E) - 1$$

Where *S* is the sensitivity and *E* is the specificity. A *J* index was calculated for every OD value and the OD value with the best index was chosen as a cut-off.

RESULTS

When testing a known panel of human *T. solium* fecal samples from taeniasis patients, heterologous gut parasitic infections, and normal controls, the new hybrid WWE-ES coproantigen ELISA was 100% specific and 96.4% (27/28) sensitive for detection of *T. solium* taeniasis (Figure 1). There were no cross-reactions with fecal samples from *T. saginata* (*N* = 33) or *T. asiatica* (*N* = 6) carriers. Similarly, fecal samples for other parasite species (*N* = 20) were also negative and remained under the ELISA cut-off value.

The cut-off value was determined by two means: 1) by averaging the negative samples OD values plus 3 SD and 2) by assessing the value with the best *J* index. The cut-off values thus calculated were 0.108 by averaging the negative samples OD values plus 3 SD and 0.104 with a *J* index of 0.964. Both cut-off values were similar (Figure 1).

To estimate the detection sensitivity of the *T. solium* WWE-ES coproantigen ELISA, ES or WWE *T. solium* adult extracts were titrated into normal human feces. Using the *J* index determined cut-off of 0.104, the minimum detectable amounts of spiked antigen the test detected in human feces was 0.6 ng/mL of adult ES antigen and 19.5 ng/mL of adult WWE antigen (Figure 2).

The performance of the new WWE-ES ELISA for *T. solium* coproantigen detection was also compared with the standard WWE-ELISA.⁷ For the new test, the specificity was 100% and the sensitivity was 96.4%. The specificity and sensitivity of the standard WWE-ELISA for *T. solium* was 45.5% and 92%, respectively, with the same panel of human fecal samples. Notably, cross-reaction occurred in the latter test with 97% (32/33) of *T. saginata* taeniasis and 67% (4/6) of *T. asiatica* taeniasis cases but none of the heterologous non-*Taenia* infection controls (data not shown).

DISCUSSION

The accurate detection of *Taenia solium* tapeworm carriers is crucial for epidemiologic studies as well as surveillance and intervention programs.¹ The development of an ELISA for *T. solium* coproantigen detection significantly improved the diagnosis of human taeniasis^{7,16} but was genus specific rather than species specific.⁵ Before coproantigen tests, microscopy for detection of *Taenia* eggs in fecal samples, or the recovery and identification of tapeworm proglottids, were the only means to diagnose this gastrointestinal infection. Microscopy lacked sensitivity, because the detection of eggs in samples depended on the onset of the patent period of the parasite, and also *Taenia* eggs may only be shed intermittently. Furthermore, the species-specific parasitologic identification of the tapeworm depends on the recovery of non-degraded proglottids, which is not always possible.^{5,19,26} The use of PCR to amplify DNA from proglottids recovered from feces or directly from eggs in a stool sample can provide species specificity but again

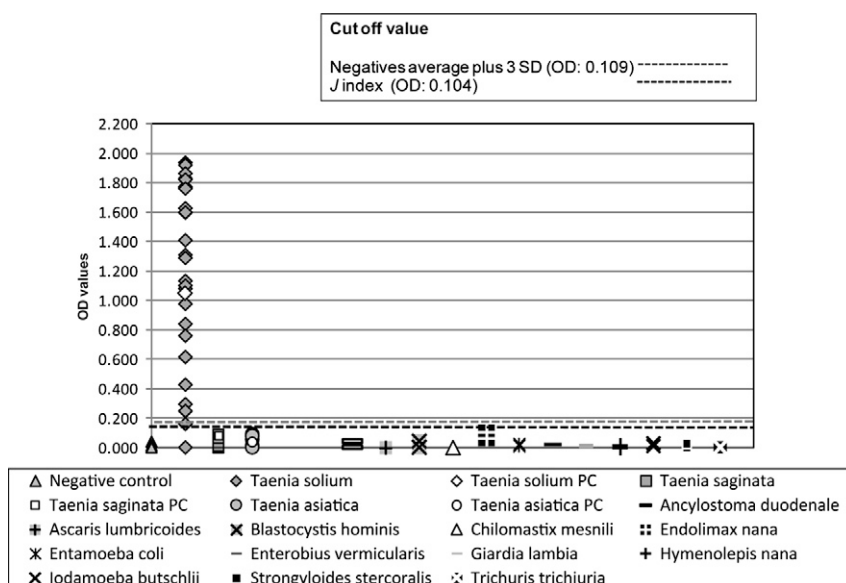


FIGURE 1. Specificity of the WWE-ES coproantigen ELISA for *T. solium* carriers (*N* = 28) versus fecal samples from *T. saginata* (*N* = 33), *T. asiatica* (*N* = 6), and a range of non-*Taenia* gut infections or normal controls (*N* = 27). PC, positive control.

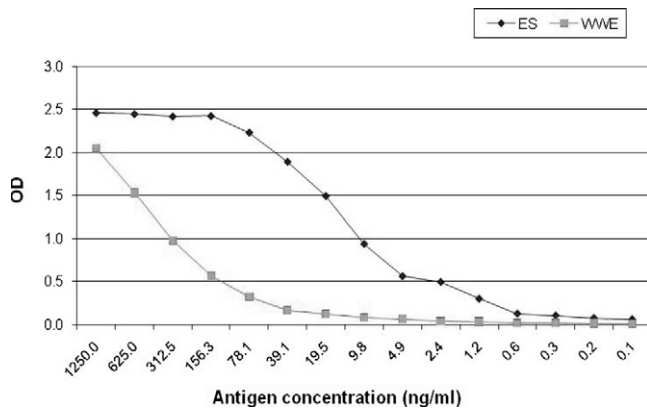


FIGURE 2. Antigen dilution curve for the *T. solium* WWE-ES coproantigen ELISA.

relies heavily on the presence of parasite material (proglottids or eggs) and is not actually easy to perform in basic laboratories.^{9,20} Detection of specific serum antibodies to *T. solium* adult tapeworm infections has been developed using immunosorbent assays, but as for any serologic assay that detects the presence of serum antibodies, such a test may not necessarily indicate a current infection.^{21,22}

The main aim of this study was to develop a more specific coproantigen ELISA test for *T. solium* taeniasis without affecting sensitivity. The coproantigen ELISA we developed used both anti-somatic and anti-ES adult antibodies and gave 100% specificity and 96.4% sensitivity for *T. solium* taeniasis detection with a parasitologically defined panel of fecal samples from human taeniasis infections as well as non-taeniid heterologous infections. Importantly, the new “hybrid” coproantigen ELISA did not show cross-reactions with samples from 33 confirmed *T. saginata* carriers or 6 *T. asiatica* carriers.

Fecal samples tested for sensitivity assessment of this new coproantigen WWE-ES ELISA were selected from a panel of parasitologically confirmed *T. solium* samples that gave different OD values in the established genus-specific WWE-ELISA. Of the 28 proven *T. solium* stool samples tested, one sample (55351, from Tumbes, Peru) gave a negative value (i.e., false negative) in both the coproantigen ELISAs. Besides this individual sample, the WWE-ES ELISA achieved a good differentiation of the *T. solium* positive and negative samples (Figure 1). Variability in the positive OD values for fecal samples from *T. solium* carriers might be caused by variation in tegumental or ES antigen turnover and/or antigen concentration in stool samples.^{15,17} It seemed that the optimal concentration of capture and conjugated antibodies, inclusion of 15% heat-inactivated fetal bovine serum diluent, and the use of TMB substrate (TMB Sureblue; KPL Inc., Gaithersburg, MD) also helped to further reduce any non-specific background OD values obtained in earlier test developments. ES products from taeniid tapeworms contain a range of low molecular weight antigenic proteins and large carbohydrate-rich surface molecules.^{5,22}

The establishment of a positive-negative cut-off for an ELISA-based diagnostic test is often relatively arbitrary. To assess the sensitivity and specificity of the new *T. solium* coproantigen WWE-ES ELISA, two methods were used: 1) take 3 SD above the mean OD negative control values and 2) the *J* index.²⁵ The *J* index is a single index value based

on the sensitivity and specificity obtained for each OD value and the highest index (maximum 1.0) is chosen as a cut-off. The advantage of using this index was that multiple cut-offs were compared against a perfect difference between a positive and negative value. In fact, the effective cut-offs selected were similar for both these optimization methods, and the *J* index for the ELISA cut-off OD value (0.104) was very high at 0.964.

Currently, two other relatively new and very specific diagnostic laboratory tools for the diagnosis of *T. solium* taeniasis infections are PCR,^{3,20} and specific antibody detection in serum,^{21,22} but both have some drawbacks. For *T. solium* PCR, the presence of eggs or tapeworm material is normally required in the fecal sample for the test to detect DNA. This can limit the sensitivity of the test and may also miss pre-patent infections. Detection of anti-adult serum antibodies has the drawback of detecting residual circulating antibodies in human serum after tapeworm infection has terminated. Recent studies indicate that *T. solium* adult antibodies could last several years, and therefore, may not be as useful for detection of current taeniasis infections (Cysticercosis Working Group in Peru, 2008, unpublished data). The combination of a serum antibody detection test and the new coproantigen detection test (using anti-somatic and anti-ES antibodies) could be used as an accurate method for epidemiologic and diagnostic studies in *T. solium*-endemic regions. The predictive value of the new coproantigen test should now be assessed on its own and in combination with the serologic test for *T. solium* taeniasis. The reproducibility of the coproantigen ELISA was good in this study, with concordant blind assessment of samples in the Lima and Salford laboratories. Furthermore, production of parasite extracts (somatic or ES) and of effective polyclonal antibodies was not problematic and can be repeated in low-tech laboratory settings if the parasite is available. The exact nature of the *T. solium* coproantigens detected in human fecal samples remains to be determined, but based on our studies of coproantigens from a related taeniid infection (*Echinococcus*),²⁷ they are likely to be large molecular weight carbohydrate molecules (hence, in this study, not degraded by formalin stool preservation) of cestode tegumental origin and consequently not very amenable to conventional immunoblot analysis.²⁷

In conclusion, the new coproantigen ELISA described in this study seemed to be highly specific and sensitive with formalin-fixed stools, was relatively easy to develop, and was also easy to use, and thus, could be potentially useful for mass screening and post-intervention program surveillance in areas co-endemic for *T. solium* and *T. saginata* and in Asian regions where *T. asiatica* is also endemic.

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