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Original Article

Evaluation of the Ability of Antigen B Originated from *Echinococcus granulosus* Sensu Stricto and *E. canadensis* for the Diagnosis of Confirmed Human Cystic Echinococcosis Using ELISA

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Abstract

Background: Cystic echinococcosis (CE) is an important zoonotic parasitic disease worldwide. Application of species-specific antigen for serodiagnosis of human CE has not been utilized, so far. In this regard, AgB originated from *Echinococcus granulosus* sensu stricto (G1-G3) and *E. canadensis* (G6/G7) CE cysts, confirmed by molecular biology and sequencing was used for evaluation of their ability in the diagnosis of confirmed human CE.

Methods: The hydatid cyst fluid (HCF) of *E. granulosus* sensu stricto and *E. canadensis* species were separately, used for preparation of AgB during 2017-2018 in Shiraz and Tehran, Iran. A total of 45 sera samples from confirmed CE patients, 102 sera from healthy people as negative control and 44 sera from other parasitic diseases, were used for measurement of the diagnostic ability of antigen B originated from *E. granulosus* sensu stricto and *E. canadensis* species of CE, alone or in 50%:50% mixture using ELISA method.

Results: Overall, 38 (84.4%) out of 45 confirmed human CE were positive by ELISA using AgB originated from *E. granulosus* sensu stricto. This items for AgB originated from *E. canadensis* was 39 (86.6) out of 45 serum samples. A total of 39 out of 45 samples (86.6%) showed positivity by a mixture of antigen B originating from both species. The specificity of the above tests was calculated as 93.15%, 96.58%, and 93.84%, respectively.

Conclusion: Due to the diversity of the cyst species in human population, application of AgB from prevalent species alone or in combination with other species is suggested.

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Introduction

Cystic echinococcosis (CE) is an important zoonotic parasitic disease causes by the metacestode form of tapeworm *Echinococcus granulosus* sensu lato (1, 2). The disease is a major health problem all over the world more special in countries with a high number of canids infected with the adult worm of the parasite including the Middle East countries (3-8). CE is important in terms of medical, veterinary and economic loss (9, 10). Ruminants and humans are usually infected by ingestion of contaminated foods and water with the eggs of the worm. Due to the slow growth of cysts, the disease remains asymptomatic for several years and diagnosis is difficult in early-stage of the disease (11).

Generally, diagnosis of the disease is not possible based on clinical symptoms and conventional laboratory diagnosis. Furthermore, the efficiency of X-ray and ultrasound is not satisfactory for new cysts (11). Therefore, application of valid laboratory examination based on antibody detection is strictly required. Advanced imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) especially in the early stages of cyst formation are also required (11, 12). Application of other techniques such as proteomics to investigate biomarkers in patients as well as developed DNA-based methods that are able to distinguish *Echinococcus* species following clinical biopsy of suspected CE cases has been used (13-16).

Different antigens regardless their genotypic sources have previously, been applied for serodiagnosis of human CE during several years. Antigen B (AgB), a 160-kDa lipoprotein component of HCF is one of the most immunogenic and specific *E. granulosus* native antigens (17). The antigen was approved by the WHO. It has already been used for serodiagnosis of the disease (15, 16, 18-21).

As, the most prevalent genotype of CE is *E. granulosus* sensu stricto (s.s.) in different regions of the world (20, 21), it can be assumed that this genotype has been the source of the utilized native AgB in sero-epidemiological studies. On the other hand, it is considered that an antigen with the specific genotype is likely developed different immune responses in compare with the others. The most prevalent genotypes of human CE in Iran has been reported to be *E. granulosus* s.s. followed by *E. canadensis* (22-26). Application of antigen B originated from *E. granulosus* s.s. (*E. g* ss-AgB) or *E. canadensis* (*E.c*-AgB), alone could be questionable. Therefore, comparison of the ability of these two antigens individually or in mixture may be useful for diagnosis of human CE.

We aimed to compare the ability of AgB originated from *E. granulosus* sensu stricto (G1-G3) and AgB originated from *E. canadensis* (G6/G7) cyst genotypes for serodiagnosis of human CE using ELISA method.

Materials and Methods

Cyst and HCF preparation

Hydatid cyst fluid (HCF) were collected from the infected sheep and camel livers or lungs in abattoirs during 2017-2018 in Shiraz and Tehran, Iran. To remove the large particles, HCF was centrifuged at 3000 × g for 15 minutes at 4° C. Supernatant was utilized for AgB preparation.

Molecular Evaluation

The protoscoleces or germinal layer of each cyst was aseptically collected in cryotubes containing ethanol for determination of genotypes.

DNA extraction was performed using phenol-chloroform protocol (26). JB3 (5'-TTTTTTGGG-CATCCTGAGGTTTATTTTTTTGGG-

CATCCTGAGGTTTAT-3') and JB4.5 (5'-TAAAGAAAGAA CATAATGAAAATG-3') primers were utilized for Cytochrome c oxidase subunit 1 (*cox1*) gene amplification. Furthermore JB11 (5'-AGATTCGT AA GG GGCCTAATA-3') and JB12 (5'-ACCACTAACTAATTCACTTTC-3') were used for NADH dehydrogenase subunit 1 (*nad1*) gene amplification (27, 28).

PCR was carried out in the final volume of 50 µL for each genes, including, 3.5 mM MgCl2, 250 µM of dNTPs, 25 pmol. of each primer, 2.5 µl genomic DNA and 2 U of Taq polymerase under the following conditions: 40 cycles of 94 ºC for 45 s, 51 °C for 35 s, 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. Negative (no added DNA) and positive controls were included in each PCR cycle.

The amplification products were analyzed by electrophoresis in GelRed agarose gel prepared in TAE buffered medium (65 mM Tris-HCl, 22.5 mM boric acid, 1.25 mM EDTA, pH 8.3) and subsequently visualized using an UV trans-illuminator (UVitec, Cambridge, UK). The PCR amplicons for the two genes were purified using GEL/ PCR Purification Kit (Vivantis®, Malaysia) and subjected to sequencing in two directions, using the same PCR primer set (First BASE Laboratories Sdn Bhd-604944X, Malaysia). Genius and BioEdit softwares were used for sequence results to be edited and aligned (29, 30).

HCF belonged to *E. granulosus* s.s. and *E. canadensis* were assigned for AgB preparation.

Preparation of antigen B

AgB was prepared from HCF of related cyst genotypes by Oriol method (31). Briefly, 100 mL of HCF was dialyzed overnight against 5 mM of acetate buffer (pH 5) at 4 °C. Samples were centrifuged (50 000 g for 30 minutes), supernatant was removed and the pellet was dissolved in 0.2 M phosphate buffer (pH 8). Saturated ammonium sulfate was used to remove the globulins from the sample. Finally, the sample was boiled in a water bath for 15 min and centrifuged at 50 000 g for 60 min to separate heat-stable antigen B (supernatant) from other components. Concentration was determined by Bradford protein assay (32).

Serum sample collection

Overall 191 serum samples, were used in this study as follows: Group I: 45 surgically, pathologically and serologically confirmed CE samples including 22 liver, 17 lungs and 6 from other organs (Table 1). Group II: 102 sera from healthy people; Group III: 44 sera samples from patients of non-CE disease (Table 2).

Enzyme linked immunosorbent assay

ELISA was performed in flat-bottom 96-well microplates. The plate wells were coated with 5 µg/mL of AgB (100 µL/well) in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) and incubated at 4 °C overnight and the tests were carried out as described previously (20, 33). The absorbance was read at 492 nm after 30 min using automatic microplate reader.

The cut-off point was set as the mean OD492 plus 2SD for control samples (102 sera from healthy people).

All sera samples were evaluated by ELISA AgB originated from each of *E. granulosus* genotypes and mixed (50%:50%) of them (Table 1 and 2).

Ethical approval

The project was approved by National Institute for Medical Research Development, Ethical Committee**.**

Statistical analysis

The binomial test was applied for statistical analysis. Statistical characteristics were calculated using MedCalc's diagnostic test evaluation calculator (34) (Table 1).

Table 1: Statistical parameters of ELISA using (*E. g* ss- AgB), (*E.c*-AgB) and (50% *E. g* ss: 50% *E.c* AgBs) in diagnosis of human CE

Variable	***No. Cases***	***Seropositive cases with various Antigens***		
		*(*E. g ss*- AgB)	**(*E.c*-AgB)	***(*E. g ss*- and *E.c* AgBs)
Liver CE	22	20	20	20
Lungs CE	17	13	14	13
Other organs CE*	6	5	5	5
Total (CE confirmed samples)	45	38	39	39
Non-CE samples	44	10	5	9
Negative samples	102	0	0	0
Sensitivity (%)		84.44	86.67	86.67
Specificity (%)		93.15	96.58	93.84
Positive likelihood ratio		12.33	25.31	14.06
Negative likelihood ratio		0.17	0.14	0.14
Positive Predictive Value		79.17	88.64	81.25
Negative Predictive Value		95.10	95.92	95.80
Accuracy		91.10	94.24	92.15
Diagnostic the odds ratio		73.82	183.30	98.94
95%CI of odds ratio		26.33- 206.93	53.11-632.58	33.18-295.03
Significance level		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

**E. granulosus* sensu stricto antigen B (*E. g ss*-AgB)
** *E. canadensis* antigen B (*E.c*- AgB)
***Mixture of 50%:50% *E. g ss*-AgB and *E.c* -AgB

Results

Comparison of the sensitivity and specificity of ELISA using E. granulosus s.s. and E. canadensis antigens

Results of the ELISA, using two native antigens including *E. granulosus* sensu stricto antigen B (*E. g ss*-AgB), *E. canadensis* antigen B (*E.c*-AgB) and their 50%:50% mixture (*E. g* ss - *E.c*-AgBs), together with different statistical parameters are shown in Table 1.

The diagnostic odds ratio of the assay showed that, the best performance was related to (*E.c*-AgB). The sensivity of (*E. g ss*-AgB) was 84.44% and of (*E.c*-AgB) was 86.67%). However, the differences was not statistically significant ($P = 0.76$). Application of a mixture of 50%:50% of two antigens (*E. g ss- E.c*-AgBs) the sensitivity was observed as 86.67%.

The cross-reactivity with other diseases

The cross reactivity with other parasitic diseases was tested using 44 sera from other parasitic diseases (Table 2).

There was no cross reaction in 102 healthy people sera tested with *E. granulosus* sensu stricto antigen B (*E. g ss*-AgB), *E. canadensis* antigen B (*E.c*-AgB) and their 50%:50% mixture (*E. g* ss- *E.c* -AgBs) (Table 1).

Table 2: Results of ELISA using (*E. g ss*- AgB), (*E.c*-AgB) and (*E. g ss*- *E.c* AgBs) in patients suffering from other diseases in diagnosis of human CE

variable	***Seropositive cases with various Antigens***			
	No. Cases	AgB *E. granulosus* s.s.	AgB *E. canadensis*	AgB *E. granulosus* s.s. & *E. canadensis*
Ascariasis	4	2	0	1
Fasciolosis	2	1	0	1
Giardiasis	5	0	0	0
Hymenolepiasis	8	1	1	1
Leishmaniasis	1	0	0	0
Malaria	3	0	0	0
Strongyloidiasis	6	2	1	2
Toxocariasis	3	1	1	1
Trichstrongyliasis	8	1	1	1
Taeniasis	4	2	1	2
Total	44	10	5	9

Discussion

Diagnosis of CE is mainly based on radiological methods and confirmation by seroimmunological procedures. Imaging methods are sometimes limited due to small size of the cyst and the atypical images, which are not easy to be distinguished from neoplasms or abscesses. Thus, more advanced techniques are advisable (13,15, 35-36). Several different immune tests such as ELISA, latex agglutination test (LAT), indirect hemagglutination antibody test (IHAT) and immunofluorescence antibody test (IFAT), immunoelectrophoresis (IEP), counter current immunoelectrophoresis (CCIEP)) utilizing various crude and recombinant antigens with different accuracy have been used for many years. ELISA is among the most sensitive of the currently available immunodiagnostic techniques in detecting antibodies (14, 15, 35, 37-38).

The main antigenic source for the immunodiagnosis of human CE is HCF. For clinical practice, crude HCF has a high sensitivity, ranging typically from 75% to 95% (35). However, its specificity is often unsatisfactory and cross-reactivity with sera from patients suffering from other cestode, trematode and nematode infections, malignancies, liver cirrhosis and even in presence of anti-P1 antibodies is commonly reported (5, 12). Other antigens such as somatic and excretory products of protoscoleces or adult worm were also utilized during different studies (16, 20, 33).

Given the genetic diversity of *E. granulosus*, different immunological responses to the antigens with different genetic source are expected. Therefore, *E. g ss*- AgB, *E.c*-AgB and a mixture of 50% of *E. g ss* AgB together with 50% of *E.c* AgB were used for detection of anti-hydatid cyst antibodies. Our results showed a higher performance of *E.c*-AgB in compare with *E.g ss*-AgB. We believed that, an appropriate immunodiagnostic test depends on different aspects including the protocol of the tests and nature of the applied antigens (37-38). The serodiagnosis sensitivity and specificity of the immunological and advanced tests are usually varied based on antigen type, methodology, the geographical region, and cross-reaction (16, 39-40).

In a field trial or its application in endemic areas where more than one genotype of *E. granulosus* is responsible for CE, application of one type antigen could be doubtful. On the other hand, the serological responses to hyda-

tid antigens are considerably different, depends on the host species and the location of the parasitic cysts. So that antigens obtained from lung cysts has shown very low responses (41). Nevertheless, other locations such as the liver has shown good or acceptable serological responses (42-44). With respect to immunodiagnosis, it has been suggested that different "genotypes" of the parasite in genetically different sheep may give varying results (37, 45). However, they have not carried out any evaluation in this regard.

Application of antigen B for serodiagnosis of human CE has been evaluated many years ago (46). Its sensitivity has been reported between 60 to ≥96% (16, 20, 35, 42, 43). The nature and quality of antigen B in hydatid cyst fluid have been reported to be variable among the host species, which could be one of the reasons why different laboratories usually obtain different results when they use AgB prepared from different host species. On the other hand, the differences in the 3D structure of proteins between two species may interpret such difference between specificities of the tests (47).

Our evaluation showed that using native AgB, regardless of the genetic source could provide a high and appropriate immune response. Although the sensitivity was not so much different but specificity, consequently accuracy and diagnostic odds ratio was considerable different between two utilized antigens. The mentioned statistical parameters were also relatively desirable when mixture of two antigens were used.

Since, we did not find similar studies in which, application of species specific antigen was evaluated for serodiagnosis of human CE, more detailed comparison was not possible. As the viability of cysts utilized in preparation of antigen, the study and antigen preparation methods and serum set were similar in the current study; the differences of the result obtained from the two antigens can be due to the difference in the host and its genetic source origin.

Conclusion

Application of specific genotypes of AgB for diagnosis of human CE in endemic areas is superior. According to the cyst species diversity, application of AgB originated from different species/genotypes and mixture of them with ELISA method is suggested, especially in areas with parasite genetic diversity.

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Conflict of interest

The authors declare that they have no conflict of interest.

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