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A MONOCLONAL ANTIBODY-BASED ELISA FOR THE DETECTION OF CIRCULATING EXCRETORY–SECRETORY ANTIGENS IN *TAENIA SAGINATA* CYSTICERCOSIS

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Abstract—BRANDT J. R. A., GEERTS S., DE DEKEN R., KUMAR V., CEULEMANS F., BRIJS L. and FALLA N. 1992. A monoclonal antibody-based ELISA for the detection of circulating excretory–secretory antigens in *Taenia saginata* cysticercosis. *International Journal for Parasitology* 22: 471–477. A series of monoclonal antibodies (MoAb) produced against excretory and secretory products from 10- and 20-week-old *Taenia saginata* cysticerci were tested for their ability to detect circulating antigen in a double antibody sandwich enzyme-linked immunosorbent assay (ELISA). Two MoAb, 12G5 and 2H8, proved to be highly reactive with the tegument of viable *T. saginata* cysticerci and recognized antigenic components of 65, 87 and 100 kDa in immunoblotting. The detection limit of the assay using 12G5 as trapping antibody and 2H8 as a biotinylated indicator antibody was 0.1 ng protein per ml. Although the sensitivity of the test varied from one animal to another, the minimum number of living cysticerci, which could be detected by the ELISA, was 88. Animals harbouring only dead cysticerci gave similar reactions as non-infected control animals. Cross-reactions were only observed with taeniid parasites. The test was able to detect circulating antigen also in sheep and pigs, respectively infected with *T. ovis* and *T. solium* and in the serum samples of confirmed cases of human *T. solium* cysticercosis.

INDEX KEY WORDS: *Taenia saginata*; *Taenia solium*; cysticercosis; excretory–secretory antigen; circulating antigen; ELISA; monoclonal antibodies; cattle; pig; human; diagnosis.

INTRODUCTION

THE ante-mortem diagnosis of *Taenia saginata* cysticercosis in cattle is mainly based on the demonstration of specific antibodies, permitting, to a certain extent, the identification of cysticercosis as a herd problem. It does not allow, however, diagnosis of the infection in individual bovines nor the distinguishing between animals of those harbouring living cysticerci from those carrying dead ones (Sewell, 1987).

From the public health point of view only living metacestodes of *T. saginata* are important. A serological test, enabling the detection of animals infected with living cysticerci, would be superior to the currently used meat inspection techniques. The latter detect only a small fraction of infected animals (Geerts, Kumar & Van Den Abbeele, 1980; Kyvsgaard, Ilsoe, Henriksen & Nansen, 1990).

The purpose of the present work was the development of monoclonal antibodies against excretory and secretory (ES) products of living *T. saginata* cysticerci and their use in an enzyme immunoassay to monitor the evolution of circulating ES-products during the course of an experimental infection and to evaluate their reliability as diagnostic tools for the detection of animals harbouring living cysticerci of *T. saginata*.

MATERIALS AND METHODS

Excretory and secretory (ES) products of T. saginata *metacestodes.* Two calves were experimentally infected with *T. saginata* eggs and autopsied, respectively 10 and 20 weeks after infection. Two hundred cysticerci were collected, removed from their host capsule and immediately transferred in RPMI-1640 (Gibco), to which gentamycin (1 mg per 10 ml medium) was added. After 15 h of incubation at 37°C in a 5% CO_2 atmosphere, sterile ox-bile (1/10 v/v) was added to the medium for 1.5 h to evaginate the scolices. Then the cysticerci were washed and incubated again in protein-free medium as above. The ES-antigens produced during the next 24 h were

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collected and used as antigen in the serological tests and to immunize mice. The protein content of the ES-products in the medium was determined by a colloidal gold technique (Nano-gold, Sopar-Biochem, Brussels, Belgium).

Monoclonal antibodies (MoAb) against ES-products of 10- *and* 20-*week-old* T. saginata *cysticerci (ES*10 *and ES*20). Adult BALB/c female mice (Charles River Wiga) were immunized against ES10 or ES20 according to Holmdahl, Moran & Anderson (1985). In short, antigens were injected together with Freund's complete adjuvant in the footpad of both hindlegs of the mice. Ten days later the mice were killed by cervical dislocation. The lymphocytes of the popliteal lymph nodes were used for a fusion with myeloma cells (NSo). Cell fusion was generated using polyethylene glycol in a serum-free medium. Hybridomas were grown in RPMI-1640 with 10% FCS (Gibco) and 10% horse serum (Gibco) in a 5% CO_2 gas-phase. Supernatants of hybridomas were screened by means of an ELISA against ES10 or ES20. Positive hybridomas were cloned and injected into pristane-sensitized BALB/c mice for production of ascites. This was treated with 50% ammonium sulphate to purify the immunoglobulins. Afterwards they were dialysed for 1 week against 0.15 M-NaCl at 4°C. The isotype of the monoclonal antibodies was determined by a line immunoassay (Innogenetics, Antwerp, Belgium) using strips coated with rat monoclonal antibodies against mouse immunoglobulins.

Serum samples. Serum samples were collected from calves ($n=38$) experimentally infected with *T. saginata* eggs, as described by Geerts, Kumar, Ceulemans & Mortelmans (1981) and pigs ($n=8$) experimentally infected with *T. solium* eggs (n being the number of sera tested). After killing at the abattoir, the number of dead and living cysticerci present in the calves was estimated by carefully slicing half of the carcass. Negative control sera ($n=8$) from calves, kept in helminth-free conditions from birth, were included in addition to sera from cattle or sheep, experimentally infected with *Fasciola hepatica* ($n=3$), *Echinococcus granulosus* ($n=15$), *Taenia ovis* ($n=19$), *Taenia hydatigena* ($n=7$) and some protozoan parasites *Anaplasma marginale* ($n=1$), *Theileria parva* ($n=1$) and *Babesia bigemina* ($n=1$). Human sera from confirmed cases of *T. solium* cysticercosis ($n=13$) were also included. They originated from Spain, Cameroun, Mexico and Ecuador.

ELISA for the detection of circulating antigen. Polystyrene ELISA-plates (Nunc® Maxisorb) were coated with 100 μl purified monoclonal antibodies (20 μg ml^{-1} in borate buffer, pH 8.3). Plates were incubated for 1 h at 37°C and overnight at 4°C and then blocked with 1% bovine serum albumin (BSA) in PBS + 0.05% Tween 20 (PBS–T20) for 1 h at 37°C. After washing (three times for 5 min in physiological saline with 0.05% Tween 20) the test sera, diluted 1/4 in PBS–T20, were added and incubated for 1 h at 37°C. The plates were washed as above and biotinylated monoclonal antibodies, diluted in PBS–T20 to a concentration of 10 μg ml^{-1}, were added to each well. The biotin conjugation to the immunoglobulins was carried out according to the technique described by Bowman, Mika-Grieve & Grieve (1987). After incubation for 1 h at 37°C the plates were washed again and an avidin–biotin peroxidase complex (Dakopatts), diluted 1/500 (v/v) in Tris–HCl buffer (pH 7.6) was added and incubated for another hour. The plates were washed and finally the substrate solution, consisting of orthophenylene diamine (OPD) and H_2O_2, was added. After incubation for 15–20 min in the dark at room temperature the plate was read using an automated spectrophotometer (Titertek Multiskan EIA reader) at 405 nm. Control negative and positive serum samples were included in each assay, as well as negative bovine sera to which determined quantities of ES-protein in various concentrations were added. The cut-off level, above which a serum sample was considered positive, was calculated by comparing the optical density (O.D.) of each sample with the mean of a series of eight control negative samples at a probability level of $P=0.01$ (Sokal & Rohlf, 1981).

ELISA for antibody detection. The test was slightly modified from Geerts *et al.* (1981). ES10 diluted 1/20 in RPMI was coated onto polystyrene plates. Rabbit anti-bovine IgG peroxidase conjugate (Nordic) was diluted 1/1000 and used with OPD and H_2O_2 as substrate.

Immunoblotting. The SDS–PAGE was performed as described by Doucet, Murphy & Tuana (1990) using a 10% separating gel and a 4% stacking gel. Samples were brought to a final 1% concentration of sodium dodecyl sulphate (SDS) and heated at 100°C for 3 min. ES-products were dialysed and concentrated 100 times. The SDS–PAGE-resolved components were electrophoretically blotted onto nitrocellulose (Schleicher & Schuell, 0.22 μm) at 200 mA and 50 V for a minimum of 2 h at 4°C. Blocking was carried out using Tris-buffered saline (TBS) containing 5% skimmed milkpowder (w/v). The sheet was cut into strips and exposed to various MoAb (diluted in TBS–5% w/v skimmed milkpowder to 25 μg protein per ml). A monoclonal antibody (IgM) against Human Immunodeficiency Virus (HIV1-p24) was used as a negative control. Antigen bands with bound antibodies were visualized by a double overlay with rabbit anti-mouse IgM (Dakopatts) diluted 1/1000 and an anti-rabbit IgG–alkaline phosphatase conjugate (Sigma) diluted 1/2000. Bound phosphatase activities were visualized using nitro blue tetrazolium (BCIP/NBT) as a substrate. The molecular weights of the recognized antigens were determined in comparison with marker proteins (Rainbow protein molecular weight markers, Amersham).

Dissociation of immune complexes. In order to free parasite antigen from antigen–antibody complexes in the serum, the method of Weil & Liftis (1987) was used, slightly modified. One part of serum and three parts of 0.1 M-Na_2 EDTA (pH 4) were mixed and boiled for 5 min. After centrifugation at 9000 *g* for 3 min, the deposit was resuspended and again centrifuged at 9000 *g* for 3 min. The supernatant was recovered for use in ELISA.

Immunoperoxidase staining of histological sections. To identify the parasite structures, with which the MoAb against the ES-products of *T. saginata* are reacting, paraffin-embedded sections of living *T. saginata* metacestodes were treated basically as described by Hsu, Raine & Fanger (1981). Biotinylated MoAb were used at a concentration of 100 μg ml^{-1}. The avidin–biotin peroxidase complex (Dakopatts) was diluted 1/50 in Tris buffer (0.05 M, pH 7.6) and diaminobenzidin hydrochloride (+ H_2O_2) was used as substrate.

TABLE 1—DETECTION LIMIT OF ES-PRODUCTS OF *T. saginata* CYSTICERCI IN CATTLE SERUM USING A SANDWICH ELISA

Monoclonal antibody		Detection limit (ng protein ml^{-1})	
"Trapping"	Biotinylated indicator	ES10	ES20
	12 G5	0.1	0.1
	2 H8	0.1	0.1
	41 E1	0.5	1
12 G5	46 H11	5	5
	51 H3	> 50	> 50
	53 C5	> 50	> 50
	59 D9	> 50	> 50
	59 D10	> 50	> 50

ES10 and ES20 : excretory and secretory products of respectively 10- and 20-week-old *T. saginata* cysticerci.

RESULTS

Eight monoclonal antibodies were produced against ES-products of 10- and 20-week-old cysticerci of *T. saginata*. All were of the IgM-isotype. Different combinations of homologous and heterologous MoAb were evaluated in a double antibody sandwich ELISA. The detection limits of the assay using MoAb 12G5, which was found to be the best trapping antibody, and different biotinylated indicator MoAb are given in Table 1. Although the combination 12G5 (trapping) and 12G5 (indicator) gave as good results as 12G5 (trapping) and 2H8 (indicator), the latter one was selected. Both MoAb were prepared against ES10. All the ELISA results presented below were obtained with this combination.

Western blot analysis showed that the MoAb 12G5 and 2H8 were reacting with antigenic components of a somatic extract of adult *T. saginata* with mol. wts of 87,000 and 100,000. Immunoblotting of the ES-products of the metacestodes of *T. saginata* against the same MoAb revealed a faint band situated at a mol. wt of 65,000 (Fig. 1). Immunoperoxidase staining of sections of *T. saginata* metacestodes showed that both MoAb strongly reacted with the tegument of the parasite.

Circulating ES-antigens could be demonstrated from 5 weeks post-infection in animals harbouring living cysticerci and were not present in animals which carried only dead cysticerci (Fig. 2a, 2b). This circulating antigen remained present for about 15 weeks post-infection (w.p.i.), with a peak between the 6th and 8th w.p.i. Specific antibodies against the ES-products were detected from the sixth w.p.i. onwards and continued to increase until about 20 w.p.i., at the time of autopsy (Fig. 3).

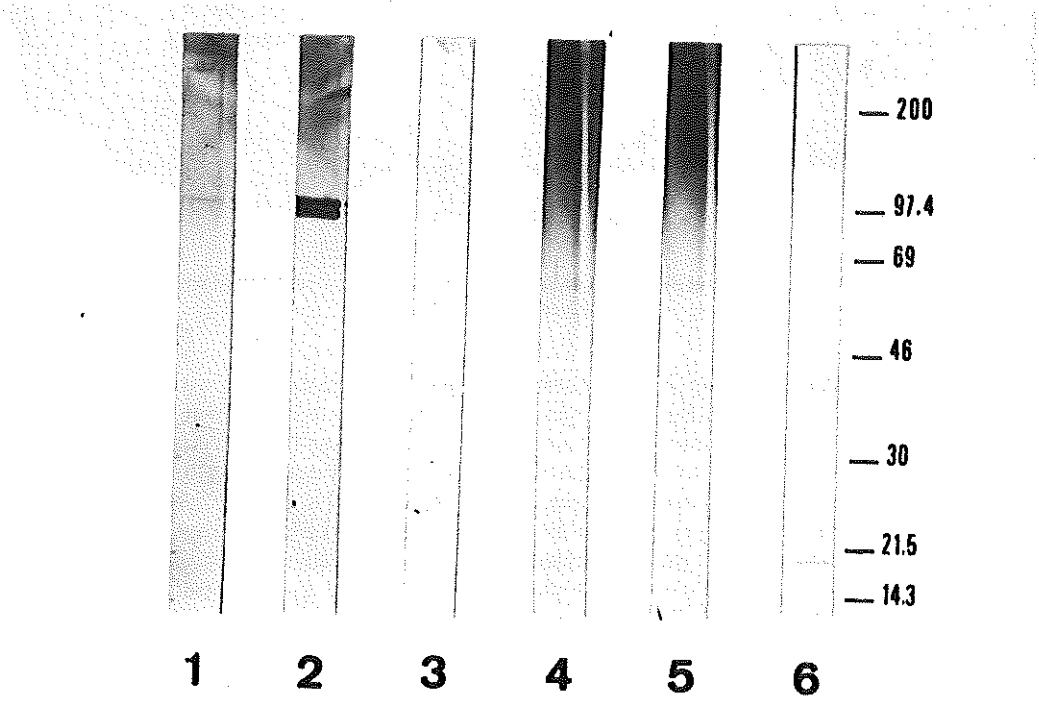

FIG. 1. Immunoblottings of *T. saginata* adult worm extract (tracks 1–3) and ES-antigen of *T. saginata* metacestodes (tracks 4–6) reacting with MoAb (IgM) 12G5 (tracks 1 and 4), 2H8 (tracks 2 and 5) and a control IgM-MoAb (anti HIV1-p 24) (tracks 3 and 6). The positions of the molecular weight standards (× 1000) are indicated on the righthand margin.

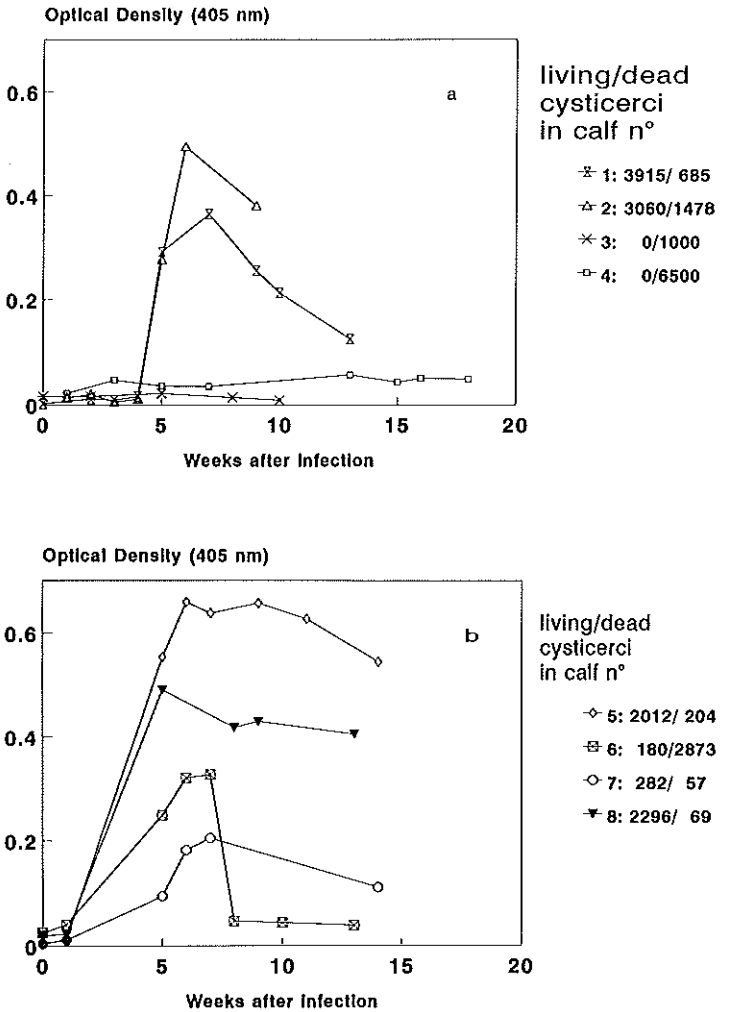

FIG. 2. ELISA values for circulating ES-antigen in sera of calves infected with *T. saginata* metacestodes.

Figure 4 shows the detection limit of the double antibody sandwich ELISA for circulating ES-antigen. This antigen was present in high amounts in animals ($n = 12$) harbouring more than 500 living cysticerci, whereas it could not be demonstrated in animals ($n = 8$) harbouring less than 50 living cysticerci in the whole carcass. Out of seven animals carrying between 50 and 200 living cysticerci three were positive in the circulating antigen test, with respectively 88, 135 and 194 living cysticerci. On the other hand, one animal, in which 281 living cysts were present, remained negative for circulating ES-products. Attempts to increase the sensitivity of the test by liberating antigen through dissociation of antigen–antibody immune complexes were unsuccessful.

Figure 5 summarizes the cross-reactivity with sera of humans and animals infected with parasites other than *T. saginata*. From the 19 sheep infected with *Taenia ovis* five harboured living cysts (< 10). Two of these showed cross-reactions. No positive reactions were obtained with the sera of animals harbouring only dead cysts. Out of 15 sera from sheep infected with *Echinococcus granulosus* three reacted positively, two of which contained a large number of cysts (428 and 655) in the liver and the lungs. Cattle infected with different protozoa, nematodes or trematodes and sheep infected with *T. hydatigena* did remain negative in the circulating antigen assay. All tested sera of pigs, infected with *T. solium* metacestodes, and nine out of 13 confirmed cases of human cysticercosis did also cross-react.

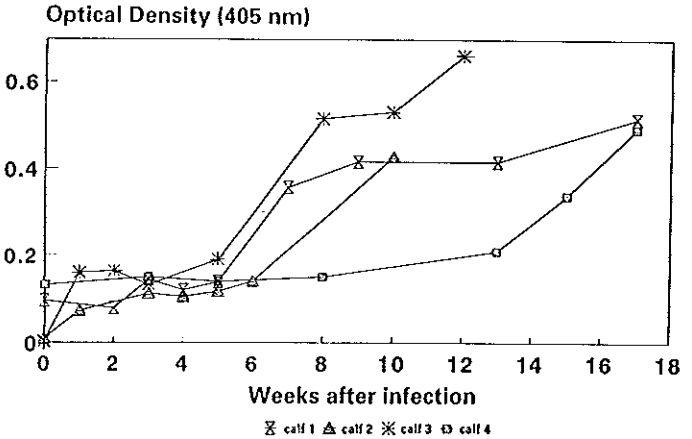

FIG. 3. Evolution of antibodies (ELISA) against ES-products in calves infected with *T. saginata* cysticerci.

DISCUSSION

The results obtained in the present work using the MoAb 12G5 and 2H8 correspond very much with the observations of Harrison, Joshua, Wright & Parkhouse (1989) working with MoAb HP10, which was produced after immunization of mice by injecting live cysticerci of *T. saginata* intraperitoneally. The latter MoAb reacted with an epitope present on a polydispersed group of molecules of 155,000–220,000 and also on a few components of lower molecular weight (< 55,000), present additionally in the surface of the somata as in the excretory–secretory antigens of *T. saginata* cysticerci. The MoAb 12G5 and 2H8, however, reacted with antigenic components of the ES-products of a different mol. wt (65,000) (Fig. 1). Immunoperoxidase staining of *T. saginata* metacestodes showed a strong reaction of both MoAb with the tegument of the parasite. A similar association with the tegument of the bladder wall was also observed for antigen B, an immunodominant protein of *T. solium* metacestodes (mol. wt 95,000 and 105,000) (Laclette, Merchant & Willms, 1987).

The sensitivity of this sandwich ELISA varied from one animal to another. The lowest number of living cysticerci which could be detected was 88, whereas another animal, harbouring 281 living cysts, escaped detection. These differences of sensitivity of the test might be related to the permeability of the host capsule around the cysticercus, which influences the amount of ES-products released in the circulation. Although attempts to free parasite antigen from antigen–antibody complexes according to the method of Weil & Liftis (1987) did not succeed, further research on the possible presence and the dissociation of circulating immune complexes might also result in an increased sensitivity of the test.

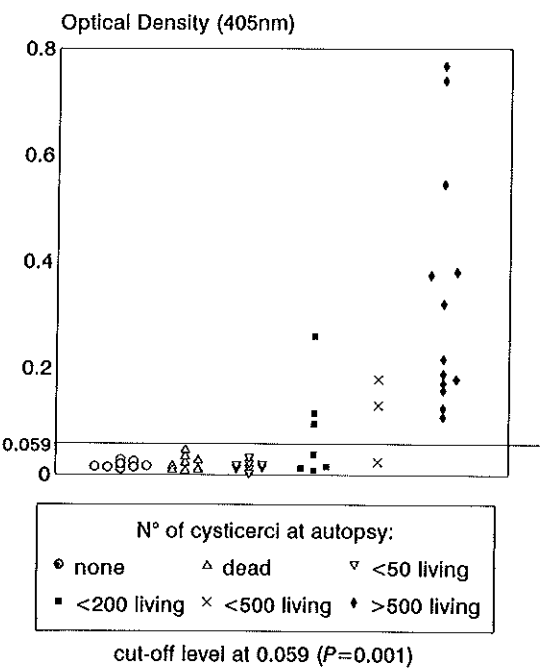

FIG. 4. ELISA values for circulating ES-antigen in calves infected with *T. saginata* metacestodes.

The evolution of circulating antigen in chronic infections is another point which needs more research, since no sera were available from animals, experimentally infected with *T. saginata* cysticerci for a period longer than 15 weeks. Available evidence from

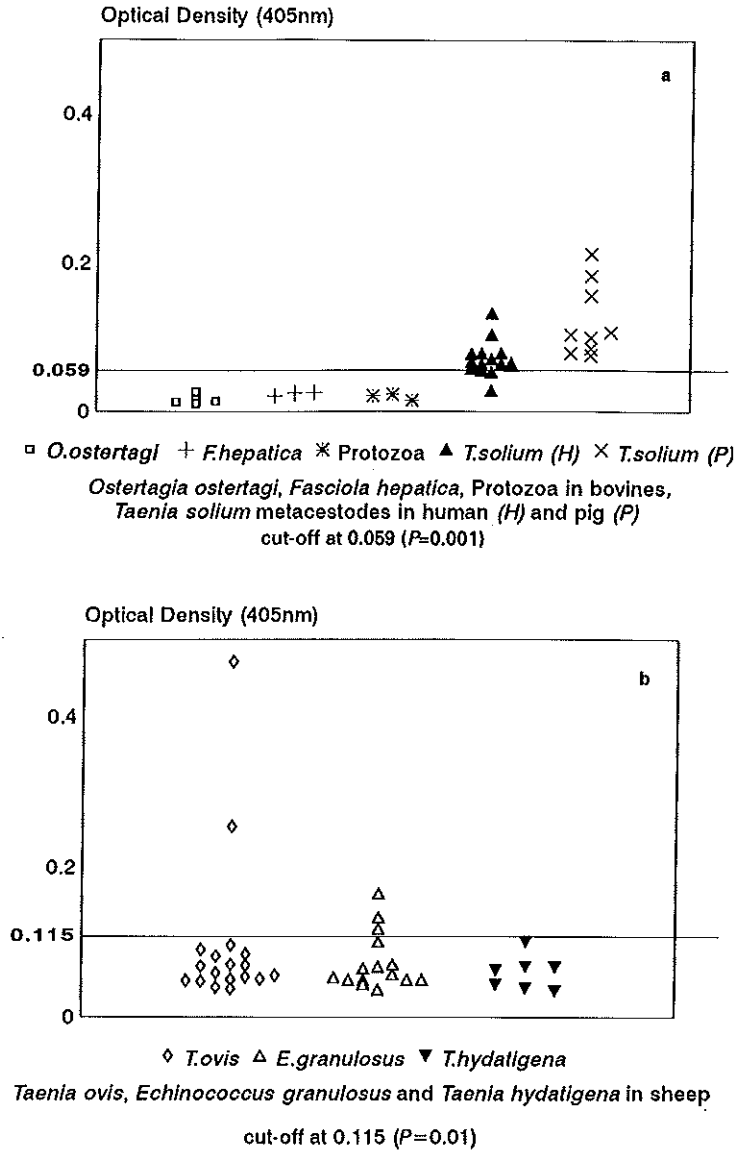

FIG. 5. ELISA values for circulating ES-antigen in humans and animals infected with parasites other than *T. saginata*.

naturally infected animals, which were frequently positive for circulating antigen, suggests that ES-products of the cysticerci remain present in the blood circulation after 15 weeks of infection.

The two MoAb 2H8 and 12G5, used in this assay, were not species-specific. Strong cross-reactions were obtained with sera of pigs and humans infected with *T. solium*. Out of the 13 sera from confirmed cases of human cysticercosis (with an unknown number of living cysticerci) nine (69.2%) could be detected using the circulating antigen assay. This is a similar sensitivity to that (72%) obtained by Correa, Sandoval, Harrison, Parkhouse, Plancarte, Meza-Lucas & Flisser (1989) using the HP10 monoclonal antibody.

The cross-reactions with sera from *T. ovis*-infected sheep are not problematic and might even open a new field of application. The positive reactions with 20% of the sera from sheep with hydatidosis suggest that the assay may cross-react with hydatidosis in cattle. Further research on sera of cattle naturally and experimentally infected with *E. granulosus* is necessary

before drawing definitive conclusions on the specificity of the assay for cattle parasites.

It can be concluded that the combination of MoAb 12G5 and 2H8 gives similar results for circulating antigen detection to the HP10 assay described by Harrison *et al.* (1989). A further increase in sensitivity will be necessary, however, in order to develop a useful field test since the average burdens of cysticerci present in whole carcasses of lightly infected animals, which are epidemiologically the most important group, vary between 3.3 and 17 (Juranek, Forbes & Keller, 1976; McCool, 1979; Kyvsgaard *et al.*, 1990; Walther & Koske, 1980).

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