Purification of specific proteins from Hydatid Cyst fluids of sheep liver and application to ELISAs

A. BURGU[1]*, O. SARIMEHMETOGLU[1], B. GONENC[1], A. DOGANAY[1], H. OGE[1], S. OGE[1], G. SAHIN[2], S. AYPAK[3], H. AVCIOGLU[4], C. S. BOLUKBAS[5], E. ARAZ[6]

[1]*Department of Parasitology, Faculty of Veterinary Medicine, University of Ankara, 06 110 Ankara, TURKEY.*
[2]*Ministry of Agriculture and Rural Affairs General Directorate of Protection and Control, Department of Public Health, Akay Street No:3 Bakanlıklar, 06 130 Ankara, TURKEY.*
[3]*Department of Parasitology, Faculty of Veterinary Medicine, Adnan Menderes University, 09 100 Aydın, TURKEY.*
[4]*Department of Parasitology, Faculty of Veterinary Medicine, Atatürk University , 25 240 Erzurum, TURKEY.*
[5]*Department of Parasitology, Faculty of Veterinary Medicine, University of Ondokuz Mayıs, 55 139 Samsun, TURKEY.*
[6]*Gülhane Military Medical Academy, 06 018 Ankara, TURKEY.*

**Corresponding author: ayse.burgu@veterinary.ankara.edu.tr*

SUMMARY

The aims of this study were to determine the sensitivity and specificity of ELISA tests using cyst fluid antigens or purified 8, 68, 116 kDa immunoreactive proteins for diagnosis of hydatid cyst in mice and humans and to compare them with the performances of 2 commercial kits available in humans. For that, serum samples were obtained, on one hand, from mice (n = 25) intraperitoneally infected with 3 000 protoscoleces from ovine liver cyst 10 months ago or from healthy mice (n = 25) and on the other hand, from 10 patients with hydatidosis (radiologically confirmed), from 6 patient with other parasite diseases than hydatidosis (serologically confirmed) and from 10 healthy patients. The sensitivity of the ELISA tests using purified proteins or the cyst fluid antigens varied in mice from 96% to 100% for the 116 kDa antigen and in humans from 90% to 100% for the 68 kDa antigen and was higher than with commercial tests (60% and 80%). The specificity determined from healthy mice was maximal (80%) with the 8 kDa and 116 kDa proteins as antigens and the specificity determined from humans not infected with hydatidosis ranged from 93.8% with one of the 2 commercial kits and 87.5% with the 8 kDa antigen to 75% with the 68 kDa antigen and the total antigen preparation and 62.5% with the other commercial kit. Some cross-reactions in human sera were observed between *Echinococcus* purified antigens and *Toxocara* or *Trichinella* at a lesser extend. These results clearly show that some purified hydatid cyst proteins, especially 8 kDa and 116 kDa proteins, can be used as antigens for ELISAs and improve the sensitivity and the specificity of the tests.

Keywords: Hydatidosis, Hydatid cyst, ELISA, Sheep, Protein, Purification, Mouse, Human, Sensitivity, Specificity.

RÉSUMÉ

Purification de protéines spécifiques des kystes hydatiques de foie de mouton et application à des tests ELISA

Les objectifs de cette étude étaient de déterminer la sensibilité et la spécificité de tests ELISA utilisant les antigènes totaux des kystes hydatiques ou les protéines immunogènes purifiées de 8 kDa, 68 kDa et 116 kDa dans le cadre d'un diagnostic de kyste hydatique chez la souris et chez l'homme et de les comparer avec les performances de 2 trousses de dosage commercialisées. Pour cela, des échantillons sériques ont été obtenus, d'une part, sur des souris intrapéritonéalement infectées (n = 25), 10 mois auparavant, par 3 000 protoscolex issus de kystes hépatiques ovins ou sur des souris saines (n = 25) et d'autre part, sur 10 patients atteints d'hydatidosis (confirmée radiologiquement), 6 patients avec une maladie parasitaire autre que l'hydatidose (confirmée par sérologie) et 10 patients sains. La sensibilité des tests ELISA utilisant les protéines purifiées ou les antigènes totaux des kystes a varié, chez la souris, de 96 % à 100 % pour l'antigène de 116 kDa et chez l'homme, de 90 % à 100 % pour l'antigène de 68 kDa et s'est avérée plus élevée que celle des kits commerciaux (60 % and 80 %). La spécificité déterminée à partir des souris saines a été maximale (80 %) avec les antigènes de 8 kDa et de 116 kDa; la spécificité déterminée sur les patients non atteints d'hydatidosis était comprise entre 93.8 % pour l'un des 2 kits commerciaux et 87.5 % avec l'antigène de 8 kDa d'un côté, et 75 % avec l'antigène de 68 kDa et 62.5 % avec le 2ème kit. Quelques réactions croisées ont été mises en évidence dans les sérums humains, entre les antigènes purifiés d'*Echinococcus* et *Toxocara* ou *Trichinella* à un moindre degré. Ces résultats montrent clairement que certaines protéines purifiées de kyste hydatique, en particulier celle de 8 kDa et celle de 116 kDa, peuvent être utilisées comme antigènes au cours d'un test ELISA, permettant ainsi d'en accroître la sensibilité et la spécificité.

Mots clés : Hydatidose, kyste hydatique, ELISA, mouton, protéine, purification, souris, humain, sensibilité, spécificité.

Introduction

Hydatidosis also known as hydatid cyst (HC), caused by larval forms of *Echinococcus granulosus* found in the small intestines of dog and other carnivores, is commonly observed in several wild and domestic mammals including man and it is associated with economic losses and health problems [1, 20].

Although the presence of HC in ruminants is determined after slaughter or necropsy, there is no available test practically used for the HC diagnosis on live animals. In humans, the diagnosis of the disease is achieved by means of clinical signs together with imaging techniques and serological assays. However, cross-reactions due to the diversity of different molecular weight antigens used in serological tests are observed between various infections initiated by other parasites (mainly cestod species) [10, 19, 22, 30]. In some studies on HC diagnosis in humans and animals, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) revealed 15 protein fractions between 8 and 116 kDa and

Western blots evidenced that produced antibodies mainly targeted the 8, 68 and 116 kDa bands. The use of these antigens could minimize cross-reactions with other infections [3, 5, 14, 17, 18]. SU and PRESTWOOD [34] and KANWAR *et al*. [14] highlighted the difficulty in preparation of specific antigens against parasites for eliminating host antigens and other common parasitic antigens. After obtaining specific bands by western blot technique (considered as a confirmation test [26]), the purification of the antigens from these bands is essential for developing easy assays such as ELISA [6, 12]. The SDS-PAGE method following by a rotofor system separates proteins according to their molecular sizes coupled to the isoelectric pH values [2, 8, 23] and this combined technique has provided the feasibility of determination and purification of specific protein fractions in a total cyst fluid [4, 27].

The aims of the present study were to determine the sensitivity and specificity of ELISA tests using total cyst fluid antigens or the 8, 68 and 116 kDa immunoreactive proteins in mice and humans and to compare them with 2 commercially available ELISA Kits widely used in human diagnostic centres in Turkey.

Materials and Methods

ANTIGEN PREPARATIONS

Cyst fluid antigens

The HC fluid obtained from ovine liver was centrifuged at 10.000 g for 30 minutes at 4°C. After pelleting protoscoleces, the supernatant fraction was filtered through 0.45 μm membrane filter and dialysed against distilled water at 4°C for 24 hours. The cyst fluid antigens were stored at -70°C until used.

Purification of immunoreactive antigens (8, 68 and 116 kDa)

The SDS-PAGE was performed as previously described by SAMBROOK *et al*. [26] using Protean II xi Cell (BIO-RAD, Italy) device. The cyst fluid antigens were analysed by SDS-PAGE (5% stacking gel and 15% separating gel) and after separation a total of 23 protein bands including the 3 targeted specific proteins of 8, 68, and 116 k Da were observed. For protein purification, a 2% ampholyte (BIO-RAD, 163-1112) within pH 3-10 range was added to total antigens (50 mL) and then the Rotofor Cell (BIO-RAD, USA) device that achieves sedimentation of proteins according to isoelectrofocusing (pI) was used. At the beginning stage, a 15 W currency was applied, initial Volt and mA were recorded. Rotofor procedure was ended at the fixation point of volt. Generally, the rotofor procedure continued for 5-6 hours, and then proteins assembled in 20 divisions were collected into 20 separate tubes by mean of a vacuum pump. Each tube sample (40 μL) was run again by SDS-PAGE for determination of molecular weight and of purity degree. With this technique, the 8, 68 and 116 kDa immunoreactive proteins were collected into separate tubes without contamination by other proteins. After measuring the protein concentration by spectrophotometry (Picodrop Microlitre UV/Vis spectrophotometer, GRI), the corresponding protein fractions were stored at -70°C until used.

ELISA TESTS

ELISA was performed essentially as previously described [11]. Briefly, ELISA plate (Bio-Hit High Binding, Finland) wells were coated with 10 μg/mL antigens and incubated at 4°C overnight. Unbound antigen was removed by washing with 0.1% PBS Tween-20. Non-specific bindings were blocked by addition of 5% fat free milk powder in PBS. During preliminary steps, the use of sera in different dilutions (from 1:200 to 1:20) showed that 1:50 serum dilution is optimal. Consequently, mouse or human sera diluted to 1:50 were added to each well. After the addition of the peroxidase conjugate anti-mouse IgG (Sigma A-4416) or anti-human IgG (Sigma A-8775) diluted to 1:3 000 to each well, plates were incubated for 2 hours at 37°C. The antibody-conjugated reaction was visualised with the enzyme substrate, the o-phenylene diamine dihydrocloride (Sigma LOT 055K 8204, P-9187). The absorbance value was measured at 405 nm using ELISA reader (BIO-RAD Model 680, Japan). The cut-off value was ≥ 0.420. The cut-off value for seropositivity was defined as mean + 2 standard deviation (SD) obtained for the negative control group in each experiment to eliminate the discrepancy between plates. The specificity and sensitivity (the proportions of true negative samples and of true positive samples respectively) of each ELISA test were determined according to the literature [9].

In parallel, the human sera were analysed with 2 commercial ELISA kits [Kit I: IBL Echinococcus IgG (LOT EG-131, IBL Immuno-biological laboratories) - Kit II: DRG Echinococcus (LOT LN457, DRG International Inc.)] commonly used for the HC diagnosis in Turkey.

SERUM SAMPLES

Mouse sera

A total of 50 Swiss albino mice, 1 month old, 25 males and 25 females, were used in the study and allotted into two groups. In the experimental group (n = 25) mice were intraperitoneally infected with 3 000 protoscoleces recovered from hydatid cysts of ovine livers whereas the other group (n = 25) served as the negative control group. Ten months after infection, blood samples (500 μL) were taken directly by heart puncture using a 21 G needle with 2 ml syringe (Hayat sterile dispossible syringe-TS-3971). Blood samples collected into sterile tubes without any coagulant were allowed to clot for up to one hour at room temperature then centrifuged at 2 000g for 10 minutes at room temperature and sera were carefully harvested and stored at -20°C until analysis. Furthermore, a necropsy was performed in all mice of the experimental group in order to verify the occurrence of hydatidosis.

Human sera

A total of 26 human sera were kindly provided by the Gülhane Military Medical Academy (Ankara, Turkey). Among them, 10 sera belonged to patients elected for surgery after radiological diagnosis of hydatid cyst, 6 sera were stemming from patients negative for hydatidosis (confirmed

by advanced imaging methods) but seropositive for other parasite diseases (2 toxocarosis, 2 trichinellosis and 2 fasciolosis) and the last 10 sera were negative for hydatidosis and other parasite diseases.

Results

As shown in Table I, the specificity and the sensitivity of ELISA tests developed with pure immunoreactive 8 kDa, 68 kDa and 116 kDa proteins and conducted on mouse sera ranged from 76% to 80% and from 96% to 100% respectively. The lowest specificity (76%) was obtained with the 68 kDa protein and the highest sensibility was achieved with the 116 kDa protein. Using cyst fluid antigens, a high sensitivity (96%) was also obtained but the specificity remained moderate (68%).

For human sera (Table II), the sensitivity of these ELISA tests was high whatever the antigens used, comprised between 90% and 100% with the 68 kDa antigen, whereas a lower sensitivity was obtained with the 2 commercial available kits (60% and 80% with the kits I and II respectively). Considering only the healthy patients, the highest specificity (100%) was recorded with the commercial kit II but the ELISA tests developed with the 8kD or the 116 kDa antigens gave also a high value (90%): except for only one sample, all negatives sera were identified (Table II). The other ELISA tests developed with the 68 kDa immunoreactive protein and with the cyst fluid antigens presented a moderate specificity

ELISA tests	HC free mice (n = 25)	Mice with HC (n = 25)*
8 kDa antigen		
Positive sera	5	24
Negative sera	20	1
Sp / Se	Sp: 80%	Se: 96%
68 kDa antigen		
Positive sera	6	24
Negative sera	19	1
Sp / Se	Sp: 76%	Se: 96%
116 kDa antigen		
Positive sera	5	25
Negative sera	20	0
Sp / Se	Sp: 80%	Se: 100%
CF antigens		
Positive sera	8	24
Negative sera	17	1
Sp / Se	Sp: 68%	Se: 96%

CF: Cyst fluid; Sp: specificity (proportions of true negative samples in %); Se: sensitivity (proportions of true positive samples in %).
**Hydatidosis was experimentally induced in mice by intraperitoneal injection of 3 000 protoscoleces recovered from hydatid cysts of ovine livers.*

TABLE I : Distribution of positive and negative mouse sera with ELISA tests developed with pure immunoreactive 8 kDa, 68 kDa and 116 kDa proteins or with cyst fluid antigens prepared from hydatid cysts of ovine liver.

(80%) and the commercial kit I gave the lowest result (60%). The specificity of the different tests measured in patients free from hydatidosis but infected by other parasites (*toxocara, fasciola* and *trichinella*) (n = 6) was 66.7% for the cyst fluid antigens, the 68 kDa and the 116 kDa proteins as well as for the commercial kit I and reach 83.3% with the 8 kDa antigen and the commercial kit II. Consequently, the overall specificity determined by considering all patients without hydatid cyst (n = 16) was maximal for the commercial kit II (93.8%), and for the ELISA test using the 8 kDa protein as antigen at a lesser extend (87.5%), intermediate for the tests using the 116 kDa antigen (81.3%) and the 68 kDa antigen or the cyst fluid antigens (75%) and weak with the commercial kit I (62.5%).

The Table III presents the individual values obtained with the different ELISA tests in humans free from hydatidosis but infected with other parasites. As shown, a serum from one patient infected with *Toxocara* produced false positive reaction using all antigens and the both commercial kits. Furthermore, a serum from one human with *Trichinella* generated false positive reactions except with the ELISA test using the 8 kDa antigen and with the commercial kit II.

Discussion

Different life stages of *Echinococcus granulosus* show various antigenic structures. The HC antigens are generally prepared from cyst fluid, membrane or protoscoleces. In prepared antigens, the involvement of host components produces a negative effect on serological tests [6, 13]. However, the use of purified antigens minimizes the influence of the host components, improving in this way the diagnostic efficiency of the serological tests. Furthermore, the concentrations of antigenic proteins vary in HC fluids according to the host species and to the localisation of the cysts. Camel and ovine cysts had higher antigenic concentrations compared to human, pig and bovine cysts and liver cysts generally contain higher antigenic materials than lung [12, 13, 21]. In this study, considering the important place of sheep among the animal population and the accessibility to flocks in Turkey, the antigens were prepared from ovine fertile liver hydatid cysts.

Electrophoresis analysis of HC fluid antigens prepared from humans and ruminants generated different number and molecular size protein bands [3, 14, 17, 18, 28]. As the hosts develop a humoral response to some of these proteins, the lack of the test standardization may lower the sensitivity and specificity of the assay [35]. Two main antigenic components within cyst fluid, named as AgB (major units are 8, 12, 16, 24 kDa) and Ag5 (major units are 57, 67, 22, 24, 38 kDa) can be used for diagnostic purposes [7, 35]. In addition, recombinant antigens were also used as antigen source [13, 35]. In sheep, the 8 kDa [12, 13, 18], 12 kDa [12, 13, 28, 31], 16 kDa [31-33], the 24 kDa [12, 13, 32, 33], the 29 kDa and the 45 kDa [32, 33], the 66 kDa [14] and the 68 kDa [32, 33] as well as the 98 and 116 kDa [32, 33] polypeptides obtained from cyst fluid were commonly used for the HC diagnosis. Among them, the 8, 68 and 116 kDa proteins appeared as

ELISA tests		HC free humans			Humans with HC
		No diseases (n = 10)	Other diseases (n = 6)*	Total (n = 16)	(n = 10)*
8 kDa antigen	Positive sera	1	1	2	9
	Negative sera	9	5	14	1
	Sp / Se	Sp: 90.0%	Sp: 83.3%	Sp: 87.5%	Se: 90.0%
68 kDa antigen	Positive sera	2	2	4	10
	Negative sera	8	4	12	1
	Sp / Se	Sp: 80.0%	Sp: 66.7%	Sp: 75.0%	Se: 100.0%
116 kDa antigen	Positive sera	1	2	3	9
	Negative sera	9	4	13	1
	Sp / Se	Sp: 90.0%	Sp: 66.7%	Sp: 81.3%	Se: 90.0%
CF antigens	Positive sera	2	2	4	9
	Negative sera	8	4	12	1
	Sp / Se	Sp: 80.0%	Sp: 66.7%	Sp: 75.0%	Se: 90.0%
Kit I	Positive sera	4	2	6	6
	Negative sera	6	4	10	4
	Sp / Se	Sp: 60.0%	Sp: 66.7%	Sp: 62.5%	Se: 60.0%
Kit II	Positive sera	0	1	1	8
	Negative sera	10	5	15	2
	Sp / Se	Sp: 100.0%	Sp: 83.3%	Sp: 93.8%	Se: 80.0%

*HC: Hydatidosis; CF: Cyst fluid; Sp: specificity (proportions of true negative samples in %); Se: sensitivity (proportions of true positive samples in %). *Hydatidosis were radiologically confirmed in 10 patients and parasite diseases other hydatidosis (2 toxocarosis, 2 trichinellosis and 2 fasciolosis) were confirmed by serological specific tests in 6 patients.*

TABLE II : Distribution of positive and negative human sera with ELISA tests developed with pure immunoreactive 8 kDa, 68 kDa and 116 kDa proteins or with cyst fluid antigens prepared from hydatid cysts of ovine liver or with commercial available ELISA tests (Kit I: IBL Echinococcus IgG - Kit II: DRG Echinococcus).

Patients	ELISA tests				Commercial kits	
	8 kDa	68 kDa	116 kDa	CF Ag.	Kit I	Kit II
Trichinellosis 1	Negative	**Positive**	**Positive**	**Positive**	Positive	Negative
Trichinellosis 2	Negative	Negative	Negative	Negative	Negative	Negative
Toxocarosis 1	**Positive**	**Positive**	**Positive**	**Positive**	Positive	Positive
Toxocarosis 2	Negative	Negative	Negative	Negative	Negative	Negative
Fasciolosis 1	Negative	Negative	Negative	Negative	Negative	Negative
Fasciolosis 2	Negative	Negative	Negative	Negative	Negative	Negative

CF Ag.: Cyst fluid antigens.

TABLE III : Results obtained with the ELISA tests developed with pure immunoreactive 8 kDa, 68 kDa and 116 kDa proteins or with total cyst fluid antigens prepared from hydatid cysts of ovine liver or with 2 commercial kits (Kit I: IBL Echinococcus IgG - Kit II: DRG Echinococcus) in human sera from patients (n = 6) exempt of hydatidosis but infected with other parasites (*Toxocara, Fasciola* or *Trichinella*).

relatively specific antigens of hydatid cyst as previously confirmed [3] and were used as antigens for ELISA tests in the present study.

The ELISA test is generally considered as economic, sensitive and specific and allows running a high number of samples in a short time span. It was reported that sensitivity and specificity for ovine HC ELISA tests were comprised between 60% and 92% and between 77% and 100%, respectively [6, 12, 13, 32, 33]. Some antigen characteristics (molecular weight, the tissue localisation of the cysts, the cyst shape and the host type) can affect the qualities of the ELISA test [6, 13, 32]. For example, the sensitivity and specificity of an ELISA test using the AgB prepared from ovine cyst reached 91.6% and 77.1% respectively, whereas they fell to 79.1% and 60.4% respectively when crude cyst fluid was used as antigen [33]. In addition, according to IBRAHEM *et al*. [12, 13], the ELISA test using the AgB prepared from camel cyst exhibited higher sensitivity (90-92%) and specificity (99-100%) than a test using the same antigen prepared from ovine cyst (88% and 95% respectively). In 70-150 kDa

crude protoscoleces antigens, sensitivity was 62.7% while specificity was 98.2% [16]. In sheep, the sensitivity and specificity of an ELISA test using the r-AgB (52 kDa) prepared from ovine CH were 28% and 95%, respectively [13] whereas using the 116 kDa antigen from ovine cyst these qualities reached 88% for the sensitivity and 84% for the specificity [32]. With the 8 kDa, 16 kDa and the 21 kDa proteins as antigens in the same ELISA test, the sensitivity was 91.4% [6]. In the present study, the use of the 116 kDa antigen prepared from ovine liver cyst has allowed the achievement of a high sensitivity (100%) and of a high specificity (80%) in mouse sera whereas in ELISA tests conducted with the 2 other selected immunoreactive proteins (8 kDa and 68 kDa), the sensitivity was 96% and the specificity was slightly lower (80% and 76% respectively). For human sera, the sensitivity and the specificity of the different ELISA tests ranged from 73% to 97% and from 84% to 97% respectively [15, 24, 25, 29]. Antigens derived from ovine HC commonly used in human produced high sensitivity and specificity both in ELISA [15, 25, 29] and in immunoblotting [24]. In this study, ELISA sensitivity was 100% with the 68 kDa antigens and 90% for the other antigens (8 kDa and 116 kDa pure proteins and crude antigens from cyst fluid). The ELISA specificity calculated for healthy humans was 90% with the 8 kDa and 116 kDa antigens and 80% with the 68 kDa protein and the crude preparation. Although using same pure immunoreactive proteins prepared from ovine liver cyst fluid, the different ELISA tests in the present study exhibited slightly variations of the sensitivity and the specificity according to the serologically targeted species (mouse versus human). These variations would probably result from differences in the affinity and the specificity of the produced antibodies by given species. Moreover, the proportions of false negative human sera were higher with the both 2 commercial kits [40% and 20% for the kit I (IBL Echinococcus IgG) and the kit II (DRG Echinococcus) respectively] than with ELISA tests using prepared antigens from ovine cyst (10%). Nevertheless, the specificity considering only healthy humans was maximal (100%) with the kit II but was markedly low with the kit I. Consequently, the Kit II in comparison to Kit I better differentiated the diseased and healthy individuals.

On the other hand, the specificity calculated on healthy patients plus patients with parasite diseases other than hydatidosis fell to 87.5% for the test using the purified 8 kDa antigen and to 75% for those using the purified 68 kDa protein or the cyst fluid antigens preparation from ovine cyst. Again, the specificity extended to patients not infected with HC was markedly higher for the commercial kit II than for the commercial kit I (93.8% for the kit II versus 62.5% for the kit I). These results evidenced that some cross-reactions can occur between *Echinococcus* and other parasites, such as *Toxocara* and *Trichinella* at a lesser extend. Indeed, one Toxocara positive human serum gave false positive results with the ELISA tests in this study whatever the antigen used, while one *Trichinella* positive human serum induced positive ELISA reactions except with the 8 kDa protein as antigen and with the commercial kit II.

As a conclusion, the ELISA tests performed with purified antigens (8 kDa, 68 kDa and 116 kDa proteins) presented a high sensitivity but a moderate specificity for the HC diagnosis in mice and humans. In humans, the relative high proportions of false positive sera resulted from antibody cross-reactivity with other parasites, especially *Toxocara* and *Trichinella*. Moreover, the sensitivity was higher for ELISA tests using purified antigens (90% - 100%) than for the 2 commercial kits (60% and 80%). Nevertheless, further studies are required for taking into account all criteria susceptible to interfere with the sensitivity and the specificity of the serological tests and for proposing a standardized procedure.

References

1. - BARIS I., SAHIN A., BILIR N., KALYONCU A.F., EMRI A.S., AKHAN O., BARIS B., COPUR A.S., SELCUK Z.T.: Hidatik kist hastalı ı ve Türkiye'deki konumu. *Türkiye Akci er Hastalıkları Vakfi Yayını* No:1 *Ankara*, 1989, pp.: 1-18.
2. - BONO J.L., LEGENDRE A.M., SCALARONE G.M.: Detection of antibodies and delayed hypersensitivity with rotofor preparative IEF fractions of blastomyces dermatitis yeast phase lysate antigen. *J. Med. Vet. Mycol.*,1995, **33**, 209-214.
3. - BURGU A., DOGANAY A., GONENC B., SARIMEHMETOGLU H.O., Kalınbacak F.: Analysis of hydatid cysts from sheep by SDS-PAGE, and determination of specific antigens in protein structure by Western blotting. *Tr. J. Anim. Sci.*, 2000, **24**, 493-500.
4. - BURGU A., SARIMEHMETOGLU O., GONENC B., OGE H., SAHIN G., AYPAK S.: Effects of immunization with purified specific proteins on the formation of secondary hydatid cysts in mice. *Deutsch. Tierarztl. Wochenschr*., 2007, **114**, 349-353.
5. - DOGANAY A., BURGU A., GONENC B., SARIMEHMETOGLU O.: Koyun kist hidatik protoskolekslerinin protein yapısının analizi ve spesifik antijenlerinin saptanması. *Ankara Univ. Vet. Fak. Derg.*, 2000, **47**, 67-75.
6. - DUEGER E.L., VERASTEGUI M., GILMAN R.H.: Evaluation of the enzyme-linked immunoelectrotransfer blot (EITB) for ovine hydatidosis relative to age and cyst characteristics in naturally infected sheep. *Vet. Parasitol*., 2003, **114**, 285-293.
7. - FERNÀNDEZ V., FERREIRA H.B., FERNÀNDEZ C., ZAHA A., NIETO A.: Molecular characterisation of a novel 8-kDa subunit of Echinococcosus granulosus antigen B[1]. *Mol. Biochem. Parasitol.*, 1996, **77**, 247-250.
8. - FURSTER C., ZHANG J., TOLL A.: Purification of a 3beta-hydroxy-delta 5 C27-steroid dehydrogenase from pig liver microsomes active in major and alternative pathways of bile acid biosynthesis. *J. Biol. Chem*., 1996, **271**, 20 903-20 907.
9. - GRINER P.F., MAYEWSKI R.J., MUSHLN A.I., GREENLAND P.: Selection and interpretation of diagnostic tests and procedures. Principles and aplications. *Ann. Int. Med.*, 1981, **94**, 553-600.
10. - HEATH D.D., LAWRENCE S.B.: Antigenic polypeptides of *Echinococcus granulosus* oncospheres and definition of protective molecules. *Paras. Immunol.*, 1996, **18**, 347-357.
11. - HIRA P.R., BAHR G.M., SHWEIKI H.M., BEHBEHANI K.: An enzyme-linked immunosorbent assay using an arc 5 antigen for the diagnosis of cystic hydatid disease. *Ann. Trop. Med. Parasitol*., 1990, **84**, 157-162.
12. - IBRAHEM M.M., CRAIG P.S., Mc VIE A.: Echinococcosus granulosus antigen B and seroreactivity in natural ovine hydatidosis. *Res. Vet. Sci.*, 1996, **61**, 102-106.
13. - IBRAHEM M.M., RAFIEI A., DAR F.K., AZWAI S.M., CARTER S.D.: Serodiagnosis of cystic echinococcosis in naturally infected camels. *Parasitology*, 2002, **125**, 245-251.
14. - KANWAR J.R., KAUSHIK S.P., SAWHNEY I.M.S., KAMBOJ M.S., MEHTA S.K., INAYAK K.: Specific antibodies in serum of patients with hydatidosis recognised by immunoblotting. *J. Med. Microbiol*., 1992, **36**, 46-51.
15. - KAUR M., MAHAJAN R.C., MALLA N.: Diagnostic accuracy of rapid enzyme linked immunosorbent assay for the diagnosis of human hydatidosis. *Ind. J. Med. Res.*, 1999, **110**, 18-21.

16. - KITTELBERGER R., REICHEL M.P., JENNER J., HEATH D.D., LIGHTOWLERS M.W., MORO P., IBRAHEM M.M., CRAIG P.S., O'KEEFE J.S.: Evaluation of three enzyme-linked immunosorbent assay (ELISAs) for the detection of serum antibodies in sheep infected with *Echinococcosus granulosus*. *Vet. Parasitol.*, 2002, **110**, 57-76.

17. - KOKSAL F., SERIN M.S., KEKEC Y., SADR Y.E.: nsan ve hayvan kökenli kist hidatik sıvılarının SDS-PAGE metoduyla analizi ve Western blot metodunun klinik önemi. *T. Parazitol. Derg*.,1995, **19**, 221-229.

18. - MADDISON S.E., SLEMENDA S.B., SCHANTZ P.M., FRIED J.A., WILSON M., TSANG V.: A spesific diagnostic antigen of *Echinococcus granulosus* with an apparent molecular weight of 8 kDa. *Am. J. Med. Hyg*., 1989, **40**, 377-383.

19. - MARCH F., ENRICH C., MERCADER M., SANCHEZ F., MUNOZ C., COLL P., PRAT S.: *Echinococcus granulosus*: Antigen characterization by chemical treatment and enzymatic deglycosylation. *Exp. Parasitol*., 1991, **73**, 433-439.

20. - MORRIS D.L., RICHARDS K.S.: Hydatid Disease. Butterworth-Heinemann Ltd. Oxford, 1992, pp.: 57-76.

21. - MUSIANI P., PIANTELLI M., LAURIOLA L., ARRU E., POZZUOL R.: *Echinococcus granulosus* specific quantification of the two most immunoreactive antigens in hydatid fluids. *J. Clin. Pathol*., 1978, **31**, 475-478.

22. - NJERUH F.M., OKELA G.B.A., GATHUMA J.M.: Usefulness of indirect haemaglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) in the diagnosis of human hydatidosis. *E. Afr. Med. J.*, 1989, **66**, 310-314.

23. - NORE B.F., HARRISON M.A., KEEN J.N., ALLEN J.F.: Partial purification of a cyanobacterial membrane protein with amino terminal sequence similarity to the N-methylphenylalanine pilins. *Acta. Chem. Scand.*, 1994, **48**, 578-581.

24. - PLANCHART S., BOTTO C., ALORCON DE NOYA B., BONIFACINO R., VIVAS L., SPENCE L., VIVAS S.: Evaluation of the double diffusion, enzyme immunoassay and immunoblotting techniques for the diagnosis of human hydatid disease in tropical areas. *Rev. Inst. Med. Trop. Sao. Paulo.*, 1994, **36**, 205-210.

25. - SADJJADI S.M., ABIDI H., SARKARI B., IZADPANAH A., KAZEMIAN S.: Evaluation of enzyme-linked immunosorbent assay, utilizing native antigen B for serodiagnosis of human hydatidosis. *Iran J. Immunol.*, 2007, **4**, 167-172.

26. - SAMBROOK J., FRITSCH E.F., MANNIATIS T.: Molecular Coloning: A Laboratory Manual. Cold Spring Harbor., NewYork, 1989, pp.: 18.47-18.76.

27. - SARIMEHMETOGLU H.O., KUBAR A., TANYUKSEL M., GUN H.: Koyun karaci er kist sıvısından 8 kDa'luk antijenin purifikasyonu. *T. Parazitol. Derg*., 1998, **22**, 137-141.

28. - SASMAZ Z.E., HASHEMPOOR G.R., BAHIAR I.H., YULUG N.: *Echinococcus granulosus*'un kar ıla tırmalı antijenik analizi. *T. Parazitol. Derg*., 1995, **20**, 83-87.

29. - SBIHI Y., RMIQUI A., RODRIGUEZ-CABEZAS M.N., ORDUñA A., RODRIGUES-TORRES A., OSUNA A.: Comparative sensitivity of six serological tests and diagnostic value of ELISA using purified antigen in hydatidosis. *J. Clin. Lab. Anal.*, 2001, **15**, 14-18.

30. - SENLIK B.: Bursa Yöresi Koyunlarda Indirekt Floresan Antikor (IFA) ve Indirekt Hemaglutinasyon (IHA) Testleriyle Hidatidoz'un Sero-Prevalansı Üzerine Ara tırmalar. Doktora Tezi, Uluda Üniv Sa lık Bilim Enst, Bursa. 1998.

31. - SHEPHERD J.C., Mc MANUS D.P.: Specific and cross-reactive antigens of *Echinococcus granulosus* hydatid cyst fluid. *Mol. Biochem. Parasitol.*,1987, **25**, 143-154.

32. - SIMSEK S., KOROGLU E.: Evaluation of enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunoelectrotransfer blot (EITB) for immunodiagnosis of hydatid diseases in sheep. *Acta. Trop.*, 2004, **92**, 17-24.

33. - SIMSEK S., UTUK A.E., KOROGLU E. : Hidatik kist sıvısından antijen B (Agb)'nin kısmi purifikasyonu ve koyun hidatidosisinin tanısındaki etkinli i. *Fırat Üniv. Sa lık. Bilim. Derg*., 2006, **20**, 337-340.

34. - SU X., PRESTWOOD A.K.: A Dot-ELISA mimicry westernblot test for the detection of swine trichinellosis. *J. Parasitol.*, 1991, **77**, 76-82.

35. - ZHANG W., Mc MANUS D.P.: Recent advances in the immunology and diagnosis of echinococcosis. *FEMS. Immunol. Med. Microbiol.*, 2006, **47**, 24-41.