An enzyme-linked immunosorbent assay for diagnostic detection of *Taenia saginata* copro-antigens in humans

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Abstract

An immunodiagnostic sandwich enzyme-linked immunosorbent assay (ELISA) was developed for the detection of soluble *Taenia saginata* antigens in stool samples (copro-antigens) of infected humans, using affinity-purified polyclonal antibodies obtained from rabbits hyperimmunized with excretory/secretory antigens derived from *T. saginata* maintained *in vitro*. Investigation of operating characteristics showed very low cross-reactivity with crude antigens from helminths other than *Taenia*, including *Dipylidium caninum* and *Diphyllobothrium latum*. The specificity of the assay was 95% when testing stool samples from 100 persons who were either infected with *Ascaris lumbricoides*, *Trichuris trichiura*, hookworms, *Enterobius vermicularis* or *Hymenolepis nana*, or who had no intestinal helminthosis detected. Analysis of diagnostic sensitivity demonstrated that in 85% of 34 samples from 23 untreated persons with intestinal *T. saginata* infection (selected by previous proglottid and/or egg detection) copro-antigens were detected by the *T. saginata* ELISA. In the same samples, *Taenia* eggs were detected in 62%. Only 41% of the samples reacted positively in a heterologous *T. hydatigena* ELISA. Post-treatment control revealed a high concentration of *T. saginata* copro-antigens for 1–4 d after administration of niclosamide or praziquantel, and negative values 9–17 d after treatment. The *Taenia* copro-antigens remained detectable by ELISA even after storage of untreated faeces at 25°C for at least 5 d.

Introduction

Infections of humans with *Taenia saginata* or *T. solium* are of medical and veterinary importance as the larval forms of these two species cause cysticercosis in cattle or in pigs and man, respectively. Efficient control of these zoonotic infections is hampered by many factors, including unsatisfactory methods of diagnosing intestinal *Taenia* infections in man; it is almost impossible to diagnose early infection before proglottids and/or eggs start to be excreted. During patency proglottids and eggs are irregularly excreted and the available techniques for faecal examination have a relatively low sensitivity. Although the perianal swab method for egg detection gives somewhat better results, the sensitivity is variable and estimated to be between 35% and 85% (HALL *et al.*, 1981; KIRICHEK *et al.*, 1986). Therefore, new diagnostic tools with a higher diagnostic sensitivity are needed for detecting the infection in individuals, for assessing efficacy of chemotherapy and for screening larger populations (FLISSER, 1985; CRUZ *et al.*, 1989; PAWLOWSKI, 1990).

The diagnostic detection of parasite antigens in stool samples (copro-antigens) by enzyme-linked immunosorbent assay (ELISA) was initially applied for the diagnosis of *Giardia lamblia* and *Entamoeba histolytica* infections in humans (GRUNDY, 1982; UNGAR *et al.*, 1984, 1985; BAUMANN & GOTTSTEIN, 1987). MACHNICKA & KRAWCZUK (1988) and ALLAN & CRAIG (1989) demonstrated the detection of *Hymenolepis*-specific antigens in faeces of rats infected with *H. diminuta*. For the detection of *Taenia* copro-antigens, a sandwich ELISA using affinity-purified polyclonal rabbit antibodies directed against excretory/secretory (E/S) antigens from adult *T. hydatigena* was developed (DEPLAZES *et al.*, 1990). This test allowed the diagnosis of prepatent and patent infections in 6 dogs with experimental *T. hydatigena* infection. The test exhibited a high degree of specificity at the genus level. Recently, ALLAN *et al.* (1991) described two ELISAs for the detection of *T. solium* and *T. saginata* antigen in faeces of golden hamsters infected with *T. solium*. Both tests, using immunoglobulin fractions directed against somatic parasite antigens, also recognized *Taenia*-specific antigens in stool samples of 11 persons infected with *T. saginata* or *T. solium*.

In this paper we describe an ELISA detecting *T. saginata* copro-antigens using affinity-purified antibodies against *T. saginata* E/S-antigens.

Materials and Methods

Stool samples

Stool samples were collected from the following groups of persons.

Group 1. 23 persons (17 from Poland and 6 from Switzerland) infected with *T. saginata*; 34 samples were collected before specific chemotherapy and 16 samples 1–17 d after therapy with niclosamide or praziquantel (for details see the Table). The patients were selected on the basis of proglottid excretion reported in their history (shortly before sampling stool specimens). Specific identification of proglottids relied upon routine parasitological criteria.

Group 2. A negative control group of 40 healthy persons (20 from Poland and 20 from Switzerland), not infected with *T. saginata* (confirmed by no report of proglottid excretion and by routine stool examination with sedimentation/flotation technique).

Group 3. 100 stool samples (40 from Swiss persons and 60 from immigrants) submitted to our Institute by physicians for routine coproscopical examination.

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Fresh stool samples were collected (groups 1 and 2) and immediately mixed in a 1:2 ratio with phosphate-buffered saline (PBS) containing 0·04% NaN_3, 0·05% bovine haemoglobin (Fluka) and 0·3% Tween 20®. These samples were stored at −20°C for 1 to 4 weeks until further processing. The thawed suspensions were ultrasonicated for 30 sec (40 W) and subsequently sedimented at 3000 *g* for 10 min before use in the ELISA. One of these negative samples (designated as control 'stool-in-buffer suspension,' SBS) was subsequently used for analysis of the sensitivity of the method in recovery experiments (briefly referred to as 'sensitivity'). Stool samples of group 3 were suspended in the buffer solution described above on arrival at the Institute (after 1–3 d in transit without any preservation or buffer solution). All supernatants were frozen at −20°C.

Coproscopical examination

The coproscopical examination was performed by an established sedimentation/flotation technique (BOCH & SUPPERER, 1983) on the sediments of the same samples (2 *g*) using zinc chloride (specific gravity 1·3) as flotation medium. Preliminary experiments had shown that the embryophore of *Taenia* eggs in SBS was not destroyed by ultrasound treatment (see above).

Antigens

T. saginata *E/S antigens*. Part of a living *T. saginata* strobila with gravid proglottids and without scolex (spontaneously excreted from a patient) was washed with PBS and maintained at 37°C with air as gas phase in a 75 cm^2 tissue culture flask (Corning Glass Works, no. 25110) containing 200 ml serum-free Eagle's Minimal Essential Medium (EMEM) (Gibco, no. 072 1100) with D-glucose (4 mg/ml), gentamycin (200 μg/ml) and Fungizone® (250 ng/ml), pH 7·2. The medium was replaced after 4, 10, 18 and 28 h of incubation, then every day up to day 10. The collected batches of tissue culture medium (TCM) (from days 3 to 6) were stored at −20°C until further processing. The viability of the strobila was judged by its motility. The sterility of the cultures was tested on day 3 of maintenance according to standard bacteriological procedures.

E/S antigens were dialysed and concentrated from the collected TCM to 0·7 mg protein per ml using an Amicon® untrafiltration unit, a YM-10 membrane and PBS. All protein concentrations were assessed by a Bio-Rad® protein assay with bovine albumin as standard. The *T. saginata* E/S antigens were used in the antigen-detecting ELISA (=*T. saginata* ELISA) as described below (see 'anti-*T. saginata* E/S hyperimmunoglobulins').

The following antigens were used for the determination of specificity.

Heterologous helminth somatic and metabolic antigens. Somatic and metabolic antigens prepared from the adult stage of various helminth species were used for the evaluation of the specificity of the test system. The various helminth species as well as the host origins are listed in Fig. 2.

Non-helminth antigens. Crude extracts were obtained from *Escherichia coli* strain Y 1089, from *Entamoeba histolytica* strain HK9 (ICN Medica Diagnostic Products, Covina, California, lot no. 4729) and from trophozoites of *Giardia lamblia* (Swiss bovine strain) (ROHRER *et al.*, 1987). Mammalian antigens included bovine and human serum diluted 1:50, cow's milk diluted 1:2 with PBS-Tween 20®, and muscle extracts of calf, chicken, and swine.

All freshly obtained materials were processed as described by DEPLAZES *et al.* (1990).

*Anti-*T. saginata *hyperimmunoglobulins*

A rabbit was hyperimmunized as described by BAUMANN & GOTTSTEIN (1987), but using *T. saginata* E/S antigens for immunization. All other steps, including the coupling of *T. saginata* E/S antigens to CNBr-activated Sepharose® 4B, the purification of antibodies by affinity chromatography, the preparation of rabbit anti-*T. saginata* E/S conjugates (alkaline phosphatase), and the preparation of normal rabbit immunoglobulin G (IgG) for control reactions were done according to the procedures described by BAUMANN & GOTTSTEIN (1987).

Enzyme-linked immunosorbent assay

A sandwich ELISA was devised for the detection of *T. saginata* antigens in human stool samples. Techniques and concepts were exactly as previously elaborated for the *T. hydatigena* copro-antigen test (DEPLAZES *et al.*, 1990), and can be summarized briefly as follows. (i) Affinity-purification of *T. saginata* copro-antigen-specific catching (solid phase) antibodies (from hyperimmunized rabbits), in parallel with inclusion of irrelevant rabbit catching (solid phase) IgG as a control; (ii) antibody-copro-antigen immune reaction; (iii) visualization of immune reaction by using affinity-purified antibody (liquid phase) as in (i) but labelled with alkaline phosphatase and the corresponding chromogenic substrate solution. The results are expressed as the absorbence at 405 nm (A_{405nm}) and controlled with reference to standard positive and negative samples run in triplicate. When the results obtained with control rabbit IgG amounted to >30% of a positive A_{405nm} value of the specific IgG reaction, the sample run in question was discarded. This *T. saginata* ELISA was evaluated as described below; the previously described *T. hydatigena* ELISA was used for comparison.

For the assessment of potential cross-reactivity and non-specific reactions in the ELISA, the antigens of various helminths, protozoa, bacteria and mammalian control tissues (Fig. 2) were tested at a concentration of 10 μg protein per ml of the control SBS; human and bovine serum were used diluted 1:50 and cow's milk 1:2. The procedure for the evaluation of the assays was carried out as listed in the following experimental design.

(i) Determination of a significant threshold in the ELISA, discriminating between positive (=*T. saginata* antigens detectable) and negative (no *T. saginata* antigen detectable) in both systems.

(ii) Determination of the sensitivity of the *T. saginata* ELISA and the *T. hydatigena* ELISA in recovery experiments using stool samples diluted 1:2 and known protein concentrations of *T. saginata* metabolic E/S antigens.

(iii) Determination of the specificity of the *T. saginata* ELISA using SBS containing known protein concentrations of somatic or metabolic antigens

obtained from various heterologous parasite species, bacteria, or mammalian control tissues.

(iv) Definition of diagnostic sensitivity and specificity, etc., by testing stool samples from persons infected with *T. saginata* before and after specific chemotherapy, from persons with other helminth infections, or from parasite-free persons.

(v) Examination of the potential stability of copro-antigens in stool samples under different storage conditions.

Results

Sensitivity of the ELISA

In order to determine the sensitivity of the ELISA, *T. saginata* E/S antigens were mixed with negative SBS and then diluted in serial 2-fold dilutions with the same SBS. These samples were tested by ELISA using anti-*T. hydatigena* and anti-*T. saginata*-antibodies (*T. hydatigena* and *T. saginata* ELISA, respectively) (Fig. 1). The threshold (cut-off point) for both assays for discriminating a negative from a positive reaction was determined by calculating the mean A_{405nm} value ($\bar{x}$) of the 40 stool specimens from the negative control group 2 plus 4 standard deviations (SD). According to this calculation, both test systems showed the same background reactions. The *T. hydatigena* ELISA (DEPLAZES *et al.*, 1990) detected at least 0·2 μg of *T. saginata* E/S antigens per ml of diluted (1:2) SBS; the newly developed *T. saginata* ELISA had a higher sensitivity, detecting a lower limit of 0·07 μg, equivalent to 140 ng/ml of stool. All control reactions with normal rabbit IgG were very low ($A_{405nm}<0·04$).

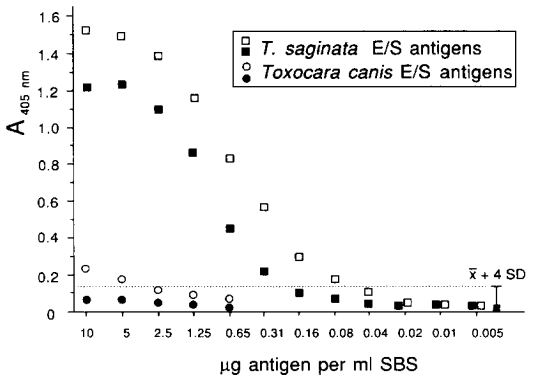

Fig. 1. Titration of (homologous) *Taenia saginata* and control *Toxocara canis* excretory/secretory (E/S) antigens artificially diluted in human control stool-in-buffer solution (SBS). Testing was carried out with *T. saginata* (□, ○) and *T. hydatigena* ELISA (■, ●). The values obtained in the control reaction with purified irrelevant rabbit control IgG were subtracted from the values obtained with specific rabbit anti-*T. saginata*- or anti-*T. hydatigena* E/S antigen Ig. The cut-off point (---) was determined in both systems by calculating the mean absorbance at 405 nm of 40 negative control samples plus four standard deviations.

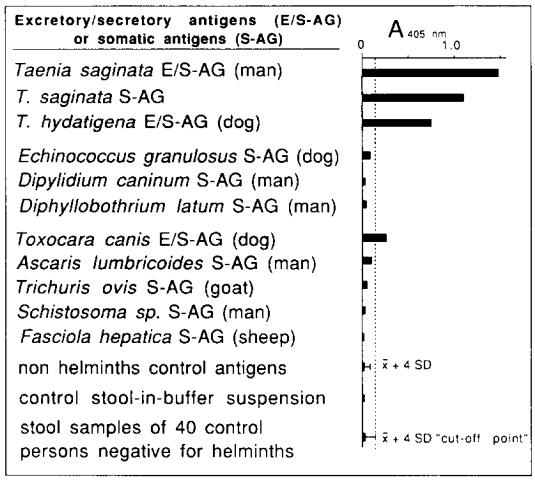

Fig. 2. Specificity evaluation of the *Taenia saginata* ELISA with 10 μg antigen from helminth and non-helminth (somatic antigens from *Giardia lamblia, Entamoeba histolytica, Escherichia coli*, calf, swine, chicken and human muscle; 1:50 diluted calf and human serum, and 1:2 diluted cow's milk) per ml of control stool-in-buffer suspension. The host origin of the antigens is given in parentheses. A_{405nm}=absorbance at 405 nm.

Specificity of the ELISA

The results of the specificity evaluation with defined protein concentrations in control SBS are shown in Fig. 2. The antibody reactivities with all non-helminth antigens were negligible compared to the *Taenia* antigens tested. *Toxocara canis* E/S antigens from adult stages showed cross-reactivity at high antigen concentrations (10–2·5 μg/ml of stool sample) in the *T. saginata* ELISA (Fig. 1) but not in the *T. hydatigena* ELISA. All antigens of the other investigated helminth species showed no significant reaction in the assay.

Diagnostic sensitivity and specificity of the ELISA

In the *T. saginata* ELISA (Fig. 3), the range of negative reactions was determined by testing 40 stool samples from a helminth-free group (group 2). The A_{405nm} values obtained in the control reactions for both assays with purified irrelevant rabbit control IgG (mean A_{405nm} value=0·02, range 0·01–0·04) were subtracted from the values obtained with rabbit anti-*T. hydatigena* E/S and anti-*T. saginata* E/S antigens, respectively. The mean values of these corrected data plus 4 SD amounted to A_{405nm}=0·14 ($\bar{x}$=0·027, SD=0·027) and A_{405nm}=0·14 ($\bar{x}$=0·025, SD=0·028), respectively, and were arbitrarily considered as the cut-off limit between specific antigen-detecting reactions and background reactions.

Twenty-three specimens from 23 persons infected with *T. saginata* were collected before treatment (only the initial sample from each person was used for the present study), examined for copro-antigens with the two ELISAs mentioned above, and investigated in parallel by a sedimentation/flotation technique for *Taenia* eggs. *Taenia* eggs were detected in 17 samples (74%); copro-antigens were detected by *T. saginata* ELISA in 20 samples (87%) (Fig. 3). One of the 3 samples was negative for copro-antigen detection, although a few *Taenia* eggs had been found by routine examination. In the same group of 23 specimens, 13 (57%) samples were positive in the *T. hydatigena* ELISA (data not shown).

Samples from group 3 were also examined for eggs and copro-antigens with the *T. saginata* ELISA (Fig.

3). Three of 40 samples from Swiss persons were positive in the *T. saginata* ELISA, and *Taenia* eggs were found in one of them. The other samples were free of helminth eggs. Three of 60 samples from immigrants (4 infected with *Hymenolepis nana*, 2 with *Enterobius vermicularis*, 4 with *Trichuris trichiura*, 11 with hookworms, 5 with hookworms and *T. trichiura*, 2 with *Ascaris lumbricoides* and *T. trichiura* and one with *A. lumbricoides* and hookworms) gave positive reactions for copro-antigens in the *T. saginata* ELISA (Fig. 3). In 2 of these cases, helminth infection could not be detected; the third case had an infection with *A. lumbricoides* and *T. trichiura*. The diagnostic specificity of the *T. saginata* ELISA was thus 95% (5

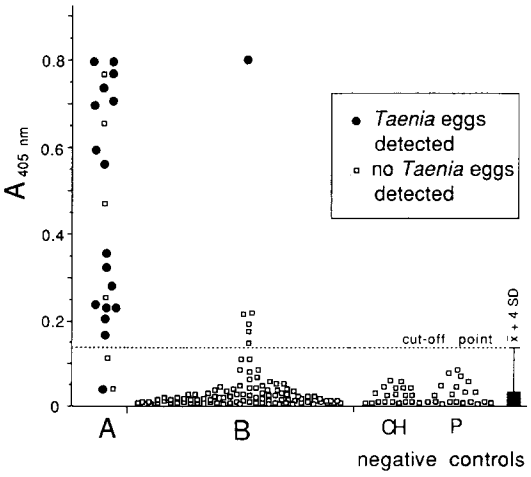

Fig. 3. Diagnostic sensitivity and specificity of the *Taenia saginata* ELISA with the following stool samples (with multiple samples, only the first was included): (A) from 23 persons with proven *T. saginata* infection (group 1); (B) from 100 persons (group 3) with the following species of eggs detected at routine coproscopical examination: *T. saginata* (n=1), *Hymenolepis nana* (n=4), *Enterobius vermicularis* (n=4), *Trichuris trichiura* (n=4), hookworms (n=11) and mixed infections with hookworms, *T. trichiura* and *Ascaris lumbricoides* (n=8). The cut-off point (---) was determined by calculating the mean absorbance at 405 nm (A_{405nm}) of 20 Swiss (CH) and 20 Polish (P) negative control samples (group 2) plus four standard deviations.

positive results without proven *T. saginata* infection among 100 samples originating from different persons).

Reproducibility of test results and antigen detection after treatment

The diagnostic reproducibility of a positive result was tested by examination of 1–3 samples per infected person before treatment (Table). The data show an acceptable degree of reproducibility in antigen detection. Considering all data, including those from a single examination and from repeated examinations, the following diagnostic sensitivities were calculated: 62% for *Taenia* egg detection; 85% for copro-antigen detection by *T. saginata* ELISA; and 41% by *T. hydatigena* ELISA. *T. saginata* ELISA was positive with 9 of 13 samples (69%) from infected persons without egg detection, and with *T. hydatigena* ELISA copro-antigens were detected in only one sample. Eleven samples from 11 persons were all positive in the *T. saginata* ELISA during 1–4 d after treatment, whereas no copro-antigen was detected in any samples 9–17 d after treatment (Table).

Storage conditions of samples

The stability of the copro-antigens in stool samples (diluted 1:2 with buffer solution) was tested with 4 positive specimens. After 5 d storage at room temperature, all samples were still positive. The mean value of A_{405nm} decreased by 9%. Four negative specimens, either fixed in buffer or without buffer solution, remained negative after the same exposure time.

Discussion

Man is the only natural final host for both *T. saginata* and *T. solium*. *T. saginata*, a relatively harmless intestinal parasite of humans, causes in its metacestode form bovine cysticercosis which induces considerable economic losses throughout the world. *T. solium*, inhabiting the intestine of humans, also has a low pathogenicity in its adult stage, but may cause cysticercosis in pigs as natural intermediate hosts and in humans as accidental hosts. Among various forms of clinical cysticercosis of man, neurocysticercosis represents the most serious form and is potentially fatal.

Table. Comparison of *Taenia* egg versus copro-antigen detection in stool samples of persons with *T. saginata* infection

Pesons with *T. saginata* infection	No. of persons	No. of samples	No. of samples with positive result: *Taenia* egg detection	Copro-antigen (*T. saginata* ELISA)	Copro-antigen (*T. hydatigena* ELISA)
Before treatment					
Examination with one sample (Switzerland)	6	6	6	6	5
Examination with one sample (Poland)	9	9	7	8	7
Examination with 2 samples (Poland)	5	10	6[a]	6[a]	2
Examination with 3 samples (Poland)	3	9	3[b]	9	0
Total	23	34	21 (62%)	29 (85%)	14 (41%)[c]
After treatment					
1–4 d after	11	11	10	11 (100%)	nd[d]
9–7 d after treatment	5	5	0	0	nd[d]

[a]2 persons with both negative egg and copro-antigen results.
[b]The 3 samples corresponded to 2 persons.
[c]All positive reactions were positive in the *T. saginata* ELISA.
[d]nd=not done.

At present, the only available practical ways to control these two zoonotic parasites are (i) the detection and treatment of carriers and (ii) socio-hygienic measures to prevent infection of man and intermediate host animals.

Immunization of intermediate host animals appears potentially feasible but vaccines are not yet available (MITCHELL, 1989). An indispensable prerequisite for the control measures listed above and for treatment of individual patients is efficient and correct diagnosis. This is at present based on the detection of proglottids and/or eggs by faecal examination or on egg detection by perianal swab. These methods are not very sensitive (see the Introduction) and, furthermore, have the disadvantage that parasites during prepatency, or during patency but lacking proglottid or egg excretion, cannot be readily diagnosed. An alternative immunological approach is the detection of antibodies in serum samples from persons serving as definitive hosts. FLENTJE & PADELT (1981), using the indirect immunofluorescent antibody test, found a diagnostic sensitivity of 56% in 200 persons infected with *T. saginata*. In general, and with respect to other cestode and host species (GASSER *et al.*, 1988; HEATH *et al.*, 1985; JENKINS & RICKARD, 1985, 1986a, 1986b), antibody detection did not allow individual diagnosis of infected definitive hosts, but it matched and reflected the parasite prevalence in populations (JENKINS *et al.*, 1990; GOTTSTEIN *et al.*, 1991).

The present paper demonstrates that, overall, 85% of 34 samples from 23 untreated persons with intestinal *T. saginata* infection contained copro-antigens detectable by a sandwich ELISA using affinity-purified polyclonal antibodies. These antibodies had been generated in rabbits by hyperimmunization with E/S antigens released *in vitro* by adult *T. saginata*. In the same group of patients, *Taenia* eggs were found in only 62% of the cases at a single examination. With the heterologous *T. hydatigena* ELISA, copro-antigens were detected in only 41% of the same samples.

There are still unknown features to be investigated—the diagnostic sensitivity/cross-reactivity independence of parasite species (i) used for generating hyperimmune antibodies or (ii) targeted in the sandwich ELISA for diagnosis, which may also be dependent upon the stage of infection (e.g. patent or not) and other criteria. ALLAN *et al.* (1991) used a *T. saginata* copro-antigen ELISA and observed cross reactions with stool specimens from human patients infected with intestinal *T. solium* (with egg excretion), whereas the same infection in hamsters, but without egg excretion, gave negative test results. In general, we found that stool specimens with large numbers of *T. saginata* eggs had very high activity in the copro-antigen ELISA (diagnostic sensitivity 95%; some specimens were still positive when diluted to 1:512; data not shown). Even with diagnostically interesting samples from patients with proven taeniasis but without detectable egg excretion, we obtained a diagnostic sensitivity of 69%. These data indicate the need to include samples from infected patients without egg excretion in trials, which more reliably reflects the diagnostic sensitivity under routine field conditions. We conclude that assessment of diagnostic sensitivity of the copro-antigen ELISA tested exclusively on infected patients excreting eggs might be too optimistic.

The specificity of the present *T. saginata* copro-antigen ELISA was high: 95% when testing faecal samples from persons infected with *Hymenolepis nana* or with various nematode species. Cross-reactivity occurred with *T. hydatigena* antigen and with faecal samples from dogs infected with *T. hydatigena* (data not shown), but this infection does not occur in humans. The specificity of the present test thus operated at the genus level; we are currently assessing its potential for diagnosing carriers of adult *T. solium*.

After having previously demonstrated that *T. hydatigena* metabolic copro-antigens were detectable during the prepatent period in experimentally infected dogs (DEPLAZES *et al.*, 1990), we have subsequently shown in the present study that the same applies to *T. saginata* copro-antigens in persons without parasite egg excretion. We are now addressing the problem of specificity by (i) identification and characterization of parasite epitopes of copro-antigens and (ii) generation of monoclonal antibodies against these epitopes for the development of species-specific tests. In this context we have to mention another option for the differential diagnosis of *Taenia* species, the use of deoxyribonucleic acid (DNA) probes and the polymerase chain reaction (HARRISON *et al.*, 1990; GOTTSTEIN *et al.*, 1991 and in press), which, however, requires proglottids or parasite eggs as a source of the diagnostic DNA template. All the new immunological or molecular biological tools discussed above clearly have the potential of being further developed to provide test kits applicable under field conditions and may prove very useful as diagnostic tools in the near future.

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