

Shared and non-shared antigens from three different extracts of the metacestode of *Echinococcus granulosus*

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Hydatid cyst fluid (HCF), somatic antigens (S-Ag) and excretory-secretory products (ES-Ag) of Echinococcus granulosus protoscoleces are used as the main antigenic sources for immunodiagnosis of human and dog echinococcosis. In order to determine their non-shared as well as their shared antigenic components, these extracts were studied by ELISA-inhibition and immunoblot-inhibition. Assays were carried out using homologous rabbit polyclonal antisera, human sera from individuals with surgically confirmed hydatidosis, and sera from dogs naturally infected with E. granulosus. High levels of cross-reactivity were observed for all antigenic extracts, but especially for ES-Ag and S-Ag. Canine antibodies evidenced lesser avidity for their specific antigens than antibodies from human origin. The major antigenic components shared by HCF, S-Ag, and ES-Ag have apparent molecular masses of 4-6, 20-24, 52, 80, and 100-104 kDa, including doublets of 41/45, 54/57, and 65/68 kDa. Non-shared polypeptides of each antigenic extract of E. granulosus were identified, having apparent masses of 108 and 78 kDa for HCF, of 124, 94, 83, and 75 kDa for S-Ag, and of 89, 66, 42, 39, 37, and 35 kDa for ES-Ag.

Key words: *Echinococcus granulosus* - hydatid cyst fluid - somatic antigens - excretory-secretory antigens - cross-reactivity

Echinococcosis caused by cestodes of the genus *Echinococcus* is one of the major zoonotic helminthiasis, causing considerable socio-economic consequences in endemic areas. Due to its world-wide distribution and its important impact in both human and animal health, *E. granulosus* is considered the most relevant species (Romig 2003). The adult worm lives in the small intestine of dogs and other canids, in intimate contact with the intestinal epithelium. The intermediate larval stage (metacestode) can grow in a wide range of mammal species including humans, that acquire infection through accidental ingestion of eggs. Currently, diagnosis of hydatidosis/echinococcosis is based on a combination of imaging techniques (ultrasonography, computerized axial tomography, X-rays) and immunodiagnostic methods such as ELISA and immunoblotting (Zhang et al. 2003).

For immunodiagnosis of human hydatidosis and dog echinococcosis, hydatid cyst fluid (HCF), somatic antigens (S-Ag) and excretory-secretory products (ES-Ag) of *E. granulosus* protoscoleces and adult worms are used as main antigenic sources. The choice of the most appropriate antigenic extract depends on the developmental stage of the worm and the host species. Thus, HCF is mainly used for the immunodiagnosis of human cystic echinococcosis, based on the detection of antigen 5 and

antigen B. The subunit of 8/12 kDa from antigen B is considered the most specific component of HCF in the genus *Echinococcus* (Verastegui et al. 1992, Zhang et al. 2003). S-Ag from protoscoleces have been used for the serodiagnosis of dog echinococcosis, as protoscoleces are the infective stage of the parasite in the definitive host. However, because of their variable diagnostic sensitivity and high cross-reactivity levels with antigens from other parasite species, S-Ag are unreliable for serodiagnostic purposes (Gasser et al. 1988, 1994, Jenkins et al. 1990, Lightowers & Gottstein 1995). In the last few years, ES-Ag from protoscoleces has become the main antigenic source for the immunodiagnosis of dog echinococcosis, based on the detection of parasite antigens in fecal samples (coproantigens) by ELISA (Jenkins et al. 2000, Benito & Carmena 2005).

Currently, there is very little information available about the recognition of antigenic components from different extracts of *E. granulosus* by sera from infected individuals of diverse species (Auer et al. 1988, Gasser et al. 1992). In this paper we present a comparative analysis of the cross-reactive antigenic components of HCF, S-Ag, and ES-Ag from protoscoleces of *E. granulosus* by ELISA-inhibition and immunoblot-inhibition assays, in order to determine the non-shared and shared antigenic components of these extracts. These data may provide valuable information for the identification and isolation of specific antigenic components from the metacestode of *E. granulosus* for immunodiagnostic purposes.

MATERIALS AND METHODS

HCF - HCF was obtained from liver and lung fertile cysts of ovine origin as described by Varela-Díaz et al. (1974). Briefly, HCF was centrifuged at 2000 × g for 45 min, then passed through a Millipore AP20 filter (Bedford, US) and dialyzed against distilled water, using dialysis tubing with a cut-off of 5 kDa (Medicell, London, UK). Finally,

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HCF was centrifuged at $6500 \times g$ for 30 min, lyophilized and stored at 4°C . To perform this study a pool of hydatid fluid from several large cysts from a single sheep lung was used. The concentration of proteins was 736 mg g^{-1} dry weight.

S-Ag - *S-Ag* were also prepared from protoscoleces obtained by aseptic puncture from fertile hydatid cysts of ovine origin, washed with phosphate-buffered saline (PBS) and stored at -25°C 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 $2300 \times g$. Supernatants were aliquotted and stored at -25°C . To perform this study a batch of protoscoleces from a single sheep liver infected with multiple hydatid cysts was selected. Protoscoleces had a viability of 97.4% at the time of their extraction from the cyst. The concentration of proteins was 2.95 mg ml^{-1} .

ES-Ag - To obtain *ES-Ag*, a total of 10 cultures of protoscoleces from sheep liver were carried out. Initial viability was assessed by morphological appearance, flame cell motility and general contractile movements (Howell 1986). Protoscoleces were cultured in PBS complemented with 10% glucose, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin at 37°C in 5% CO_2 (Carmena et al. 2002). The medium was renewed every 8 h and, after 50 h of culture, concentrated using filters with a 5 kDa pore diameter membrane (Ultrafree 15, Millipore). EDTA (5 mM) and PMSF (2 mM) were added, and the *ES* products were aliquotted and stored at -25°C . To perform this study, a batch of protoscoleces with an initial viability of 95.2% was obtained from a single liver infected with multiple cysts. The concentration of protein was 0.2 mg ml^{-1} .

Human sera - Thirty two pre- and post-surgery sera from 11 individuals with confirmed liver hydatidosis were assayed by ELISA (HCF as solid phase) for levels of *E. granulosus* specific antibodies. Sera from the five patients with the highest absorbance values in ELISA assays were pooled and used as positive control in the inhibition assays.

Dog sera - Dog sera were collected from the Council Animal Rescue Mission, Vitoria, Spain. Five sera from dogs naturally infected with *E. granulosus* were obtained. All animals were diagnosed by autopsy. Sera from the four infected dogs with the highest absorbance values in ELISA assays using *S-Ag* as solid phase (Benito et al. 2001) were pooled and used as positive control in the inhibition assays.

Hyperimmune rabbit sera - Polyclonal immunosera anti-HCF, anti-*S-Ag*, and anti-*ES-Ag* were obtained according to Gallart et al. (1985). Titration of rabbit antiserum was performed by ELISA, using their homologous antigenic extracts as solid phase.

ELISA-inhibition assays - ELISA was carried out as described by Martínez et al. (1985). Briefly, polystyrene 96-well microtitre plates (Maxisorp™, Nunc, Roskilde, Denmark) were coated with $100 \mu\text{l/well}$ of the optimal antigen concentrations determined according to Muñoz et

al. (1986) (HCF: $10.5 \mu\text{g ml}^{-1}$; *S-Ag*: $7.5 \mu\text{g ml}^{-1}$; *ES-Ag*: $20 \mu\text{g ml}^{-1}$) diluted in PBS buffer and incubated for 3 h at room temperature (human ELISA) or 15 h at 4°C (canine ELISA). After blocking with 1% BSA-0.05% Tween 20 in PBS, wells were filled with $50 \mu\text{l/well}$ of each inhibitory antigenic extract in PBS (serial dilutions ranging from: HCF: 3 to 0 mg ml^{-1} ; *S-Ag*: 1.6 to 0 mg ml^{-1} ; *ES-Ag*: 0.2 to 0 mg ml^{-1}). Fifty $\mu\text{l/well}$ of the tested pooled sera were added at dilutions that had previously been optimized and incubated for 3 h at 37°C . Dilutions of the first antibodies were 1:800 for pooled sera from hydatid patients and 1:50 for pooled sera from dogs with *E. granulosus* infections. Wells were then incubated with peroxidase-conjugated rabbit anti-human IgG (Dako, 1:2500 dilution) or anti-dog IgG (Sigma, 1:1000 dilution) in 0.05% Tween 20-PBS for 15 h at 4°C . After washing, plates were developed with MBTH-DMAB solution at room temperature for 20 min. The reaction was stopped with $50 \mu\text{l/well}$ of 2N H_2SO_4 and the OD was measured at 620 nm. Assays were performed in triplicates. OD values from tested sera versus inhibitor concentrations were graphically depicted and percentages of inhibition were calculated with the following formula:

$$\% \text{ inhibition}_{[x]} = 100 - (\text{OD}_{[x]} / \text{OD}_{\text{max}} \times 100)$$

where $\text{OD}_{[x]}$ is the absorbance value corresponding to the assayed inhibitor concentration, and OD_{max} is the absorbance value corresponding to an inhibitor concentration equal to zero. In addition, values of Ag_{50} (antigen concentration required to inhibit 50% of the specific sera antibodies assayed), coefficient of linear correlation, slope of the regression line, and theoretical maximum percentage of inhibition were also calculated for each inhibition reaction (Martínez et al. 1985).

Immunoblotting and immunoblot-inhibition assays - Proteins from HCF, *S-Ag*, and *ES-Ag* were fractionated by 12.5% SDS-PAGE under reducing conditions according to Laemmli (1970) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore), according to Towbin et al. (1979). After washing and blocking, membranes were incubated with the sera in 20 mM Tris-buffered saline (pH 7.4), 8% skimmed milk (TBS-M) for 15 h at 4°C . Optimal sera dilutions used were: rabbit anti-HCF: 1:1000; rabbit anti-*S-Ag*: 1:1000; rabbit anti-*ES-Ag*: 1:500; pooled sera from hydatid patients: 1:800; pooled sera from dogs with *E. granulosus* infections: 1:100. Peroxidase-conjugated swine anti-rabbit total Ig (Dako, Copenhagen, Denmark), rabbit anti-human IgG (Dako) and rabbit anti-dog IgG (Sigma) were used at 1:1000 dilution in TBS-M buffer for 4 h at room temperature. Membranes were revealed by adding 4-chloro-1-naphthol solution. The immunoblotting inhibition assays were performed according to Asturias et al. (1999). Sera used in these assays were previously preabsorbed with each inhibitory antigenic extract (HCF and *S-Ag*: 1 mg ml^{-1} ; *ES-Ag*: 0.2 mg ml^{-1}) for 30 min at 37°C with gentle shaking, and then added to the membranes as described before. Non-inhibited sera were used as positive control. Sera that were inhibited with the same extract utilized as solid phase were used as negative control. Resulting antigenic band pat-

terns were analyzed and apparent molecular masses were estimated by measuring the relative mobility of each band of interest, and interpolating the data in the standard curve. Proteins which were identified by at least four of the five inhibited antisera assayed (considering each immunoserum used in the inhibition reaction independently) were regarded as overall shared components.

RESULTS

ELISA-inhibition assays - Table I summarizes the ELISA-inhibition results obtained for the pooled serum from hydatid patients and for the pooled serum from dogs infected with *E. granulosus* both of them being inhibited with the antigenic extracts HCF, S-Ag, and ES-Ag. Values corresponding to correlation coefficients (r), slopes of the regression lines, Ag_{50} , and theoretical maximum percentages of inhibition are shown. According to the analysis of these data, high cross-reactivity was mainly observed for S-Ag and ES-Ag. Furthermore, the pool of dog sera showed considerably higher Ag_{50} values than the pool of human sera, when assayed under the same conditions.

Immunoblotting and immunoblot-inhibition assays - Fig. 1 shows the antigenic profiles of HCF, S-Ag, and ES-Ag when using the different non-inhibited and inhibited immunosera. For better comprehension, the patterns of the protein bands are also presented in a schematic figure. Thirteen polypeptides ranging from 9 to 108 kDa were identified in the HCF when the non-inhibited rabbit serum against HCF was used (Fig. 1-A). Antigens of 108 and 78 kDa were specific for HCF (rabbit antibodies against these polypeptides did not bind to any S-Ag or ES-Ag components). The non-inhibited rabbit antiserum against S-Ag recognized 13 antigenic components ranging from 6 to

124 kDa when S-Ag was used as solid phase. Polypeptides of 124, 94, 83, and 75 kDa were found to be specific for this antigenic extract (Fig. 1-B). Finally, the non-inhibited rabbit serum against ES-Ag reacted specifically with at least 14 components from the ES-Ag, some of which (89, 66, 42, 40, 37, and 35 kDa) are neither shared by S-Ag nor by HCF (Fig. 1-C). In addition, immunoblot-assays were carried out using each rabbit antiserum against its heterologous antigenic extracts as solid phase.

Table II summarizes the molecular masses of the major antigenic proteins shared by HCF, S-Ag, and ES-Ag, considering each immunoserum used in the inhibition reaction independently. The antigenic components identified as shared components had apparent molecular masses of 4-6, 20-24, 52, 80, and 100-104 kDa, including doublets of 41/45, 54/57, and 65/68 kDa.

DISCUSSION

Cyst hydatid fluid and somatic antigens of protozoocoles are the best characterized antigenic extracts of *E. granulosus*, and the main antigenic sources used for immunodiagnosis of human and dog echinococcosis (Rickard & Lightowers 1986, Lightowers & Gottstein 1995). During recent years, ES-Ag have acquired a role in the diagnosis of infections in the definitive host, based on the detection of these antigens in faeces by ELISA (Fraser & Craig 1997, Jenkins et al. 2000, Benito & Carmena 2005). So far, little information is available in regard to the description of ES-Ag. We have recently carried out the biochemical characterization of this antigenic extract, evaluating its potential for immunodiagnosis of human cystic echinococcosis and dog echinococcosis (Carmena et al. 2004, 2005). However, until now no studies have been performed to determine the homology degree among

TABLE I

Results of the ELISA-inhibition assays for the pool of human sera from individuals with confirmed hydatidosis and for the pool of sera from dogs naturally infected with *Echinococcus granulosus*

Sera	Solid phase	Inhibitor extract	r	Slope	Ag_{50} (mg/ml)	Theoretical % inhibition
Pooled human serum	HCF	HCF	0.9822	11.8	0.106	100
		S-Ag	0.9848	16.0	0.416	85.6
		ES-Ag	NA	NA	NA	NA
	S-Ag	HCF	0.9817	12.4	0.420	65.2
		S-Ag	0.9900	11.7	0.051	100
		ES-Ag	0.9948	14.2	0.123	92.6
	ES-Ag	HCF	0.9920	7.5	1.247	45.8
		S-Ag	0.9720	7.9	0.119	72.5
		ES-Ag	0.9857	13.1	0.060	100
Pooled dog serum	HCF	HCF	0.9891	9.9	0.119	100
		S-Ag	0.9913	13.4	0.572	87.1
		ES-Ag	0.9538	9.9	0.676	74.6
	S-Ag	HCF	0.9919	5.6	3.459	59.3
		S-Ag	0.9987	10.0	0.354	100
		ES-Ag	0.9723	6.8	0.931	74.2
	ES-Ag	HCF	0.9860	6.7	1.104	100
		S-Ag	0.9951	5.8	3.715	99.8
		ES-Ag	0.9951	4.9	1.349	100

HCF: hydatid cyst fluid; S-Ag: somatic antigens; ES-Ag: excretory-secretory antigens; NA: not available

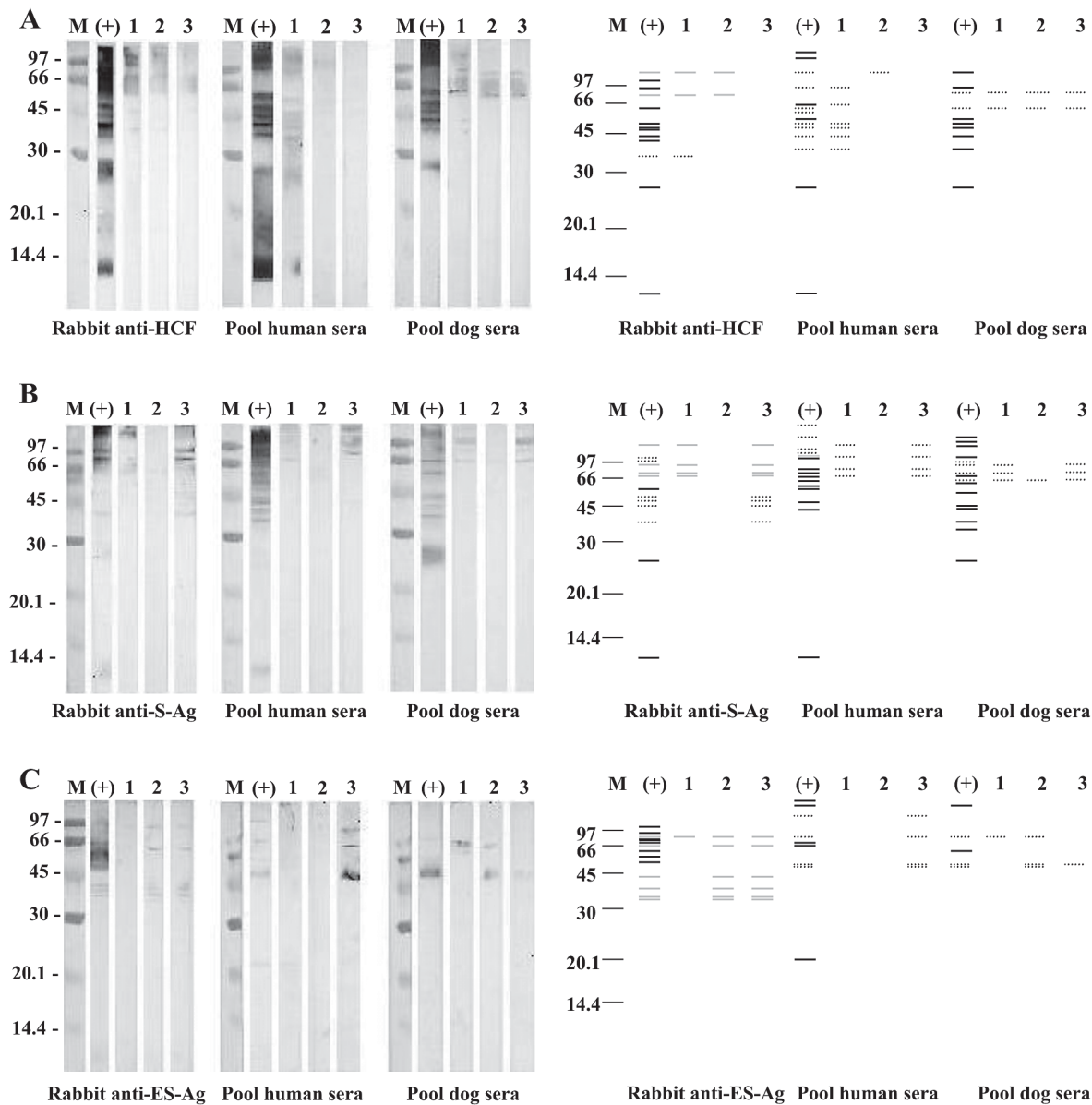


Fig. 1: immunoblotting profiles and schematic figures of the protein bands obtained when using inhibited and non-inhibited immunosera. A: hydatid cyst fluid (HCF); B: somatic antigens (S-Ag); C: excretory-secretory antigens (ES-Ag); M: molecular mass marker, expressed in kDa; (+): non-inhibited serum; 1: inhibited serum with ES-Ag; 2: inhibited serum with S-Ag; 3: inhibited serum with HCF. Non-shared components are indicated as grey bands. Shared components are indicated as black bands.

the antigenic components of HCF, S-Ag, and ES-Ag, and to find out their non-shared and shared proteins.

ELISA-inhibition results using both the pool of human sera from individuals with confirmed hydatidosis and the pool of sera from dogs infected with *E. granulosus* have shown a great resemblance with regard to the slopes of the regression lines corresponding to each antigenic extract used as solid phase. This fact indicates that there are antigens shared by HCF, S-Ag, and ES-Ag. The highest cross-reactivity was observed between S-Ag and ES-Ag. These results are supported by Ag_{50} values, since inhibition of 50% of the specific serum antibodies in each

inhibition reaction test required higher concentrations of HCF antigens than of S-Ag and ES-Ag. Interestingly, the pool of dog sera showed considerably higher Ag_{50} values than the pool of human sera, when assayed under the same conditions. These results demonstrate that dog antibodies have a lower avidity for their specific antigens than antibodies of human origin. This phenomenon may be explained by the concept of antibody affinity maturation through the course of species evolution. It is known that less evolved species have antibodies with lower affinity for their specific epitopes than higher evolved species (Du Pasquier 2001, Frank 2002). We consider that this

TABLE II

Major antigenic components shared by hydatid cyst fluid (HCF), protoscoleces somatic antigens (S-Ag), and excretory-secretory antigens (ES-Ag) recognized by the different used antisera by immunoblotting and blotting-inhibition. Molecular masses are expressed in kDa

Anti-HCF	Rabbit sera		Pool of human sera	Pool of dog sera
	Anti-S-Ag	Anti-ES-Ag		
-	-	-	133-135	130-133
117	113	-	-	115/122(D)
-	-	-	-	104-110
100	99-100	100-104	-	-
-	-	93/99(D)	-	97
89-91	91	-	-	-
84	86	86-88	-	-
79	80	80/83(D)	81	-
-	-	76-77	-	76
-	-	70-71	-	-
-	65-69	67	65/68(D)	-
62-65(D)	-	63-65	-	62-64
-	-	-	59-60	-
-	57/62(D)	57	56	56-57
54/57(D)	55	-	-	54
52	52	52	52	-
49	-	-	49	48
41/45(D)	44/46(D)	41/45(D)	41/44(D)	41/43(D)
-	37	-	38	38-39
-	-	-	34	34
-	32	30	-	-
23	21-23	24	20-23	21-22

D: doublet

may be one of the several reasons why a lower sensitivity was observed in assays for the serodiagnosis of dog echinococcosis as compared to assays for the immunodiagnosis of human cystic echinococcosis.

Immunoblotting and immunoblot-inhibition assays were carried out in order to describe the profiles of the non-shared and shared antigenic components among the different extracts of *E. granulosus* studied: HCF, S-Ag, and ES-Ag. Each antigenic extract was assayed against its homologous and heterologous rabbit antisera, the pool of human sera from individuals with confirmed hydatidosis and the pooled sera from dogs infected with *E. granulosus*, with and without inhibition. It is necessary take into consideration that the reported molecular mass estimations can have slight inaccuracies as a consequence of the limitations of the measurement method used. Among the shared components of the three antigenic extracts, the polypeptide of 4-6 kDa corresponds very likely to the 8 kDa subunit of the AgB. This component is strongly recognized by the non-inhibited pool of sera from patients infected with cystic echinococcosis and the rabbit sera anti-HCF/S-Ag, but not by the non-inhibited pool of sera from dogs infected with *E. granulosus* (Fig. 1-A, B). This fact confirms that AgB is present in the metacestode of the parasite, but not in the adult stage. Similarly, the polypeptide of 20-24 kDa may correspond to subunits of Ag 5 or Ag B, and the component of 41 kDa may match the major subunit of Ag 5 (Lightowlers et al. 1989, Ortona et al. 1995, González et al. 1996). Some of the products

identified in HCF by the pool of human sera in the present study may correspond to those previously described by other authors using the same antigenic extract. This may be the case of the protein of 34 kDa (recently identified by Poretti et al. 1999), and the protein of 110 kDa, probably related with the component of 100 kDa described by Shambesh et al. (1995), the protein of 110-120 kDa (Shapiro et al. 1992), and the protein of 116 kDa (Kanwar et al. 1992).

Some of the immunoblot-inhibition assays showed some bands in the negative controls, indicating that the inhibition reaction was not complete. This fact was detected more frequently when ES-Ag were used as inhibitory extract, and may probably be due to a lower concentration of protein used in the inhibition reaction.

Non-shared antigenic components of each extract from *E. granulosus* (HCF, S-Ag, and ES-Ag) were determined by analysing the profiles obtained by immunoblotting and comparing them with profiles that were obtained when the antisera were inhibited with the different antigenic extracts tested. HCF evidenced two non-shared components of 108 and 78 kDa, respectively. S-Ag showed four non-shared polypeptides with apparent molecular masses of 124, 94, 83, and 75 kDa. On the other hand, ES-Ag showed non-shared antigenic components of 89, 66, 42, 40, 37, and 35 kDa. The protein of 89 kDa has already demonstrated specificity for immunodiagnosis of human cystic echinococcosis and dog echinococcosis in previous reports (Carmena et al. 2004, 2005). This component

may be responsible for the higher specificity shown by ES-Ag in comparison to S-Ag in ELISA assays used for serodiagnosis of dog echinococcosis.

In summary we can conclude that HCF, S-Ag, and ES-Ag share an important proportion of antigens, which explains the high level of cross-reactivity found in ELISA-inhibition and immunoblot-inhibition assays when these extracts were used. The identification of shared and non-shared immunogenic components of HCF, S-Ag, and ES-Ag may provide information that could prove very useful when searching for specific components or antigens with potential for the immunodiagnosis of human cystic echinococcosis and dog echinococcosis.

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