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Original Article

Comparison of Native Hydatid Cyst Fluid (HCF), Lyophilized HCF, Antigen B (AgB) and Lyophilized AgB (LAgB) Originated from *Echinococcus granulosus* Sensu Stricto for Sero-Diagnosis of Active, Transitional and Inactive Human Liver Cystic Echinococcosis

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Abstract

Background: Cystic echinococcosis (CE) is an important zoonotic parasitic disease caused by the larval stage or metacestode of the tapeworm *Echinococcus granulosus* sensu lato. Due to treatment protocols for different liver cysts, diagnosis of cyst stages is very important. Different antigens have been used for CE diagnosis. However, each one is more sensitive and effective for the diagnosis of specific CE stages is not known well. We aimed to compare Native Hydatid Cyst Fluid (HCF), Lyophilized Hydatid Cyst Fluid (LHCF), antigen B (AgB) and Lyophilized antigen B (LAgB) originated from *E. granulosus* sensu stricto (G1-G3) genotype, for sero- diagnosis of active, transitional and inactive human liver CE using ELISA technique.

Methods: The HCF was collected aseptically from liver CE cysts of sheep slaughtered from 2018 to 2019 in Shiraz slaughterhouse, Southern, Iran. The cysts were characterized by PCR and sequencing for genotype specification. Four types of antigens were used: HCF, LHCF, AgB and LAgB originated from *E. granulosus* sensu stricto (G1-G3) genotype. Thirty-three serum samples from active, transitional, and inactive human cysts were collected. Overall, 48 samples from other parasitic diseases and 60 samples from healthy subjects as negative controls were checked using four antigens by ELISA method.

Results: The best diagnostic sensitivity with 96.97% was observed by anti-LHCF IgG ELISA test. The best specificity with 95.37% was observed in ELISA test using LAgB.

Conclusion: Simultaneous test of sera with anti-LHCF IgG ELISA and anti-LAgB IgG ELISA would be the best in the diagnosis of human liver cystic echinococcosis.

Keywords: Cystic echinococcosis; Ultrasonography; *Echinococcus granulosus*; Antigens; Human

Introduction

Cystic echinococcosis(CE) a zoonotic neglected parasitic disease, is caused by the metacestode of *Echinococcus granulosus* sensu lato (1). The disease is a major health problem all over the world including in Iran (2,3). Many cases of CE patients are routinely reported in different hospitals and different regions of the country (2,4).

Diagnosis of CE is mainly based on imaging methods and serological tests (5). For newly formed cysts, the efficiency of X-ray and ultrasound are not satisfactory. However, CE accompanying different imaging features, according to growth stages, relevant complications, and tissue tropism. The radiologic presentations are a variety of different features from purely cystic to completely solid mass appearance. Ultrasound is the most important imaging route used in liver CE that clearly shows the floating membranes, daughter cysts, and hydatid sands which are characteristic of purely cystic lesions (6). The radiologist's familiarity with the CE imaging findings is very crucial for earlier diagnosis and an appropriate treatment. There are several classification schemes for liver CE based on their ultrasound appearances. The classification by the WHO is the most commonly preferred (5,7). Based on this classification, the liver cysts are classified into active (CE1 and CE2), transitional (CE3) and inactive cysts (CE4 and CE5) (5,7,8). However, this classification has changed with the long-term results of the medical and percutaneous treatment and high-field magnetic resonance spectroscopy (MRI).

While sero-negativity is observed in 20% of patients with CE (9), those with multiple cysts are usually seropositive. The rate of sero-negativity has been reported to be higher in patients with CE1, CE4, and CE5 cyst types as compared to those with CE2 and CE3 types (8,10). The transitional type of CE cysts or CE3 types, are divided into CE3a with separated endocysts and, CE3b with solid type containing daughter vesicles (6,11–13).

If CE1 or CE2 are ruptured, the risk of spoilage of protoscoleces, new cyst formation (recurrent cyst) increases (5). Therefore, diagnosis and treatment of these types of cysts are very essential. In the case of inactive cysts, watch and wait is suggested. (14). On the other hand, a number of patients' sera may remain positive for several years after treatment (≥10years) (10). This may lead to unnecessary treatment (such as surgery) and consequently unnecessary costs (13). Clinical management of the disease is mainly based on the cyst stage according to the WHO Informal Working Group on Echinococcosis (WHO-IWGE) applicable to liver cysts, ultrasound cyst classification, and other clinical factors (5,7,15) and the treatment approach is based on surgery, percutaneous drainage, benzimidazoles administration, and "watch and wait" (16).

In the field of serological methods different native and recombinant antigens have been used for CE sero-diagnosis (4,17–20). Lyophilization has been used for a part of some studies (21-22). None of them has compared the effect of lyophilization on the efficacy of antigen. Moreover, application of different antigens has not clarified stage specific serodiagnosis of liver CE especially for CE1 or CE2 cases (14).

We aimed to test four types of antigens of *E. granulosus* sensu stricto (G1-G3 genotype) prepared from sheep hydatid cysts including native hydatid cyst fluid (HCF), lyophilized hydatid cyst fluid (LHCF), native Antigen B (AgB) and lyophilized antigen B (LAgB) that were used for sero-diagnosis of active, transitional, and inactive human liver cystic echinococcosis.

Materials and Methods

Hydatid cyst fluid

The hydatid cyst fluid (HCF) was collected aseptically from fertile sheep liver hydatid cysts from 2018 to 2019 in Shiraz slaughterhouse, Southern, Iran. The cysts were characterized by PCR and sequencing for genotype specification. The HCF of the cysts with *E. granulosus* sensu stricto genotype (G1-G3) were centrifuged at 3000×g in order to

remove the protoscoleces and stored at −20 °C until use.

Antigen B

Antigen B was prepared from HCF as described by Oriol et al. (23). Overall, 100 mL of HCF was dialyzed overnight against 5 mM of acetate buffer (pH 5) at 4°C. Centrifugation of sample was carried out with 50000 g for 30 min followed by supernatant removal and, the sediment was dissolved in 0.2 M phosphate buffer (pH 8). To remove the globulins from the sample, saturated ammonium sulfate was used. Finally, to separate heat-stable antigen B from other components, the sample was boiled in a water bath for 15 min and centrifuged at 50 000 g for 60 min. Bradford protein assay was used to determine its concentration (24).

Lyophilization

A part of both antigens (HCF and AgB) were lyophilized according to the standard methods (24).

Sera

The study was approved by the Regional Ethics Committee of National Institute for Medical Research Development, Iran (IR.NIMAD.REC. 1397.215).

Overall, 141 serum samples were used in this study including 33 radiologically, surgically, pathologically and serologically confirmed liver CE samples (CE1=12, CE2=8, CE3=6, CE4=5, CE5=2), collected from 2019-2020 in Shiraz, Iran. Sixty sera from healthy people and 48 samples from non-CE patients were collected from Shiraz and Tehran. CE classification were done by experienced radiologists. Cystic echinococcosis stages CE1 and CE2 were grouped as "active"; CE3 as transitional and CE4 & CE5 cysts (i.e., with solid appearance) were grouped as "inactive". Non-CE samples were collected from patients suffering from amoebiasis (*E. coli*) (N=1), ascariasis(N=4), blastocystosis (N=2), enterobiasis (N=1), fasciolosis (N=7), giardiasis (N=8), hymenolepiasis (N=5), leishmaniasis (N=1), malaria (N=5), strongyloidiasis (N=2), taeniasis (N=5), toxocariasis (N=4), toxoplasmosis (N=2), and trichostrongyliasis (N=1).

Enzyme-Linked Immunosorbent Assay

ELISA was carried out in flat-bottom 96-well microplates (Nunc, Maxisorp, Roskilde: Denmark) as previously described (17-18). ELISA was carried out in flat-bottom 96-well microplates according to previous studies. The absorbance was read at 492 nm after 30 min using an automatic microplate reader. All sera were checked using the four mentioned antigens by the ELISA method.

Statistical Analysis

The data were analyzed using SPSS software Version 22, (IBM Corp., Armonk, NY, USA). The student's t-test was applied and a *P*-value of <0.05 was considered significant. The cut-off was also calculated as $X \pm 2SD$.

Results

Four antigens including HCF, LHCF, AgB and LAgB were tested and evaluated with individual sera from CE patients, patients with other parasitic infections and, healthy control individuals using anti-IgG ELISA. The results are presented in Tables 1 and 2, Figs1a-d.

Table 1: Diagnostic performance of four different antigens (hydatid cyst fluid [HCF], lyophilized hydatid cyst fluid [LHCF], antigen B [AgB] and lyophilized antigen B [LAgB]) in IgG ELISA for sero-diagnosis of liver cystic echinococcosis (CE)

Variable		*Type of Antigen* HCF [Positive /total (No/%)]	LHCF [Positive /total (No/%)]	AgB [Positive /total (No/%)]	LAgB [Positive /total (No/%)]
Type of sera	Pathologically positive sera	30/33 (90.9)	32/33 (96.97)	27/33 (84.38)	27/33 (84.38)
	Sera of healthy people	2/60 (3.33)	3/60 (5)	1/60 (1.66)	1/60 (1.66)
	Sera of other diseases	4/48 (8.33)	5/48 (10.42)	7/48 (14.58)	4/48 (8.33)
Statistical analysis	Sensitivity Value / 95% CI	90.91/75.67 to 98.08	96.97/84.24 to 99.92	81.82/64.54 to 93.02	81.82/64.54 to 93.02
	Specificity Value / 95% CI	94.44/88.30 to 97.93	92.59/85.93 to 96.75	92.59/85.93 to 96.75	95.37/89.53 to 98.48
	Positive Likelihood Ratio Value / 95% CI	16.36/ 7.46 to 35.88	13.09/6.70 to 25.57	11.05/ 5.56 to 21.93	17.67/ 7.40 to 42.22
	Negative Likelihood Ratio Value / 95% CI	0.10 / 0.03 to 0.28	0.03/0.00 to 0.23	0.20 / 0.10 to 0.41	0.19 / 0.09 to 0.39
	Disease prevalence Value / 95% CI	23.40/16.69 to 31.27	23.40/16.69% to 31.27	23.40/16.69 to 31.27	23.40/16.69 to 31.27
	Positive Predictive Value Value / 95% CI	83.33/69.52 to 91.64	80.00/67.19 to 88.65	77.14/62.96 to 87.02	84.38/ 9.33 to 92.81
	Negative Predictive Value Value / 95% CI	97.14/92.03 to 99.01	99.01/93.55 to 99.86	94.34/88.97 to 97.18	94.50/89.26 to 97.26
	Accuracy Value / 95% CI	93.62/88.23 to 97.04	93.62/88.23 to 97.04	90.07/83.90 to 94.46	92.20/86.47 to 96.04

Table 2: Results of ELISA using four different antigens (hydatid cyst fluid [HCF], lyophilized hydatid cyst fluid [LHCF], antigen B [AgB] and lyophilized antigen B [LAgB]) for sero-diagnosis of liver cystic echinococcosis (CE) according to non-relative sera (sera of other diseases)

Variable		*Type of Antigens* HCF No. reacted/' total No.	LHCF No. reacted/ total No.	AgB No. reacted/ total No.	LAgB No. reacted /total No.
Type of sera	Amoebiasis (*E.coli*)	0/1	0/1	0/1	0/1
	Ascariasis	0/4	0/4	1/4	0/4
	Blastocystosis	0/2	0/2	0/2	0/2
	Enterobiasis	0/1	0/1	0/1	0/1
	Fasciolosis	3/7	2/7	2/7	1/7
	Giardiasis	0/8	0/8	1/8	1/8
	Hymenolepiasis	0/5	0/5	0/5	0/5
	Leishmaniasis	0/1	0/1	0/1	0/1
	Malaria	0/5	0/5	1/5	0/5
	Stronyloidiasis	0/2	0/2	0/2	0/2
	Taeniasis	1/5	2/5	1/5	1/5
	Toxocariasis	0/4	1/4	1/4	1/4
	Toxoplasmosis	0/2	0/2	0/2	0/2
	Trichostrongyliasis	0/1	0/1	0/1	0/1

Hydatid cyst fluid [LHCF], antigen B [AgB] and lyophilized antigen B [LAgB]) for serodiagnosis of liver cystic echinococcosis (CE) according to non-relative sera (sera of other diseases)

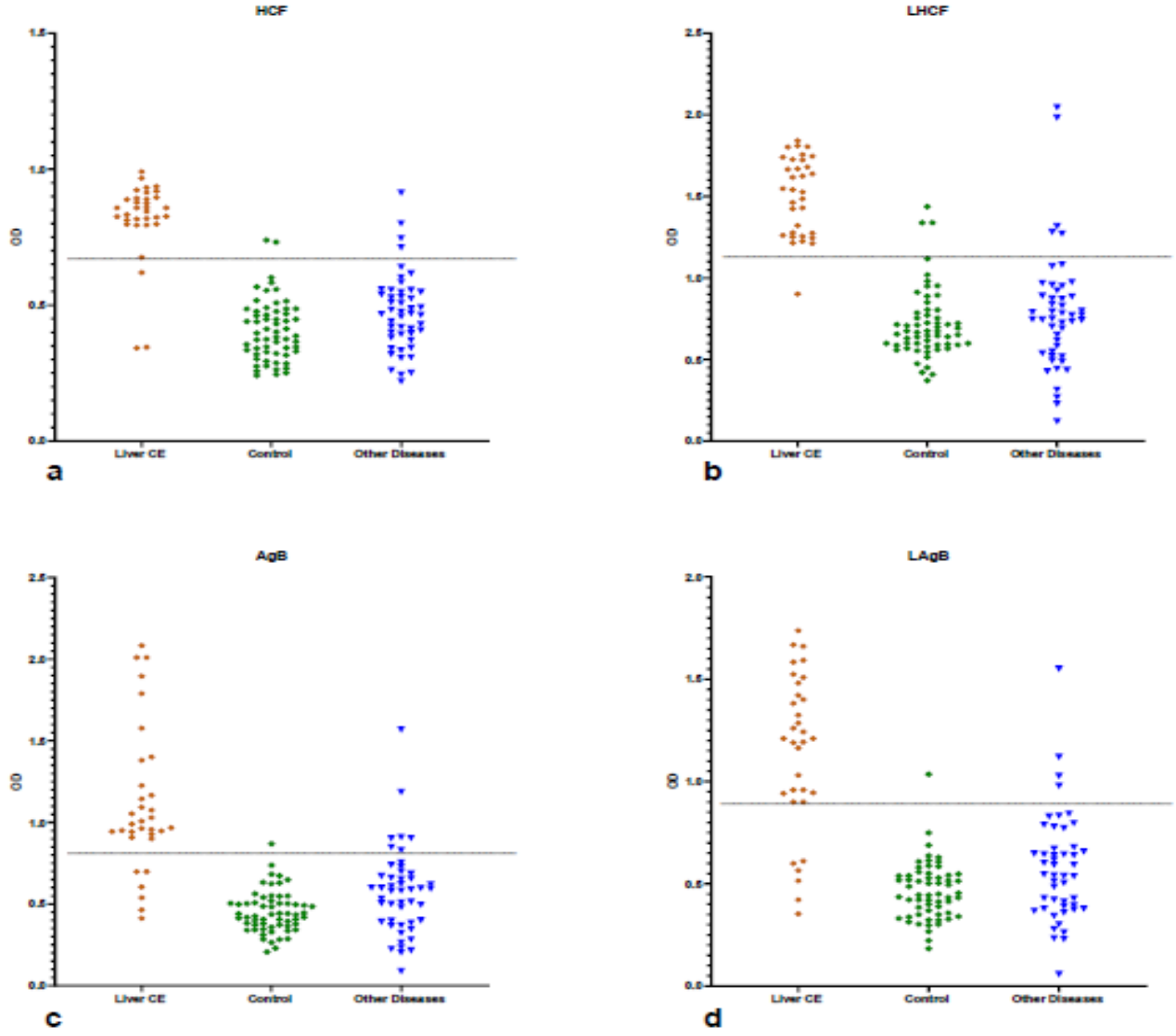

Fig. 1: Scatter plots presentation to compare four antigens a) native hydatid cyst fluid (HCF), b) lyophilized HCF, c) antigen B (AgB) and, d) lyophilized AgB (LAgB) originated from *E. granulosus* sensu stricto (G1-G3) genotype of sheep hydatid cyst for sero-diagnosis of human liver cystic echinococcosis (N: 33), control (N:60), other diseases (N: 48)

Anti-LHCF IgG ELISA showed the best diagnostic sensitivity of 96.97% with a 95% confidence interval (84.24% to 99.92%) using sera obtained from 33 confirmed CE patients. The best specificity (95.37%) with a 95% confidence interval (89.53% to 98.48%) was simultaneously related to LAgB with cross-reactions by nonrelative sera including taeniasis, fascioliasis, giardiasis, and toxocariasis (Tables 1-2, Figs.1a-d).

Diagnostic performance of four antigens was compared according to the type of cysts (Table 3). LHCF had the best performance for diagnosis of all type of cysts. So that, it was able to diagnose the most cases of active cysts.

Table 3: Diagnostic performance of four different antigens (hydatid cyst fluid [HCF], lyophilized hydatid cystfluid [LHCF], antigen B [AgB] and lyophilized antigen B [LAgB]) for sero-diagnosis of liver cystic echinococcosis (CE) according to the cyst stages

Variable			***Type of Antigens***			
			HCF	LHCF	AgB	LAgB
Cyst stage [No (%)]	Active	CE1	11/12(91.67)	12/12(100)	11/12(91.67)	11/12(91.67)
		CE2	7/8 (87.5)	7/8 (87.5)	5/8 (62.5)	5/8 (62.5)
	Transi-tional	CE3	5/6 (83.3)	6/6 (100)	5/6 (83.3)	5/6 (83.3)
	Inac-tive	CE4	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)
		CE5	2/2 (100)	2/2 (100)	1/2 (50)	1/2 (50)

Discussion

Different antigens including native, semi purified or recombinant have been used for CE diagnosis (16,19, 25-29). They have been used for both diagnostic and sero-epidemiological purposes (26, 30-34). Although, lyophilized antigens have been used in limited studies (21,22), but the validity of lyophilized HCF and AgB has not been evaluated and compared. Moreover, the use of antigens in the different stages of liver CE has not been performed, so far. In this regard, the present study was designed to compare the diagnostic efficacy of four hydatid antigens with different preparation methods; Crude and lyophilized HCF, native and lyophilized AgB in the detection of IgG antibodies using ELISA test, for diagnosis of liver CE. Moreover, these antigens were used for diagnosis of stage specific liver CE based on sonography outcomes.

Among different antigens crude, semi-purified and purified hydatid cyst fluid were evaluated in several investigations for diagnosing of CE. A 38-mer peptide (p176) of the AgB/1 subunit has been applied in an Elisa test with a higher sensitivity (80%) and specificity (94%) than native AgB, Ag5, or any other peptide antigen tested (15). In another study AgB has preferred to HCF in initial laboratory studies, with 94% sensitivity and 90.3% specificity for diagnosing of *Echinococcus* infections (20). Iranian AgB has higher sensitivity (96.4%) in compare with Chinese AgB, HCF and recombinant antigens that utilized (35). During a study, specific IgG ELISA AgB (antigen B-rich fraction) was the most sensitive test (96.5%) in compare to latex agglutination, immunoelectrophoresis (IEP), and specific IgE ELISA tests (36). In a preliminary comparative investigation with four commercially ELISA assays, native Ag5 showed higher sensitivity for detecting clinically positive CE patients (21). Verastegui et al. have utilized lyophilized HCF from different host and compared the accuracy of the antigens in ELISA, EITB (enzyme-linked immunoelectrotransfer blot) and DD5 (double-diffusion). According their results, the EITB assay offers greater sensitivity and specificity than do the ELISA and the DD5 test (22).

Our results showed that sensitivity of HCF (lyophilized /non-lyophilized) is high and is parallel to the results of Nasrieh and Abdel-Hafez (37) but in contrast to the earlier findings that showed purified antigens to be preferable to crude cyst fluid (38). Moreover, lyophilized antigens using IgG ELISA provided more accurate than non-lyophilized ones. As respect, the carriage and keeping of LHCF is simpler than other types of antigens, and according to our reliable results, lyophilization could be an effective method for providing antigens in different laboratories. Cross-reactivity of crude HCF antigen with human fascioliasis, which is endemic in certain areas of Iran and in parts of the world with endemic fasciolosis, is important. LAgB showed less cross-reactivity, particularly with fascioliasis and other prevalent helminthic

diseases in Iran. This could be an additional advantage of using LAgB over other *E. granulosus* antigens, especially where CE and fascioliasis coexist. The concentration and purity of antigen as well as the quality and quantity of host immune responses in various endemic regions and in different patient groups also may effect on the sensitivity and specificity of the tests (27). On the other hand, at different stages of the metacestode development a variation in the composition of AgB is observed which could not be ruled out (38). Fascioliasis, taeniasis, and toxocariasis are the most important helminthic diseases which have a cross-reactivity with sera of CE considered well (27,39).

The possible genotype variation of CE cysts, host immune response, CE cyst location in the body, the number and cyst stage, and role of immune complexes should be considered (39). In addition, false-negative results may be related to small size cysts, and CE1, CE4, and CE5 cyst stages (40).

An attempt has also been used for different serological tests based on liver cysts stages for diagnosis of CE by ultrasonography (7). The results were promising such that application of HCF (Crude or L lyophilized) and LAgB presented higher sensitivity and specificity for diagnosis of liver CE respectively. However, according present information, more samples are needed to evaluate the diagnostic performance of antigens based on cyst stage, which is the main limitation of such studies.

Conclusion

Using anti-LHCF IgG ELISA for initial screening of liver CE is recommended. However, as the LAgB has higher specificity, application of both antigens for better performance in the diagnosis of liver CE stages in an ELISA test is suggested.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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