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An Enzyme-linked Immunosorbent Assay (ELISA) for the Detection of *Echinococcus granulosus* Coproantigens in Dogs

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Abstract

A sandwich ELISA, using polyclonal antibody against excretory/secretory (ES) antigens of adult *Echinococcus granulosus* and a monoclonal antibody (MoAb), designated as EmA9 (Kohno *et al.*, 1995), against the somatic antigen of adult *E. multilocularis*, was used for diagnostic detection of *E. granulosus* coproantigens in dogs. The antibodies used for ELISA recognized heat-resistant antigens, thus all fecal samples could be heated to render it safe for handling before the test is performed.

By using sandwich ELISA, *E. granulosus* coproantigens could be detected during the initial phase of the infection in dogs experimentally infected with this cestode. In dogs inoculated with protoscoleces from only one cyst, recovered worms showed almost same developmental stage and the coproantigens detected in samples from these dogs showed a similar pattern. It is suggested that the release of ES antigens in the feces may be synchronized depending on the developmental stage.

Antibody response of dogs against *E. granulosus* antigens was also examined. However, antibody levels of dogs infected with a large number of worms did not rise, while only one dog infected with few worms showed apparently high antibody levels.

This sandwich ELISA was also used for the diagnosis of 59 dogs in Uruguay. Dogs were treated with arecoline hydrobromide and the excreted parasites were identified. The diagnostic probability of true-positive cases was unexpectedly low (37.5%) and that of false-positive ones was 14%.

Key words: *Echinococcus granulosus*; coproantigen; immunodiagnosis; dog; ELISA; Uruguay.

Introduction

Hydatidosis is one of the most important zoonoses caused by larval *Echinococcus* infection. The infection is acquired by ingesting eggs excreted from infected definitive hosts and the larvae multiply particularly in the liver or lung of the intermediate hosts. *E. granulosus*, one of the causative organisms, is distributed all over the world and maintains the domestic life cycle through dogs as definitive host and domestic ungulates (e.g., sheep, cattle, pigs, goats and horses) as intermediate hosts. The infection in domestic animals causes serious economic loss because of decreased growth rate of infected animals and inferior quality of animal products, especially in wool of sheep and condemnation of infected viscera, etc (FAO, 1982). In addition, the infection in man is also a serious problem because it is often fatal. Consequently, hydatid disease control programmes have been implemented in endemic areas and accurate diagnosis of *E. granulosus* infection in dogs has always been one of the most important components for establishing epidemiologic parameters of this disease.

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For diagnosis of infected dogs, microscopic examination of excreted eggs in feces or arecoline purging and subsequent fecal examination has been widely used in endemic areas (FAO, 1982). However, these techniques have some disadvantages. Parasites during pre-patency seldom excrete eggs and their excretion in feces during patency is irregular. Arecoline treatment sometimes have no effect in some dogs, while others exhibit undesirable side-effects such as vomiting or a sudden fall in blood pressure (Rickard and Arundel, 1985; Wachira *et al.*, 1990). In addition, both techniques are potential biohazards. To circumvent these problems, some immunological methods have been proposed for the diagnosis of *E. granulosus* infected dogs (Craig, 1993). Detection of serum antibodies using ELISA in infected dogs has been carried out (Jenkins and Rickard, 1986; Gasser *et al.*, 1988, 1992, 1993). Results of these studies indicated that serodiagnosis is useful for epidemiological studies, however antibody detection does not always reflect current infection of *E. granulosus* adults. On the other hand, coproantigen detection corresponds to the presence of worms in the intestine and hence is a good tool for diagnosis of infected dogs (Allan *et al.*, 1992; Deplazes *et al.*, 1992). A sandwich ELISA developed for detection of coproantigens using polyclonal antibodies against *E. granulosus* somatic or excretion/secretion (ES) antigens as capture (primary) and enzyme labelled (secondary) antibodies, showed high specificity and sensitivity (Deplazes *et al.*, *1992).* Because of its merits, coproantigen detection has been used for prevalence surveys of canine echinococcosis in endemic areas (Craig *et al.*, 1995; Deplazes *et al.*, 1994).

Recently, Kohno *et al.* (1995) produced monoclonal antibodies (MoAbs) against *E. multilocularis* somatic antigens for detection of *E. multilocularis* coproantigens. Furthermore, Sakashita *et al.* (1995) suggested that sandwich ELISA using MoAb EmA9 is a good tool for detection of *E. multilocularis* coproantigen in infected dogs. In the present study, we attempted to use this method for detection of *E. granulosus* coproantigens in experimentally infected dogs. In addition, the test was used for diagnosis of 59 dogs in Uruguay. These dogs were subsequently treated with arecoline hydrobromide and the excreted parasite species were identified. The results of arecoline survey were compared with coproantigen detection using this method and the availability of the test for diagnosis of *E. granulosus* infected dogs was evaluated.

Materials and Methods

Parasite infection

E. granulosus protoscoleces were obtained from bovine lung cysts abandoned in the dog food factory.

Four dogs with unknown clinical histories were used for the experiment. These dogs (three males and one female) previously treated with praziquantel were infected with different numbers of protoscoleces orally. The feces and sera obtained from these dogs at various periods after the infection were used for the present examination.

These dogs were sacrificed on day 32–33 postinfection (PI) and the small intestine was isolated by ligation. The small intestine was divided into three parts (anterior, middle, posterior), placed in Hank's balanced salt solution and incubated for various hours. During the time, adult worms were released from the gut mucosa. Recovered worms from each part were counted and were used for preparation of ES and somatic antigens.

ES antigens

Equipment, Hank's solution and medium were all sterilized. *E. granulosus* adult worms were thoroughly washed with Hank's solution (pH 7.2) containing antibiotics (gentamycin 200 μg/ml) and were subsequently washed with Medium 199 (Gibco Laboratories, New York, USA) supplemented with glucose (4.0 g/l) and antibiotics (gentamycin 200 μg/ml) at pH 7.2. Approximately 10,000 *E. granulosus* tapeworms were cultivated in 125 ml medium. The medium was replaced after 6, 15, 24, 36 hours and the collected culture medium was stored at –20°C until further processed.

ES antigens were prepared by concentration of the collected culture medium using an Amicon ultrafiltration unit and a YM-10 membrane (Amicon, Inc., Beverly, USA) and dialysed in phosphate buffered saline (PBS). Protein concentrations were assessed by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, USA) using bovine γ-

globulin as a standard protein.

Somatic antigens

E. granulosus somatic antigens were prepared for detection of specific antibodies in sera. Approximately 4,000 worms were washed several times with PBS and were broken up by repeated strokes of the homogeniser in PBS, containing protease inhibitors and chelating agent [3 mM L-*trans*-epoxysuccinyl-leucylamido-(4-guanidinobutane) (E-64), 1 mM Pepstatin, 1 mM phenylmethanesulfony fluoride (PMSF), 2.5 mM ethylenediaminetetraacetic acid (EDTA)]. Subsequently, the material was ultrasonicated by ultrasonic homogenizer (Cole-Palmer, Model CP50, Chicago, USA) for 3 min (40 W) and stirred at 4°C overnight. The suspension was centrifuged at 10,000 g for 30 min at 4°C. Supernatants containing the somatic antigens were stored at –80°C until used.

Fecal samples

Fecal samples were collected from four dogs prior to infection and then after infection everyday until day 32–33 PI. All fecal samples were immediately treated as follows and stored at –80°C: One flask containing 8 ml PBS and two flasks, each containing 8 ml 1% formalin were prepared. To each flask, 2 g feces were added and shaken vigorously. One flask with formalin was heated at 70°C for at least 12 hours. Each suspension was subsequently centrifuged at 10,000 g for 15 min. The supernatant was stored at –80°C until used. Fecal samples from 7 dogs not infected with *E. granulosus* were collected for negative control of coproantigens and prepared as described previously.

Fecal samples were also collected from 59 dogs in San Gregorio, Department of Tacuarembó where arecoline survey was also carried out. All fecal samples were mixed with 20 ml of 1% formalin solution in the collection vial with screw cap and heated for at least 12 hours at 70°C. Subsequently each vial was weighed and fecal weight was calculated. To each vial, additional 1% formalin solution was added to make a ratio of 1:4 (fecal weight: formalin solution). After shaking vigorously, one part of each suspension was taken out and centrifuged at 10,000 g for 15 min. Supernatant was used for the detection of the *E. granulosus* coproantigen by ELISA as described below. Fecal samples from 10 dogs not infected with *E. granulosus* were used for negative control of coproantigens.

*Rabbit anti-*E. granulosus *ES polyclonal antibodies*

Anti-*E. granulosus* polyclonal antibodies were produced by immunization of a rabbit with *E. granulosus* ES antigens as follows: ES antigen solution containing 50 μg proteins was emulsified with Freund's complete adjuvant (Sigma, St. Louis, USA) in a 1:1 ratio and injected to a rabbit subcutaneously. Two weeks later, same solution was emulsified with Freund's incomplete adjuvant (Sigma) in the same ratio and injected as previously. The rabbit was bled 14 days after the booster injection. Subsequently, same antigen solution diluted with saline was injected and the rabbit was bled 14 days after the injection. Immunoglobulins were purified by a protein A affinity column according to the manufacturer's instructions (Nippon Bio-Rad Laboratories KK, Tokyo, Japan). Immunoglobulin concentrations were assessed as described above.

Monoclonal antibody

Monoclonal antibody, EmA9, against *E. multilocularis* adult worm somatic antigens produced by Kohno *et al.* (1995) were used in the present study.

Detection of coproantigens using sandwich ELISA

Flat-bottomed microtitre plates (Greiner, Frickenhausen, W. Germany) were used for ELISA. Each well was coated with 50 μl of affinity purified γ-globulin fraction of rabbit anti-*E. granulosus* ES serum at a concentration of 25 μg/ml in 0.05 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) and left overnight at 4°C. The remaining procedures are similar to that described by Kohno *et al.* (1995). The assay was read at OD 490 nm.

Detection of circulating antibodies in sera

ELISA for detection of circulating antibodies in sera of dogs was basically performed as described by Sakashita *et al.* (1996), and is summarized briefly as follows; Each flat-bottomed microtitre plate well was coated with *E. granulosus* somatic antigens at a concentration of 10 ng/50 μl in 0.05 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) and left overnight at 4°C.

For blocking, 1% BSA-PBS (100 μl) was incubated for 1 hour at room temperature. 1:500 dilution of each test serum was added to each well, duplicately. 1:2000 dilution of peroxidase labelled goat anti-dog IgG (ORGANON TEKNIKA, Durham, USA) was added for detection of antibodies that reached to the *E. granulosus* antigen. Finally, substrate solution (o-phenylene diamine) was added and the plates were read at OD 490 nm.

Results

E. granulosus *recovered from experimentally infected dogs*

The number of worms recovered from infected dogs is shown in Table 1. Adult worms recovered from dog No. 4 and dog No. 1, which were infected with protoscoleces obtained from only one cyst, showed similar stage of development in which the terminal segment of mature worms had undeveloped uterus. On the other hand, those from dog No. 2, which was infected with protoscoleces obtained from various cysts, showed various stages of development in which worms consisted of adults with well-developed uterus, immature stages and adults with undeveloped uterus.

E. granulosus *coproantigen detection in an experimental infection*

In order to evaluate the feasibility of coproantigen detection for diagnosis, sandwich ELISA previously developed by Sakashita *et al.* (1995) was carried out using fecal samples from experimentally infected dogs. Rabbit anti-*E. granulosus* ES antibodies were coated on ELISA plate to capture the coproantigen.

At first, fecal samples obtained from dog No. 4 were treated differently with PBS, 1% formalin, or 1% formalin and heating. These three different treatments did not make a significant difference (Fig. 1), which means that there was no loss in antigenicity of the coproantigen following formalin or formalin and heat treatment.

The ELISA OD values of coproantigens in heat-treated samples were compared in all infected dogs and they seem to be correlated to the number of intestinal worms although the actual numbers of worms were not confirmed at different intervals postinfection until they were sacrificed at the end of the experiment. The coproantigens from dog No. 2 became positive on day 11 PI and those from dog No. 4 became positive on day 13 PI. In samples from dogs with relatively low worm burdens, coproantigens appeared 3 to 4 weeks after infection (Fig. 2a–2d).

Antibody detection in experimental infection

E. granulosus somatic antigens were used for detecting antibodies in the sera of infected dogs. In sera of dog No. 3 which was infected with the lowest number of worms, the antibody levels were relatively high even before the infection, and it strongly responded 2 weeks after infection. However, serum antibody levels in other dogs were not responsive during the infection, while worms were developing in the intestine (Fig. 3).

Diagnosis of the natural infection with E. granulosus *by the coproantigen detection*

Fifty nine fecal samples from dogs in San Gregorio

Table 1 Number of recovered worms from the small intestine of dogs experimentally infected with *E. granulosus*

	Number of inoculated protoscoleces	Number of recovered worms from different portions of the small intestine			
		anterior	middle	posterior	Total
Dog No. 1*	30,000	1,408	1,479	9	2,896
Dog No. 2	140,000	11,600	17,270	2,300	31,170
Dog No. 3	100,000	2	0	1	3
Dog No. 4	200,000	49,230	8,100	175	57,505

*Dogs were sacrificed on day 32–33 postinfection.

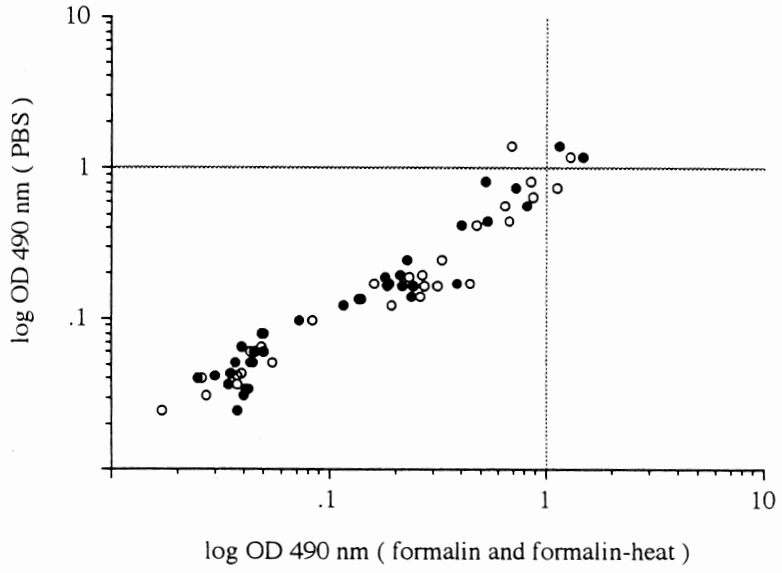

Fig. 1 Correlation analysis of ELISA OD values of PBS treated coproantigens versus OD values of formalin and formalin-heat treated coproantigens. OD values of PBS-treated coproantigens show significant correlation ($p<0.0001$) with both OD values of formalin treated (○) and formalin-heat treated (●) coproantigens.

were collected and examined for the purpose of confirming the diagnostic value of coproantigen detection in natural infection with *E. granulosus* (Fig. 4). A positive reaction could be detected in 3 of 8 samples from *E. granulosus* infected dogs. However, a positive reaction was also detected in 7 of 51 samples from non-*Echinococcus* infected dogs. Thus, the probability of pointing out true infection was 37.5% (3/8) and that of giving false-positive was 14% (7/51).

Discussion

In endemic areas, intestinal *Echinococcus* infection has been diagnosed by microscopic detection of eggs and/or proglottids in feces. However, microscopic examination is useless during the prepatent period and it is often inaccurate during patency. Arecoline purging also has some disadvantages such as, having no effect or eliciting undesirable side-effect in some dogs (FAO, 1982; Rickard and Arundel, 1985). In addition, the examiner is always in danger of infection in both techniques.

In this study, we described a sandwich ELISA using MoAb EmA9 directed against *E. multilocularis* for detecting *E. granulosus* coproantigens. In the experimental infection of dogs with *E. granulosus*, coproantigens could be detected during the prepatent period, and heat-treatment of fecal samples as well as formalin-treatment did not influence detection. Sakashita *et al.* (1995) also demonstrated the heat-stability of the epitope of *E. multilocularis* coproantigens detected by EmA9 and suggested that the recognized epitope may be a carbohydrate moiety of glycoconjugates of the worms. Thus, this method is not only useful for diagnosis but also prevents accidental infection by heating fecal samples to kill eggs. In addition, fecal samples can be preserved in 1% formalin since ELISA sensitivity is not influenced by directly using the fecal solution with formalin in the test. This may provide a good means of preserving fecal samples during field surveys. In the present study, ELISA OD values were shown to be correlated to the number of infecting worms. In case of infection with only a few worms like dog No. 3, coproantigens could not be

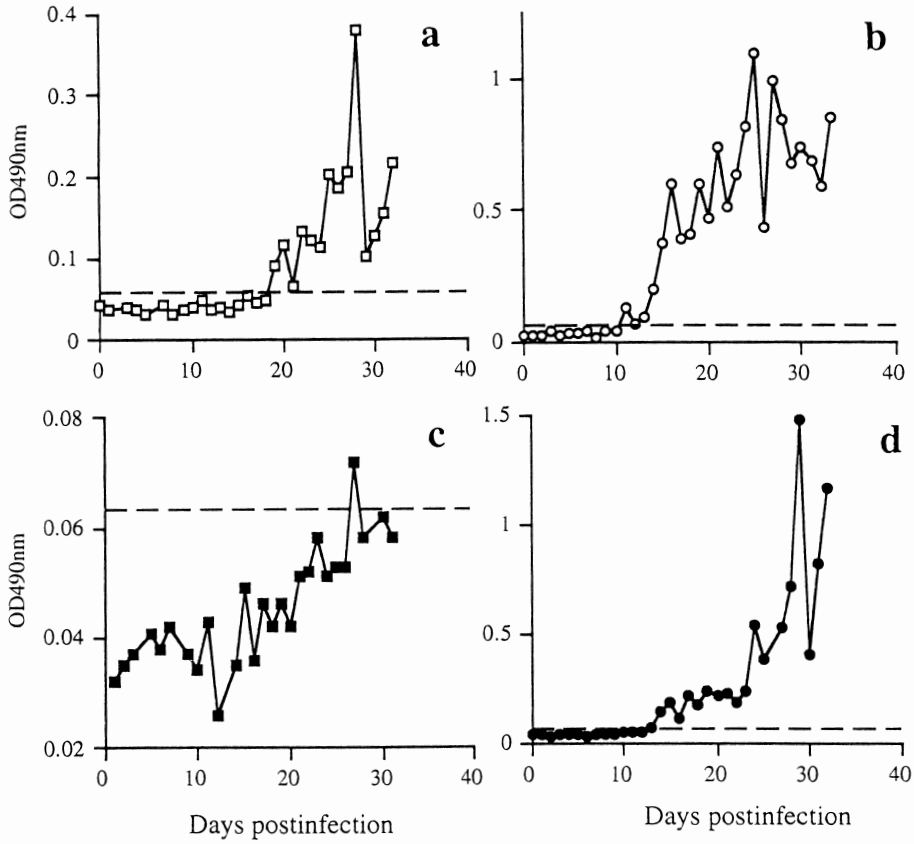

Fig. 2 Changes of coproantigen titers in fecal samples from four experimentally infected dogs after the infection. Coproantigen titers of dog No. 1(a), 2(b), 3(c) and 4(d) as described in Table 1. Various ranges of OD values were illustrated in this figure to compare the changing pattern of individual animals. Broken line is the cut-off line, which was determined by control data obtained from 7 uninfected healthy dogs (mean+3SD).

detected, although the OD value of ELISA slightly increased as days PI increased (Fig. 2c). Since the mean worm numbers of naturally infected dogs is usually low, i.e., 200–300 (Gemmell, 1990), the sensitivity of the diagnostic test has to be increased by purification or concentration of coproantigen in fecal samples.

The adult worms recovered at 32 days PI from dog No. 1 and dog No. 4 which were infected with protoscoleces from only one cyst showed similar degree of development, and changes of coproantigen levels in their feces from these dogs showed a similar pattern. In these dogs (i.e., No. 1, 4), there was a rapid rise of coproantigen levels at the 3rd week and subsequent decrease at the 4th week after infection (Fig. 2a, d). Antigen release may depend on parasite growth. If this is true, it has to be clarified whether a decrease of antigenic level reflect the death of developing worms in the host intestine. Coproantigen levels of all dogs in this experiment fluctuate on a day to day basis. The fluctuation of coproantigen levels was also observed previously in dogs experimentally infected with *E. multilocularis* (Kohno *et al.*, 1995; Sakashita *et al.*, 1995) and *Taenia pisiformis* (Allan *et al.*, 1992). If a decrease of antigen release occasionally occurs during infection, living worms may secrete very low levels of coproantigen for appropriate detection at certain

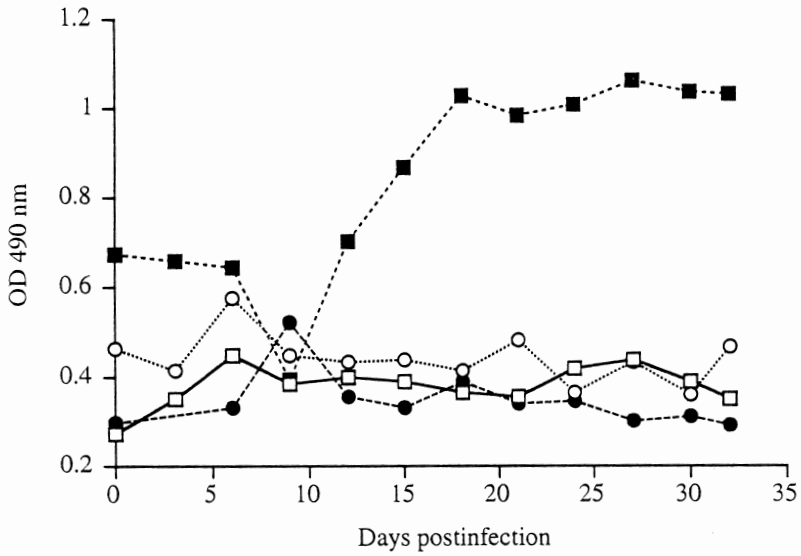

Fig. 3 Serum antibody responses of *E. granulosus* infected dogs against *E. granulosus* somatic antigens. Dog No. 1 (□), Dog No. 2 (○), Dog No. 3 (■), Dog No. 4 (●).

developmental stage of the worms, and coproantigen levels could be severely low in infections with a few adult worms.

Antibody detection in the sera of infected dogs was of limited use for the diagnosis of intestinal infection. In spite of the large numbers of infecting worms in dog No. 2 and dog No. 4, no serum antibody response was observed. Gasser *et al.* (1993) and Craig *et al.* (1995) have also examined antibodies to *E. granulosus* protoscolex in sera from many infected dogs in Uruguay and found that only low titers of IgG antibodies were detected in some of them. They suggested that the low responsiveness of the dogs may be due to "immunological unresponsiveness" caused by excess antigen presentation in the host or antigenic differences of genetic variants of the parasite in Uruguay. However, serum antibody response of dog No. 3 which was infected with a few worms was apparently high. Dog No. 3 might be previously infected with *E. granulosus* because of a relatively high titer of antibody detected before the experimental infection (Fig. 3). If this is so, memory cells might have been stimulated by the experimental infection to raise the antibody levels. In addition, the fact that a few number of worms were recovered from dog No. 3 may be due to the influence of humoral immunoreaction against the worms.

The results of coproantigen detection of naturally infected dogs in the present study were unsatisfactory, due to the limited number of dogs examined and the inaccessibility of the animals to determine parasite infection. The number of infected worms excreted by arecoline administration in positive dogs was not counted. Coproantigen-negative results in the parasite-positive dogs might be due to a small number of infecting worms as described by Craig *et al.* (1995). Furthermore, MoAb EmA9 used in the test was produced against *E. multilocularis* somatic antigens and may not be sensitive enough to detect *E. granulosus* ES antigens in feces of dogs infected with a few number of *E. granulosus*. On the other hand, coproantigen-positive results in the parasite-negative dogs can be explained by the fact that about 50% of infected dogs could not expel the worms after arecoline treatment (Rickard and Arundel, 1985). In addition, Wachira *et al.* (1990) demonstrated that arecoline survey can detect only as much as one tenth of infected dogs.

In the future, fecal samples should obtained from

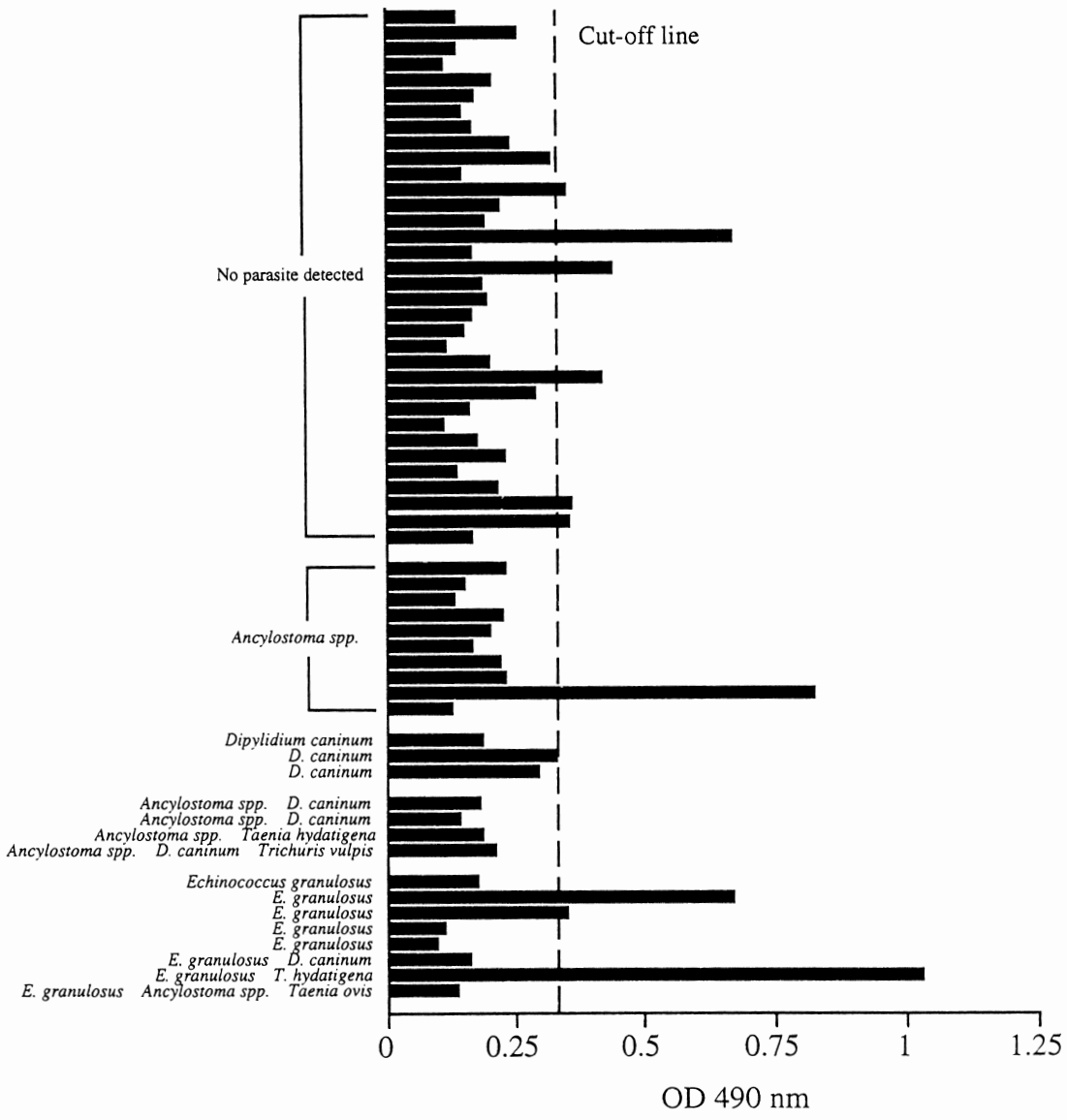

Fig. 4 Coproantigen detection in 59 samples obtained from dogs in San Gregorio. Parasites found after arecoline treatment are enumerated on the left side. The cut-off line was determined by control data from 10 uninfected dogs (mean+3SD).

necropsied dogs to determine the correlation between the presence of the parasite and coproantigen. The sensitivity of fecal antigen detection from infected dogs can be improved by renewal of MoAbs or partial purification of the carbohydrate-antigenic epitope. For the improvement of this test, other approaches are also necessary for the establishment of a practical diagnostic method for dogs infected with *E. granulosus*.

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