Preliminary study of the presence of antibodies against excretory-secretory antigens from protoscoleces of *Echinococcus granulosus* in dogs with intestinal echinococcosis

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The aim of the present study was to analyze the antibody response against excretory-secretory antigens (ES-Ag) from Echinococcus granulosus *protoscoleces, using sera from dogs infected with* E. granulosus *and other helminths. ES-Ag were obtained from the first 50 h maintenance of protoscoleces in vitro. Immunochemical characterization was performed by immunoblotting with sera from dogs naturally infected with* E. granulosus *(n = 12), sera from dogs infected with helminths other than* E. granulosus *(n = 30), and helminth-free dog sera (n = 20). These findings were compared to those obtained from a somatic extract of protoscoleces (S-Ag). ES-Ag only showed four cross-reacting proteins of 65, 61, 54, and 45-46 kDa. Antigens with apparent masses of 89 and 50 kDa in ES-Ag and of 130 and 67 kDa in S-Ag were identified by sera of dogs infected with* E. granulosus *only, whereas a protein of 41-43 kDa was recognised by the majority of the sera from dogs with non-echinococcal infection. Employing ELISA to study the same sera, S-Ag revealed higher immunoreactivity than ES-Ag, but also showed higher cross-reactivity levels when sera from dogs with non-echinococcal infection were assayed in immunoblotting.*

Key words: *Echinococcus granulosus* - excretory-secretory antigens - protoscoleces - intestinal echinococcosis - parasite antigens

Cystic hydatid disease in man is a zoonosis caused by infection with the larval stage of the dog tapeworm *Echinococcus granulosus*. The adult worm lives in the small intestine of dogs and other canids, in intimate contact with the intestinal epithelium. Parasite eggs (the infective stage for the intermediate host) are excreted in faeces, and may thus contaminate soil, grass and water. Detection of *E. granulosus* adults in dogs is very important in order to evaluate its prevalence and to develop surveillance and control programmes for hydatidosis/echinococcosis (WHO 2001). Diagnosis of taeniid cestode infections in dogs can be carried out using arecoline hydrobromide, but this technique has a highly variable sensitivity, is time consuming and some dogs suffer undesired side-effects (Wachira et al. 1990). Coprological exams, however, have low sensitivity since excretion of the eggs occurs sporadically. Furthermore, the eggs of taeniid cestodes are morphologically indistinguishable by light microscopy.

In an attempt to improve the sensitivity of *E. granulosus* diagnostics, different serological tests have been developed, mainly based on the detection of specific IgG antibodies against the adult parasite. Somatic extracts of protoscoleces (S-Ag) are the most commonly used antigenic source. However, ELISA results showed highly variable sensitivities, ranging from 40 to 90% (Jenkins et al. 1990, Gasser et al. 1992, 1993, 1994). These studies also demonstrated that 25-60% of the sera from dogs infected with *E. granulosus* did not show significant levels of specific antibody (Jenkins et al. 1990, Gasser et al. 1994), and that cross-reactivity with other parasite species may occur (Gasser et al. 1988).

During recent years, diagnosis of intestinal echinococcosis has been mainly based on the detection of excretory-secretory products of *E. granulosus* protoscoleces in dog faeces using ELISA (Allan et al. 1992, Deplazes et al. 1992, Jenkins et al. 2000). This technique considerably improves both diagnostic sensitivity and specificity, permits the detection of the parasite during the prepatent period and shows the current status of the infection (Fraser & Craig 1997).

Although characterization of somatic antigens of *E. granulosus* protoscoleces for diagnostic purposes has been described in several reports (Gasser et al. 1989, 1991, 1992, Rafiei & Craig 2002), very little information is available about excretory-secretory products of protoscoleces (Gasser et al. 1992, Carmena et al. 2004). In this preliminary work we show the presence of antibodies that recognize excretory-secretory antigens of *E. granulosus* protoscoleces in sera from dogs infected with *E. granulosus* and other helminths. Some of the described components could be promising candidate antigens for immunodiagnosis of dog echinococcosis. These results were compared with those obtained for somatic extracts, the antigenic source that has mainly been used in the serodiagnosis of intestinal echinococcosis.

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MATERIALS AND METHODS

Somatic antigens (S-Ag) - *E. granulosus* parasite material was isolated from sheep hepatic hydatid cysts collected from an abattoir in La Rioja, Spain. Somatic antigens were obtained from protoscoleces removed by aseptic cyst puncture as described by Smyth and Davies (1974), washed with phosphate-buffered saline (PBS) and stored at –20ºC with proteolytic enzyme inhibitors (2 mM PMSF and 5 mM EDTA). Protoscoleces were thawed and sonicated (10 cycles of 12 s at 60 Hz frequency), freeze-thawed once more and centrifuged for 35 min at 2300 × g. Supernatants were aliquotted and stored at –20ºC.

Excretory-secretory antigens (ES-Ag) - To obtain excretory-secretory products, protoscoleces with viability higher than 90% were selected. Viability was assessed by morphological appearance, flame cell motility and general contractile movements (Smyth & Davies 1974, Howell 1986). Protoscoleces were cultured in PBS complemented with 10% glucose, 100 U ml-1 penicillin and 100 µg ml-1 streptomycin at 37ºC in 5% CO_2, which promoted parasite survival for several days (Carmena et al. 2002). Every 8 h the medium was removed and replaced with fresh medium. Protein recovery from the media was achieved by using Ultrafree 15 filters with a 5 kDa pore diameter membrane (Millipore, Bedford, US). EDTA (5 mM) and PMSF (2 mM) were added, and the ES products were aliquotted and stored at –20ºC. Protein concentration was determined by the bicinchoninic acid method (Sigma-Aldrich, Dorset, UK). In total, 8 cultures of hepatic protoscoleces from sheep were carried out. Medium corresponding to the first 50 h of culture was subsequently used. The concentration for proteins obtained for each preparation was: ES-Ag: 0.2 mg ml^{-1} and S-Ag: 2.95 mg ml^{-1}.

Enzyme linked immunosorbent assay (ELISA) - ELISA was carried out as described by Benito et al. (2001). The following optimal antigen concentrations were used: ES-Ag: 20 µg ml^{-1} and S-Ag: 7.5 µg ml^{-1}. Microtitre plates (Maxisorp™, Nunc, Roskilde, Denmark) were coated with antigenic extract diluted in PBS buffer (100 µl/well) and incubated overnight at 4ºC. Blocking was carried out with PBS-1% bovine serum albumin (BSA, 200 µl/well) for 1 h at 37ºC. Dog sera were tested in PBS-0.5% BSA buffer (100 µl/well) using serial dilutions ranging from 1:50 to 1:200 in duplicate. The plates were incubated for 1 h at 37ºC. Peroxidase-conjugated rabbit anti-dog IgG (Sigma) was used at 1:1000 dilution in PBS-4% SAB-0.05% Tween 20 buffer for 1 h at 37ºC. Binding was visualised with 5-aminosalicilic acid. The reaction was stopped by adding 25 µl/well NaOH 1N, and the absorbance value was measured at 450 nm.

SDS-PAGE and immunoblotting - Proteins were separated by 12.5% SDS-PAGE under reducing conditions according to Laemmli (1970). For immunoblotting, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore), according to Towbin et al. (1979). Primary sera were used at 1:50 in 20 mM Tris-buffered saline (pH 7.4), 8% skimmed milk (TBS-M), incubating overnight at 4ºC. Peroxidase-conjugated rabbit anti-dog IgG (Sigma) was used at 1:1000 in TBS-M buffer for 4 h at room temperature, and binding was visualised with 4-chloro-1-napthol.

Dog sera - Include: a) dog sera obtained from the Council Animal Rescue Mission of Vitoria, Spain. All animals were diagnosed by autopsy, but their previous infection history was unknown. Five sera from dogs naturally infected with *E. granulosus* were obtained, with worm burdens ranging from 2 to 155 helminths. Thirty sera were obtained from dogs that harboured infections with *Taenia* spp. (including *T. hydatigena* and *T. pisiformis*), *Mesocestoides* spp., *Dipylidium caninum*, *Toxocara canis*, *Toxascaris leonina*, *Trichuris vulpis*, *Uncinaria stenocephala*, and *Ancylostoma caninum*. Sera from 20 helminth-free dogs that were under 2 years of age at the time of autopsy were also used; b) seven serum samples from farm dogs naturally infected with *E. granulosus* in the Chubut Province (Argentina). These samples were diagnosed by identifying worms following arecoline purgation, and were kindly provided by Dr Eduardo Fernández, Laboratorio de Control de Patologías Prevalentes, Hospital Zonal de Trelew, Chubut, Argentina; c) 20 sera from 5 dogs experimentally infected with *E. granulosus*, kindly provided by Dr P Spinelli, Instituto de Higiene, Montevideo (Uruguay). Animals had been orally infected with 60,000 protoscoleces (Spinelli et al. 1996) and four serum samples were obtained at day 0 and days 5, 15, and 29 post-infection. A pool of sera from dogs with *E. granulosus* natural infections was also used as a positive control in ELISA assays, with an assigned value of 8000 arbitrary units ml^{-1} (Benito et al. 2001).

Statistical methods - ELISA results were analyzed by means of variance analysis (ANOVA), using SPSS 11.5 statistical software.

RESULTS

Immunoblotting - Fig. 1 shows an analysis of recognition of individual components of ES-Ag and S-Ag with the 12 sera from dogs naturally infected with *E. granulosus*. These sera identify seven major components in the ES-Ag, standing out those of 89, 46, and 50 kDa. With regard to S-Ag, the same sera recognize 15 antigenic proteins, the most prominent being those of 67, 55, 48, 43, 39, and 24 kDa. Figs 2 and 3 show the antigenic profiles of the ES-Ag and S-Ag identified with sera from dogs infected with other helminths, as well as with sera from helminth-free dogs. Proteins of 41-43 kDa and 85 kDa, both shared by ES-Ag and S-Ag, were recognized by many of these sera. Table I summarizes the data obtained by immunoblotting.

ELISA - ELISA results for sera from dogs naturally infected with *E. granulosus* are shown in Fig. 4. Using ES-Ag as solid phase, sera from dogs with higher worm burdens revealed lower specific antibody levels than sera from dogs infected with low numbers of *E. granulosus*. Similar results were obtained with S-Ag: the highest antibody titre was reached by the serum from dog number 4, where only two worms were found at autopsy. However, no significant differences were found between the means of the absorbance values obtained for both antigenic extracts ($P < 0.05$).

Fig. 5 summarizes the ELISA results for the 20 sera from dogs experimentally infected with *E. granulosus*. The highest specific antibody levels were observed for the bleeds obtained at days 15 and 29 post-infection from dogs 1 and 2. S-Ag was the most immunoreactive extract and also showed more homogeneous absorbance values among the studied sera than the ES-Ag. Regarding the sera from dogs naturally infected with *E. granulosus*, no significant differences were found between the means of the absorbance values obtained for both antigenic extracts ($P < 0.05$).

DISCUSSION

Excretory-secretory products from scoleces and adults of *E. granulosus* have been previously assayed as an antigenic source for immunodiagnosis of canine echino-

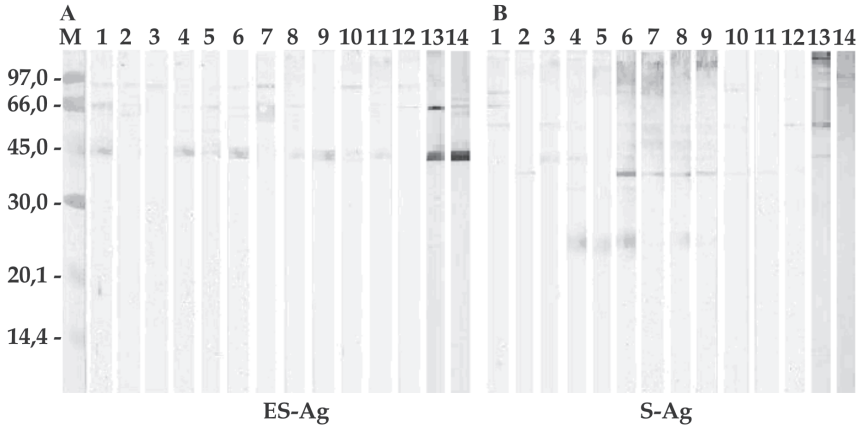

Fig. 1: immunoblotting profiles using the sera from dogs naturally infected with *Echinococcus granulosus* (lanes 1-12). Lane 13: serum from dog infected with *Taenia hydatigena* (control). Lane 14: serum from helminth-free dog (negative control). M: molecular mass marker (kDa). A: excretory-secretory antigens (ES-Ag); B: somatic antigens (S-Ag)

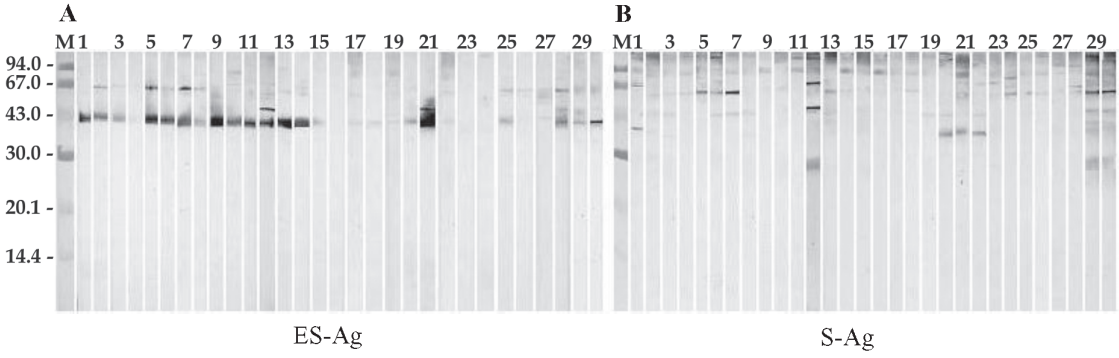

Fig. 2: immunoblotting profiles for excretory-secretory antigens (ES-Ag) and somatic antigen (S-Ag) using sera from dogs infected with helminths other than *Echinococcus granulosus*. *Taenia* spp.: lanes 1 to 8; *Mesocestoides* spp.: lane 9; *D. caninum:* lanes 10 to 14; *T. canis:* lanes 15 to 19; *T. leonina:* lanes 20 to 22; *T. vulpis:* lane 23; *U. stenocephala:* lanes 24 to 27; *A. caninum*: lanes 28 to 30. M: molecular mass marker (kDa). A: ES-Ag; B: S-Ag

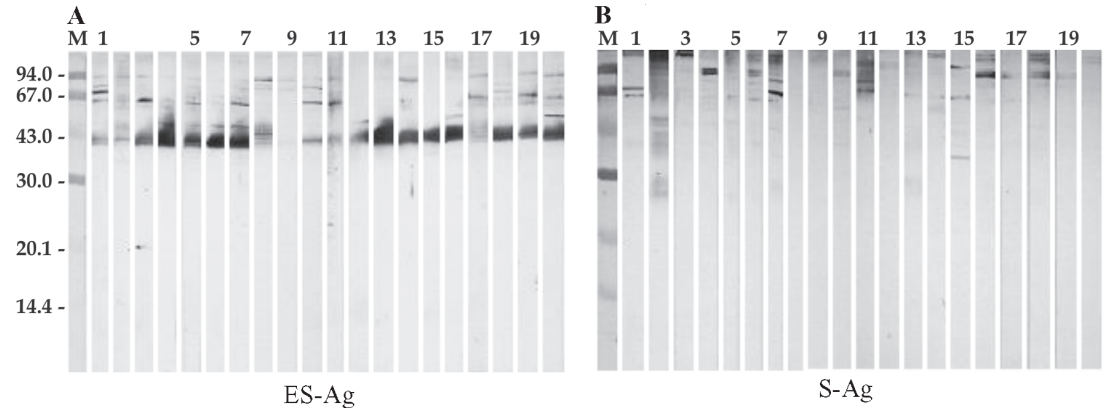

Fig. 3: immunoblotting profiles for excretory-secretory antigens (ES-Ag) and somatic antigens (S-Ag) using sera from helminth-free dogs. M: Molecular mass marker (kDa). A: ES-Ag; B: S-Ag

coccosis by ELISA and/or immunoblotting (Jenkins & Rickard 1986, Gasser et al. 1992). However, information about the characterization of ES-Ag from this parasite is very rare, which can be partially due to difficulties in obtaining enough amounts of ES-Ag. Recently, our laboratory has identified over 20 major protein components in the ES-Ag which could be distinguished by 1-dimensional SDS-PAGE and revealed apparent masses between 9 and 300 kDa (Carmena et al. 2004). In this study, we present preliminary data about the immunochemical characterization of ES-Ag from protoscoleces of *E. granulosus*, which may help to identify new candidate antigens with potential for immunodiagnosis of dog echinococcosis.

Secreted products of *E. granulosus* adults have been characterized by immunoblotting using sera from dogs naturally infected with this cestode (Gasser et al. 1992). These authors identified three antigenic components larger than 94 kDa, two triplets of 68/94 and 39/43 kDa, and seven proteins smaller than 30 kDa. In our study, we have identified seven secreted components from *E. granulosus* protoscoleces ranging from 46 to 133 kDa. When S-Ag from protoscoleces was used, Gasser et al. (1992) found antigenic proteins of 94, 76, 35, 27, and others smaller than 14 kDa. In our work, we have recognised 15 antigenic components ranging from 24 and 130 kDa in the same extract. Only three of them had similar molecular masses (76, 34, and 24 kDa) compared to the components described by Gasser et al. (1992).

We have evaluated by immunoblotting the possible cross-reactivity of S-Ag with sera from dogs infected with

TABLE I

Immunoblotting results. Components of excretory-secretory antigens and somatic antigens recognized by different dog sera. The percentage of sera which bound to each antigenic component is expressed in brackets. Molecular masses are expressed in kDa

Excretory-secretory antigens			Somatic antigens		
With *E. granulosus*	Other parasitoses	Helminth-free	With *E. granulosus*	Other parasitoses	Helminth-free
-	140 (20%)	-	-	137 (27%)	137 (20%)
133 (58%)	-	-	130 (33%)	-	-
-	-	-	115/122 (D, 83%)	112/120 (D, 70%)	113 (55%)
-	-	-	103 (58%)	104 (20%)	-
-	-	-	-	94 (53%)	98/104 (D) (50%)
89 (92%)	-	-	-	-	-
-	85 (20%)	85 (70%)	85 (42%)	-	85 (30%)
-	-	-	-	82 (33%)	81 (30%)
-	76 (17%)	77 (65%)	76 (42%)	-	78 (25%)
-	-	71 (20%)	-	70 (30%)	-
-	-	68 (60%)	67 (25%)	-	-
65 (67%)	65 (10%)	-	65 (17%)	65 (17%)	-
61 (25%)	61 (43%)	61 (25%)	-	61 (57%)	60 (30%)
54 (17%)	54 (37%)	-	55 (67%)	52 (10%)	-
46/50 (D) (68%)	45 (17%)	46 (40%)	48 (33%)	46 (40%)	-
-	41-43 (80%)	41 (100%)	41/43 (D) (50%)	41 (20%)	-
-	-	-	39 (67%)	38 (10%)	39 (10%)
-	-	-	34 (25%)	36 (17%)	-
-	-	-	24 (42%)	26 (10%)	-

D: doublet; *E*: *Echinococcus*

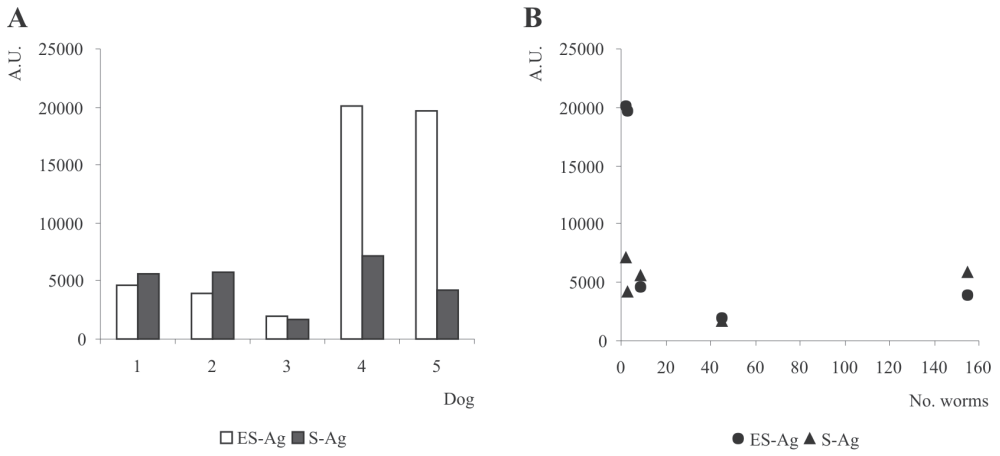

Fig. 4-A: ELISA results for different antigenic extracts using sera from dogs naturally infected with *Echinococcus granulosus;* B: ELISA results for different antigenic extracts considering the worm burden found at autopsy. ES-Ag: excretory-secretory antigens; S-Ag: somatic antigens; A.U.: arbitrary units

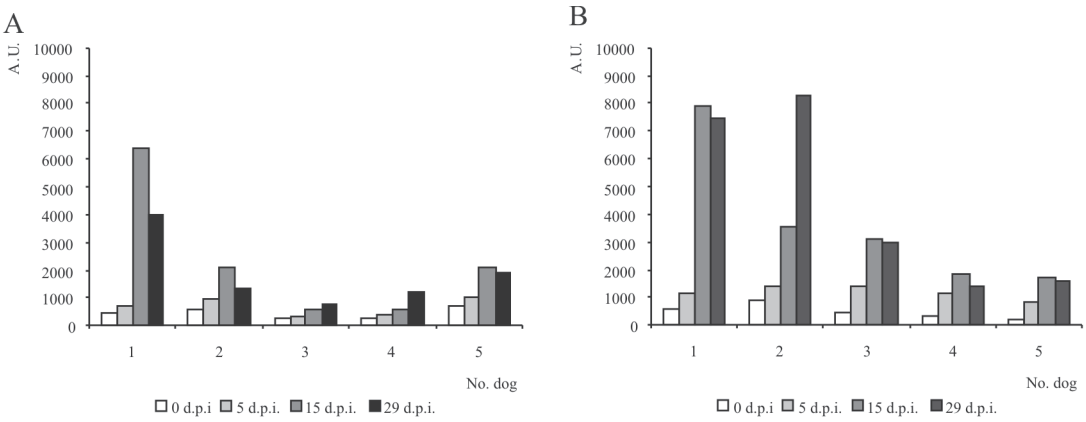

Fig. 5: ELISA results for different antigenic extracts using sera from five dogs experimentally infected with *Echinococcus granulosus*. Sera were obtained at different time points of infection on days 0, 5, 15, and 29. A: excretory-secretory antigens (ES-Ag); B: somatic antigens (S-Ag); A.U.: arbitrary units; D.p.i.: days post-infection

helminths other than *E. granulosus* as well as with sera from helminth-free dogs. Using S-Ag, 40% of sera from dogs with non-echinococcal infections reacted specifically with a 46 kDa protein, and 20% with a 41 kDa polypeptide, which were also recognized by sera from dogs naturally infected with *E. granulosus*. Additional cross-reacting proteins of 113, and 85 kDa were identified by 55%, and 30% of helminth-free dog sera, respectively, and to a lower degree, proteins of 78, and 39 kDa. S-Ag with molecular masses of 130 and 67 kDa may have diagnostic potential, since they were only identified by sera from dogs infected with *E. granulosus*, but not by sera from dogs with non-echinococcal infections.

On the other hand, only 4 components from ES-Ag (polypeptides of 65, 61, 54, and 45-46 kDa) showed significant cross-reactivity levels. These proteins were recognized by 10-43% of sera from dogs infected with helminths other than *E. granulosus* or by sera from helminth-free dogs. In this antigenic extract, 2 proteins of 89 and 50 kDa, respectively, were recognized by sera from dogs infected with *E. granulosus* only. Interestingly, the component of 89 kDa was identified by 92% of sera that were tested. This fact suggests that this protein might be a promising diagnostic antigen that could be used in immunological tests for the detection of *E. granulosus* in dogs. These data demonstrate that, although S-Ag showed the highest specific antibody levels in ELISA, ES-Ag is more specific than S-Ag for immunodiagnosis of dog echinococcosis.

It was difficult to highlight specific recognition patterns attributable to individual infections, except in the case of the sera from dogs infected with *Toxascaris leonina*. All of them showed a specific protein of 36 kDa when using S-Ag (Fig. 2B, lanes 20 to 22).

The lack of specificity of the 41-43 kDa component from the excreted products of *E. granulosus* adults has been previously described by Gasser et al. (1992). In our work, we have corroborated this result, identifying this antigenic protein both in ES-Ag and S-Ag. Recently, we have also found the 41-43 kDa component in the ES-Ag

by immunoblotting with non-hydatidic human sera, including sera from individuals with other parasitoses, from individuals with non-parasitological pathologies and from healthy donors (Carmena et al. 2004).

ELISA results for sera from dogs infected with *E. granulosus* showed that highest worm burdens are not always corresponding with highest specific antibody levels, especially when ES-Ag is used (Gasser et al. 1992, 1993). This finding seems to demonstrate that there is no relationship between the number of worms in the intestine and the circulating antibody level, confirming previous results of Jenkins and Rickard (1985), Gasser et al. (1988, 1992, 1994) and Jenkins et al. (1990, 1991). This fact may either be due to the sequestration of antibodies and the formation of circulating immunocomplexes (Gasser et al. 1988, 1993, Spinelli et al. 1996), or to immune evasion mechanisms of the parasite (Gasser et al. 1992, 1994), or to a low immune response of the host (Gasser et al. 1988, 1993, 1994). Host nutritional status may also have an impact on the antibody levels (Jenkins et al. 1991, Gasser et al. 1992). On the other hand, the broad differences of antibody levels found among the studied dog sera also may reflect the influence by physiological and environmental factors, like re-infection or co-infection with other parasite species.

In ELISA, sera from dogs experimentally infected with *E. granulosus* showed less variability regarding the levels of specific antibodies than sera from dogs with natural infections (Figs 4 and 5). This may be due to the standardized environmental conditions under which these animals were maintained. The highest specific antibody levels were reached using S-Ag, especially when sera from dogs experimentally infected were assayed. These results are in agreement with those obtained by Benito et al. (2001), who found higher discrimination levels between positive and negative controls using S-Ag than with ES-Ag or hydatid cyst fluid as antigenic extracts.

We conclude that ES-Ag from protoscoleces of *E. granulosus* contain potential diagnostic antigens to be used in the immunodiagnosis of canine echinococcosis. In particular, the protein of 89 kDa has evidenced the most promising features. However, a more comprehensive analysis will be necessary to determine more accurately the significance of these observations, since only a comparatively low number of sera was available for the present study. Further studies to clone and express individual protein components from secreted products of protoscoleces which display both high immunoreactivity and specificity may be of great interest for the diagnosis of dog echinococcosis.

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