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Detection of *Echinococcus* coproantigens by enzyme-linked immunosorbent assay in dogs, dingoes and foxes*

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Abstract. An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of *Echinococcus* coproantigens in fecal samples from dogs, dingoes or foxes infected with either *E. granulosus* or *E. multilocularis*. The ELISA was based on protein-A-purified polyclonal antibodies [anti-*E. granulosus* excretory/secretory (E/S) antigens]. The specificity of the assay as determined in 155 samples derived from carnivores that were free of helminth infection ($n=37$) or infected with non-*Echinococcus* cestodes ($n=76$) or with various nematodes ($n=42$) was found to be 98% overall. The diagnostic sensitivity was strongly dependent on the homologous worm burden. All 13 samples from foxes harboring >1,000 *E. multilocularis* worms and 13 of 15 (87%) samples from dogs or dingoes containing >200 *E. granulosus* worms were ELISA-positive, whereas 34 of 46 samples from foxes harboring <1,000 *E. multilocularis* and 9 of 10 samples from dogs or dingoes bearing <200 *E. granulosus* tested negative. Experimental prepatent infections of dogs with *E. granulosus* revealed positive ELISA reactions within the prepatent period (10–20 days post-infection) for six animals bearing >1,000 *E. granulosus* each; a low worm burden (<1,000 tapeworms/animal) resulted in ELISA positivity in only 2 of 3 animals at 30 days post-infection at the earliest. All five dogs that had been experimentally infected with *E. multilocularis* tested positive in the coproantigen ELISA as early as on day 5 post-infection.

The established methods for the diagnosis of intestinal *Echinococcus* infections in carnivores include (a) the identification of proglottids or whole parasites eliminated spontaneously or after purging (of dogs) with arecoline hydrobromide or (b) the examination of the small intestine at necropsy (Eckert et al. 1984). The detection of eggs on the skin of carnivores using the Scotch-tape technique (Deplazes and Eckert 1988) or in fecal samples by flotation techniques is indicative of infection with *Taenia* or *Echinococcus*, but the eggs of these genera cannot be differentiated by microscopy.

Serodiagnosis has been alternatively proposed in recent reports, as definitive hosts infected with intestinal stages of cestodes obviously exhibit the potential to develop a parasite-specific serum antibody response. Experimental investigations have been performed for the diagnosis of *E. granulosus* infections in dogs (Jenkins and Rickard 1985, 1986; Gasser et al. 1988) of *E. multilocularis* infections in foxes (Gottstein et al. 1991a). Unfortunately, serological studies demonstrated that antibody detection did not always reflect a current intestinal *E. granulosus* or *T. hydatigena* infection in carnivores (Jenkins et al. 1990, 1991). In areas endemic for *E. multilocularis*, prevalence rates of serum antibodies against Em2-antigen (Gottstein 1985) in fox populations reflected previous or continuing exposure to *E. multilocularis* antigen, most likely related to contact with eggs/oncospheres (Gottstein et al. 1991a), but did not enable the determination of the actual intestinal infection status in individual animals. However, on a population basis, the antibody prevalence showed a close correlation to the parasitological prevalence of *E. multilocularis* (Gottstein et al. 1991a).

Two alternatives have been proposed for the diagnosis of intestinal echinococcosis in definitive hosts; both methods involve the detection of parasites on a molecular level. One was defined as an antibody-sandwich enzyme-linked immunosorbent assay (ELISA; Deplazes et al. 1990, 1991) after it had been demonstrated that coproantigens were detectable by precipitation reactions with hyperimmune rabbit serum (Babos 1962). Such an ELISA used affinity-purified polyclonal antibodies raised against excretory/secretory (E/S) antigens of adult *T. hydatigena* or *T. saginata*. Coproantigens were reliably assayed and detected in fecal samples from dogs infected with *T. hydatigena*. Diagnostic genus specificity was underlined by the absence of cross-reactions related

* Dedicated to Prof. K.T.F. Friedhoff on the occasion of his 60th birthday

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to infections with *Echinococcus* spp. or other cestodes or nematodes. Sequential analyses revealed that *T. hydatigena* coproantigens were demonstrable during prepatency beginning at day 18 post-infection (p.i.). Another assay enabled the reliable diagnostic detection of *T. saginata* coproantigens in 85% of 34 fecal samples from 23 patients with taeniosis, whereas diagnosis by egg detection was only possible in 62% of the same individuals (Deplazes et al. 1991). In this respect, the potential of detecting coproantigens has become a general focus in the diagnosis of infection with intestinal cestode stages (Allan and Craig 1989; Allan et al. 1990; Deplazes et al. 1990, 1991). Alternatively, infection by adult cestodes could also be demonstrated and identified by the detection of parasite-specific DNA fragments originating either from parasite eggs or from cells or tissue debris of adult tapeworms. This approach has rapidly attracted attention, especially since the advent of highly sensitive techniques such as the polymerase chain reaction, and examples in this respect have been described elsewhere (Gottstein and Mowatt 1991; Gottstein et al. 1991 b).

This paper describes an ELISA designed for the detection of *Echinococcus* coproantigens in fecal samples obtained from definitive hosts infected with *E. granulosus* or *E. multilocularis*.

Materials and methods

Experimental design

A sandwich ELISA was developed for the diagnostic determination of *Echinococcus* antigens in fecal samples obtained from dogs, dingoes and foxes. The test, which uses protein-A-purified polyclonal hyperimmune antibodies, was evaluated in the following steps:

1. Determination of an ELISA threshold value, which enables the reliable discrimination between "positive" (detectable *Echinococcus* coproantigens) and "negative" (no detectable *Echinococcus* coproantigens) reactions; this step included the determination of the test's specificity against heterologous intestinal helminth infections (groups I and III for dogs and dingoes and group IV for foxes; see below)
2. Determination of the test's ability to detect coproantigens during the prepatent period of experimental *E. granulosus* or *E. multilocularis* infections in dogs (group II; see below)
3. Determination of the test's diagnostic sensitivity in fecal samples from dogs, dingoes or foxes naturally infected with *E. granulosus* or *E. multilocularis* (groups III and IV)
4. Assessment of specificity parameters based on samples obtained from uninfected animals or from animals showing experimentally or naturally acquired heterologous helminth infections (from groups I–IV)

Animals: fecal samples and intestinal contents

Fecal samples (FS) or intestinal contents (IC) from the rectum were collected from the following groups of animals as described below. Group I consisted of 13 dogs (*Niederlaufhunde*; 7 females and 6 males aged between 1 month and 6 years) kept under cestode-free conditions (13 FS). This group also included 7 dogs experimentally infected with *Taenia hydatigena* (3–6 adult *T. hydatigena* tapeworms were recovered per dog at necropsy); 2 fecal samples were collected per animal (on different days) during patency (14 FS). All samples were positive at the same time in a *T. hydatigena* coproantigen ELISA that was performed as previously described by Deplazes et al. (1990). In addition, this group contained 28 dogs of various ages and breeds, all of which showed no indication of an infection with *Taenia* or *Echinococcus* spp. However, 5 were infected with *Mesocestoides corti*; 4, with *Dipylidium caninum*; 5, with *Trichuris vulpis*; 6, with *Toxocara canis*; 3, with *Toxascaris leonina*; 3, with hookworms; 1, with *T. leonina* and hookworms; and 1, with *T. canis* and *T. leonina* (28 FS).

Group II consisted of 9 dogs (*Niederlaufhunde*; 4 males and 5 females aged between 1 and 2 years) raised under helminth-free conditions. The dogs were experimentally infected with approximately 0.6 ml sedimented (1 *g*) *E. granulosus* protoscolex suspension per os (infection mode according to Eckert et al. 1989). The protoscoleces were isolated from the following sources: a liver cyst from a naturally infected Swiss horse (6 dogs), a liver cyst from a naturally infected pig from Poland (2 dogs), and a lung cyst from a naturally infected Swiss cow (1 dog). All dogs were necropsied on day 30 p.i. This group also contained 5 female dogs (*Niederlaufhunde*) aged 2 years that were raised under helminth-free conditions. Each dog was experimentally infected with 20 g minced *E. multilocularis* metacestode tissue (obtained from *Meriones unguiculatus* experimentally infected with the parasite isolate SLI-144) containing approximately 0.5×10^6 protoscoleces. All dogs were necropsied on day 25 p.i. Fecal samples were collected at different intervals during prepatency (93 FS). The recovery and counting of (live) *E. granulosus* and *E. multilocularis* tapeworms were done according to Eckert et al. (1989).

Group III consisted of 50 stray dogs from the Logrono area of Spain. Parasitological examination of the intestine and the recovery and counting of *E. granulosus* tapeworms were performed after necropsy using conventional parasitological methods. The respective results are shown in Fig. 4 (50 IC). This group also included 16 dingoes shot in the Bondo State Forest in central southeastern New South Wales, Australia, where *E. granulosus* is known to be prevalent (Jenkins and Morris 1991). The parasitological results are shown in Fig. 5 (16 IC). In addition, this group contained 12 foxes free of *E. granulosus* infection. These foxes were shot in Taralga, New South Wales, Australia, all were infected with *Uncinaria stenocephala*, and some were additionally infected with *T. canis* ($n=4$) and *D. caninum* ($n=1$). *E. granulosus* has been found in foxes in the Blue Mountains National Park adjacent to this area (Jenkins, personal communication). The parasitological examination of the dingo and fox intestines and the recovery and counting of *E. granulosus* tapeworms (12 IC) were done according to Jenkins et al. (1990).

Group IV consisted of 100 red foxes shot in the Canton of Zürich, Switzerland, over a 5-month period in 1990. Necropsy was performed after the carcasses had been frozen at −80° C for 5 days (a procedure that kills *Echinococcus* eggs; Eckert et al. 1991). The recovery and counting of *E. multilocularis* tapeworms (100 IC) were done according to Eckert et al. (1989). This group also included 40 Swiss red foxes free of *E. multilocularis* infection; 14 were parasitologically free of helminths at necropsy, 5 were infected with other non-*Echinococcus* cestodes, 12 were infected with intestinal nematodes, and 9 displayed simultaneous infections with non-*Echinococcus* cestodes and nematodes (40 IC). In addition, this group contained 60 *E. multilocularis*-infected Swiss red foxes grouped according to the numbers of *E. multilocularis* tapeworms recovered per animal (28 foxes harboring 1–100, 18 containing 101–1,000, and 14 bearing >1,000 adult-stage *E. multilocularis*). Among these 60 foxes, 21 harbored no other intestinal helminths, 4 additionally bore other intestinal non-*Echinococcus* cestodes, 14 had intestinal nematodes, and 21 harbored non-*Echinococcus* cestodes and nematodes simultaneously (60 IC).

From all animals in groups I–IV, fresh FS or IC were collected and mixed at a 1:4 (v/v) ratio with a buffer solution [phosphate-buffered saline (PBS) containing 0.04% NaN_3, 0.05% bovine hemoglobin (Fluka) and 0.3% Tween-20]. All samples were stored or transported at −20° C until further processed. The samples were thawed and the fecal suspensions were ultrasonicated for 30 s

(40 W) and subsequently sedimented at 3,000 *g* for 10 min prior to their use in ELISA.

Echinococcus granulosus antigens

Living immature adult *E. granulosus* tapeworms were recovered from the intestines of six dogs (experimentally infected with a horse parasite isolate) on day 30 p.i. (methodology according to Eckert et al. 1989). Approximately 2,000 *E. granulosus* tapeworms were cultivated in a 30-ml plastic tissue-culture flask (Falcon) containing 30 ml Eagle's minimal essential medium (EMEM; Gibco, catalogue number 072 1100) supplemented with D-glucose (4 mg/ml), gentamycin (200 µg/ml) and Fungizone (250 ng/ml) at pH 7.2. The medium was replaced after 4, 14, 22 and 34 h in vitro maintenance and then every 2nd day until day 14. The aliquots of tissue-culture medium (TCM) collected from day 3 until day 14 were stored at −20° C until further processed. The viability of the worms was judged by their motility and morphology as viewed with an inverted microscope (Laborvert FS, Leitz). 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 and pooled TCM to a volume containing 0.1 mg protein/ml PBS using an Amicon ultrafiltration unit with a YM-10 membrane. All protein concentrations were assessed by a Bio-Rad protein assay using bovine serum albumin as the standard. The *E. granulosus* E/S antigens were used for the elaboration of the *Echinococcus* coproantigen-detecting ELISA as described below.

Anti-E. granulosus hyperimmunoglobulins

Anti-*E. granulosus* hyperimmunoglobulins were generated by the immunization of a rabbit with *E. granulosus* E/S antigens (100 µg/injection) emulsified in Ribi adjuvants according to the manufacturer's recommendations (Ribi Immunochem Research Inc., Hamilton, Montana, USA). Immunoglobulins were purified on a protein A-Sepharose CL-4B column according to the manufacturer's instructions (Pharmacia Fine Chemicals, Uppsala, Sweden). Serum from a non-immunized rabbit was processed in exactly the same way to obtain "irrelevant" negative control antibodies. All further steps, including the preparation of rabbit anti-*E. granulosus* E/S conjugate (alkaline phosphatase-labeled), were performed according to the procedure of Baumann and Gottstein (1987).

Enzyme-linked immunosorbent assay

The concept for the development of a sandwich ELISA for the detection of fecal *Echinococcus* antigens in dogs, dingoes and foxes was based (with few modifications) on that previously decribed for the detection of *T. hydatigena* coproantigens in dogs (Deplazes et al. 1990) and can be summarized briefly as follows. Protein-A-purified rabbit anti-*E. granulosus* E/S IgG was used in the solid phase as a coproantigen-specific catching antibody (at 20 µg protein/ml coating solution), with an irrelevant rabbit (solid-phase) IgG being included in parallel as a negative control. Parasite-specific antibodies enabled the complexing of coproantigens from diagnostic samples; visualization of the immune reaction was obtained by using liquid-phase (protein-A-purified) rabbit anti-*E. granulosus* E/S IgG labeled with alkaline phosphatase and the corresponding chromogenic substrate solution (4-nitrophenyl phosphate). The results were expressed in corrected $A_{405\ nm}$ values (the value obtained for the specific reaction minus that found for the control reaction using the irrelevant antibody) and were monitored in reference to standard positive and negative control samples. When the results obtained using the irrelevant control IgG exceeded 30% of the positive $A_{405\ nm}$ value found for the specific IgG reaction, the sample run in question was excluded.

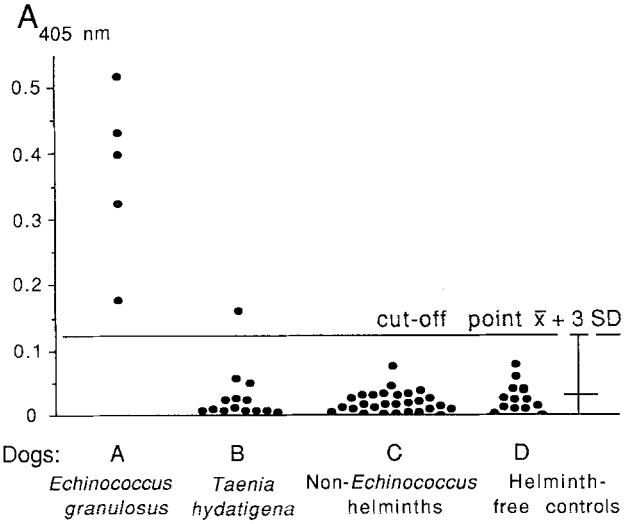

Fig. 1. Arbitrary determination of a discriminating threshold in the ELISA for *Echinococcus* coproantigen detection in dogs. The cut-off point (−) was determined by calculating the mean $A_{405\ nm}$ value +3 SD for group I animals (*B*, *C*, *D*). *A*, Preselected *E. granulosus*-positive reference controls (5 naturally infected dogs from Spain); *B*, 14 samples from 7 dogs with patent *Taenia hydatigena* infections; *C*, 28 samples from dogs with other non-*Echinococcus* helminth infections; *D*, samples from 13 helminth-free control dogs

Results

The order of the results reported below corresponds to the scheme described in Experimental design and are summarized in Figs. 1–5.

Determination of a diagnostic cut-off value

In the ELISA, a cut-off value was determined by calculating the mean $A_{405\ nm}$ value + 3 SD for animal groups with no *Echinococcus* infection and for those with heterologous helminth infections capable of producing cross-reacting antigens. The results are shown in Fig. 1. In all dogs that exhibited no helminth infection or were infected with non-taeniid helminths the findings were closely grouped in the same range of low $A_{405\ nm}$ values. Using these results together with the values obtained for dogs infected with *Taenia hydatigena*, the respective cut-off value was calculated, resulting in negative test interpretations for all non-taeniid samples. One of two samples from a dog infected with 7 adult *T. hydatigena* reacted above the cut-off value (positive reaction).

Antigen detection in prepatent infections of E. granulosus or E. multilocularis

The results obtained for dogs experimentally infected with *E. granulosus* or *E. multilocularis* (group II animals) are shown in Fig. 2. Analysis of the small intestine after necropsy (on day 30 p.i.) revealed a wide range of worm burdens (between 200 and >10,000 worms/dog for *E.*

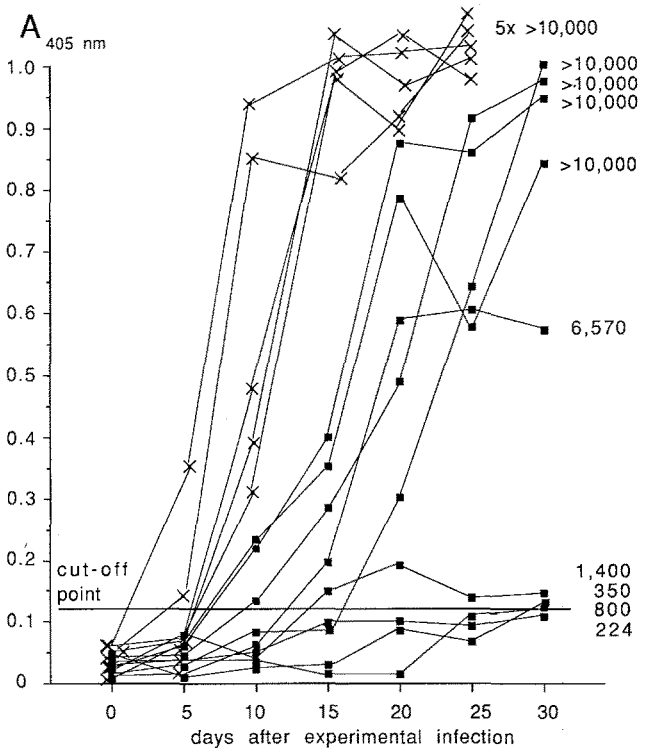

Fig. 2. Detection of coproantigens by ELISA during the prepatent period of experimental *Echinococcus granulosus* (■) or *E. multilocularis* (×) infections in 9 and 5 dogs, respectively (group II animals). Right-hand numbers indicate recovered worm burden. The cut-off point (−) was determined as described in Fig. 1

granulosus and between 10,000 and 100,000 worms/dog for *E. multilocularis*). Reliable detection of coproantigens was correlated to the number of worms detected in the dogs. The first positive reactions were detected at 10 days p.i. in dogs showing burdens of >10,000 *E. granulosus* worms and at 5 days p.i. in all dogs harboring burdens of >10,000 *E. multilocularis*. By 20 days p.i., all dogs with worm burdens of >1,000 *E. granulosus* had become positive. Dogs harboring <1,000 *E. granulosus* exhibited only weakly positive ($n=2$) or negative ($n=1$) reactions at 30 days p.i.

Antigen detection in natural infections with E. granulosus or E. multilocularis

The intestines of necropsied Spanish dogs and Australian dingoes and foxes (all animals came from areas that are endemic for *E. granulosus* and are not known to be endemic for *E. multilocularis*) were parasitologically examined (group III animals) and the intestinal content was tested in parallel by ELISA.

A positive reaction could be detected in 1 of 31 samples from Spanish stray dogs that were infected with different numbers of *T. hydatigena*, *T. pisiformis* and *Dipylidium caninum* but harbored no *E. granulosus*, demonstrating the high specificity of the test (Fig. 3). All 9 dogs containing ≥200 *E. granulosus* worms showed a positive reaction in the ELISA; on the other hand, coproantigens could be detected in only 1 of 8 samples from dogs infected with 3–70 *E. granulosus*.

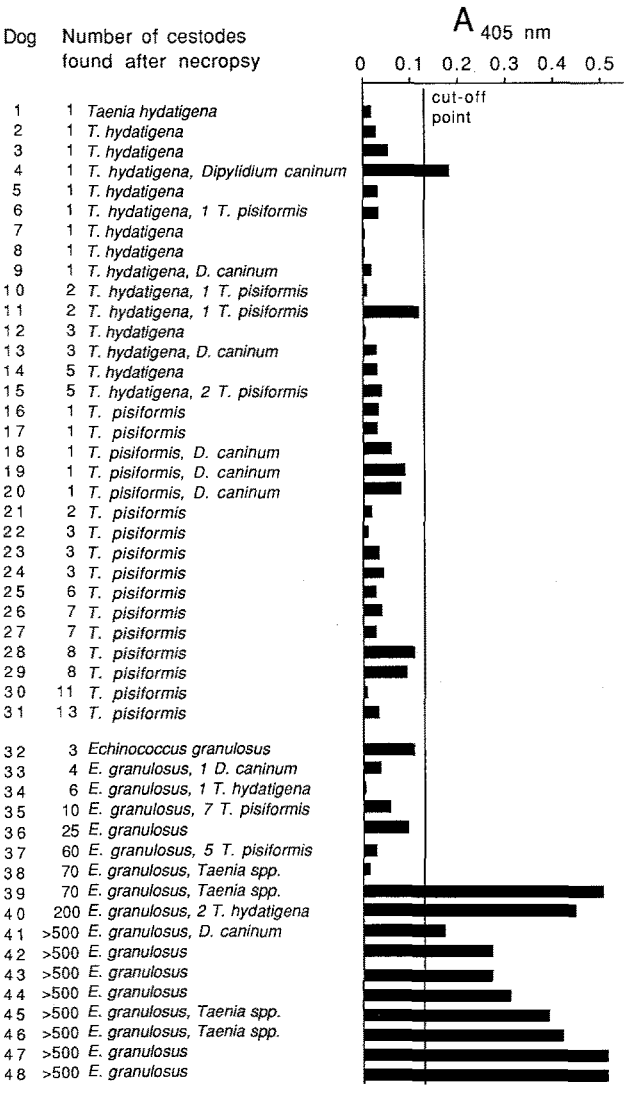

Fig. 3. Detection of *Echinococcus granulosus* coproantigens by ELISA in samples obtained from stray Spanish dogs (from group III). The cut-off point (−) was determined as described in Fig. 1

The results obtained for samples from dingoes (Fig. 4) again demonstrated the dependence of a positive ELISA reaction on the number of *E. granulosus* worms recovered. Cross-reactions occurred in one sample from a dingo with a *T. hydatigena* infection; all other samples showing no *E. granulosus* infection were negative. Australian foxes ($n=12$) were negative for *E. granulosus* in parasitological examinations as well as in the ELISA. Frequently occurring heterologous helminth infections underlined the high specificity of the ELISA.

Figure 5 shows the results of investigations of samples from foxes that were selected on the detection of adult-stage *E. multilocularis* in the intestine (group IV animals). The findings, grouped according to the worm burden of the animals, confirmed both the strong correlation between ELISA positivity and the number of worms recovered and the high specificity (100%) of the test for samples from animals showing no *Echinococcus* infection.

The overall diagnostic sensitivity of the ELISA for

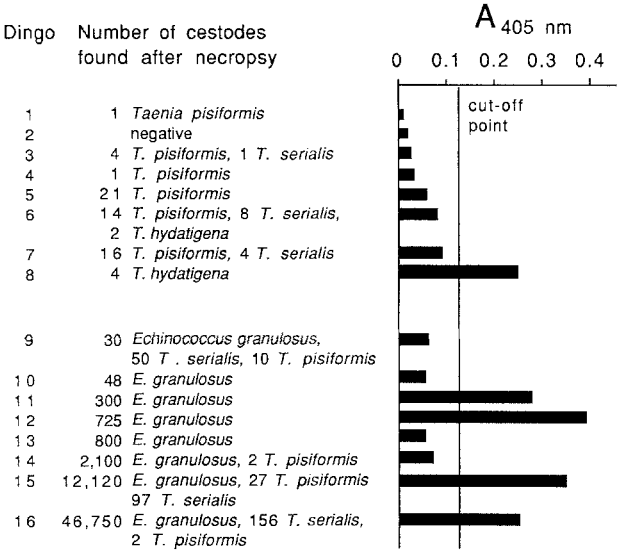

Fig. 4. Detection of *Echinococcus granulosus* coproantigens by ELISA in samples obtained from Australian dingoes (from group III). The cut-off point (—) was determined as described in Fig. 1

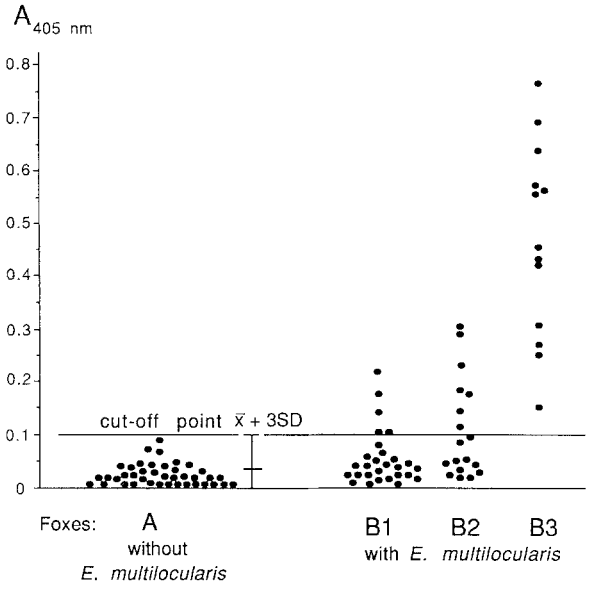

Fig. 5. Detection of *Echinococcus multilocularis* coproantigens by ELISA in samples obtained from Swiss foxes. Evaluation of the sensitivity and specificity of the assay in samples obtained from group IV animals: *A*, foxes ($n=40$) with no *E. multilocularis* infection; *B1*, foxes ($n=28$) with 1–100 *E. multilocularis*; *B2* foxes ($n=18$), with 101–1000 *E. multilocularis*; *B3* foxes ($n=13$) with >1000 *E. multilocularis*. The cut-off point (——) was determined by calculating the mean $A_{405\ nm}$ value + 3 SD for group A

the detection of *Echinococcus* spp. was 46% for *E. granulosus* infections in dingoes and 56% for infections in dogs; for animals harboring ≥200 worms, the sensitivity was 87%. The diagnostic sensitivity was 42% for *E. multilocularis* infections in foxes (18% for animals harboring <100 worms, 39% for those bearing 100–1,000 worms and 100% for those containing >1,000 worms).

Assessment of the overall test specificity

The overall specificity of the ELISA as determined using 155 samples obtained from carnivores that were free of infection or harbored experimentally or naturally acquired heterologous helminth infections (group I, II, III and IV) was found to be 98%. The samples were collected from animals that were not infected with *Echinococcus* spp., animals showing no helminth infection ($n=37$) and animals that were infected with non-*Echinococcus* cestodes ($n=76$) or with various nematode species ($n=42$). Cross-reactions were detected in two samples taken from dogs (Figs. 1, 3) and in one obtained from a dingo (Fig. 4); the animals had been infected with 7, 4 and 1 *T. hydatigena*, respectively. Of the total of 324 samples used in the present study, only 3 could not be accurately assessed due to a strong reaction with the control IgG; these samples had been collected from a fox infected with >1,000 *E. multilocularis* and from two stray dogs that showed no *Echinococcus* infection.

Discussion

This report addresses the diagnostic potential of an ELISA for the detection of soluble metabolic, intestinal-stage antigens of *Echinococcus granulosus* or *E. multilocularis* in feces. The use of hyperimmune antibodies enabled the development of a test with high specificity (98%), which represents a marked improvement as compared with coproscopy, in which eggs of taeniid cestodes are morphologically indistinguishable. The test is easier to perform than other immunological approaches for the identification of the generic origin of taeniid eggs via the use of monoclonal antibodies directed against oncospheres hatched in vitro (Craig et al. 1986). In contrast to serological methods, whereby specific antibodies persist after the elimination of the parasite, coproantigens are excreted only during or shortly after the termination of a current infection. The potential danger to investigators working with feces contaminated with *Echinococcus* eggs is minimal in comparison with that to workers engaged in coproscopy. Risks can be further reduced if the eggs are first killed by freezing of fecal samples at −70° to −80° C for at least 48 h (Blunt et al. 1991; Eckert et al. 1991). We especially addressed the logistical approach to the detection of E/S *E. granulosus* antigens, as we assumed that these would be more constantly released than somatic antigens. For this purpose, worms with immature eggs were cultivated in a defined medium in vitro. As described by Thompson and Eckert (1982), no apolysis of proglottids was observed during the in vitro cultivation, suggesting that the parasite antigens in the supernatant were mostly metabolic products.

The diagnostic sensitivity of this ELISA was found to be closely dependant on the *Echinococcus* worm burden in natural and experimental infections, which presents problems for the sensitivity of the test that have not yet been solved. In this context, it has been shown that *Taenia hydatigena* coproantigens remain stable for at least 5 days in feces at 25° C (Deplazes et al. 1990).

Our experimentally infected dogs were fed daily and regular defecation was observed. In contrast, wild carnivores are known to have irregular and very different feeding habits; this may indirectly affect the digestive activity and the time required for passage of the ingested meal through the gut. Such physiological factors could reduce the stability of E/S antigens and may influence the sensitivity of the test system.

In experimental infections with high numbers of worms, coproantigens could first be detected on day 5 p.i. for *E. multilocularis* and on day 10 p.i. for *E. granulosus*. At this time, the length of *E. granulosus* does not exceed 0.7 mm (Thompson 1975) and the lateral excretory canals become conspicuous with the first visible signs of proglottid development (Thompson 1986). Little is known about the metabolic activity of *Echinococcus* at different stages of its development in the gut. During the prepatent period (up to day 35 p.i.), a steady growth of the worms has been described (Thompson 1975). However, there is no information on worm growth or the rate of proglottisation as indicators of metabolic activity during the patent period. In one study, after the first appearance of *E. granulosus* eggs in the feces of experimentally infected dogs, the subsequent release of eggs was delayed by 4–6 weeks (Yamashita et al. 1956). Such latent periods involving a lack of egg or proglottid excretion have also described in experimental infections of dogs with *T. hydatigena* (Deplazes and Eckert 1988). In the same investigation, the patent periods were reduced after reinfection and were often followed by latent periods during which no eggs and/or proglottids were excreted for several months.

The high specificity of the present ELISA suggests good diagnostic potential, provided that technical developments improve its relatively low sensitivity. This may be approached by the molecular identification and purification of the crucial antigenic parasite components, which could then be followed by the generation of the respective monoclonal antibodies to be used in the assay.

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