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## Comparison of the efficacy of in-house-produced AgB with a domestic commercial kit for the serodiagnosis of human cystic echinococcosis by the ELISA method

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### ABSTRACT

Cystic echinococcosis (CE) is a common zoonotic infection caused by the metacestode form of *Echinococcus granulosus*. The disease is found worldwide, and Iran is considered an endemic area. Depending on the affected organ, the disease presents with different clinical signs and symptoms. Humans are regarded as dead-end intermediate hosts. CE cysts commonly develop in the liver and lungs, while they are less frequently found in other organs. Timely diagnosis of this disease is essential in humans. Diagnosis relies on imaging methods (X-ray, ultrasound, MRI, and CT scan), complemented by serological testing. The present study used the indirect ELISA method to compare the efficacy of in-house-produced antigen B (AgB) and a domestic commercial kit. Hydatid cyst fluid (HCF) was extracted from the liver of sheep infected with a cystic echinococcosis cyst, followed by the preparation of AgB. It was used in the indirect ELISA test. A total of 142 sera, consisting of 36 samples from patients with pathologically confirmed CE, 46 samples from patients with other parasitic diseases, and 60 samples from healthy individuals, were examined using in-house-produced AgB and a domestic commercial ELISA kit. Our findings revealed the sensitivity and specificity of the indirect ELISA using in-house AgB to be 86.11% and 95.28%, respectively. The commercial kit demonstrated a sensitivity of 77.78% and a specificity of 99.06%. Although both the in-house-produced AgB and the commercial kit showed considerable and relatively similar diagnostic efficiency, the study results showed a higher sensitivity and validity of AgB compared with the commercial ELISA kit.

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## 1. Introduction

Cystic echinococcosis (CE) is a global zoonotic disease caused by the larval form of *Echinococcus granulosus sensu lato* (1). This parasite is mainly transmitted between dogs as the definitive hosts, while various livestock species (sheep, cattle, goats, and camels) serve as intermediate hosts. Humans serve as accidental intermediate hosts of this parasite. Infection in humans and intermediate hosts occurs through the accidental intake of *E. granulosus* eggs, usually present in the feces of infected canines, leading to CE infection (2). The disease is prevalent in various regions globally, particularly in the Mediterranean, Middle East, South America, Central Asia, Australia, Chile, Eastern Europe, New Zealand, and West Africa (3). Iran is regarded as an endemic area for *E. granulosus sensu lato*, encompassing different species and genotypes (4).

The disease causes substantial economic losses for both livestock farming and human health. In Iran, the average annual financial impact of CE is estimated to exceed \$232.3 million (5). The disability-adjusted life years (DALY) of CE in 2018 were estimated to be 1,210 years in Iran (700 years in men and 510 years in women) (6). Timely diagnosis of this disease is very important in humans. CE is asymptomatic in most cases (about 65%) and is occasionally discovered following an examination for the diagnosis of other diseases (7). Achieving a conclusive diagnosis requires the integration of multiple methods, and careful consideration of clinical symptoms, epidemiological data, and the patient's medical history is essential.

Imaging methods such as CT scans, ultrasound, MRI, and X-ray radiography, along with immunological tests like ELISA, Western blot, indirect hemagglutination, indirect immunofluorescence, latex agglutination tests, and counter-current immunoelectrophoresis (CCIEP), constitute the primary methods used for diagnosing CE (4,8). The World Health Organization endorses the use of the international ultrasonography classification for staging CE through stage-specific imaging (9).

During recent years, different native and recombinant antigens with various sensitivity and specificity have been utilized for setting up immunological tests in definitive or intermediate hosts (10-13). Hydatid cyst fluid (HCF) is the most common antigen used in this field. The sensitivity of this antigen can vary between 50 and 98%, depending on factors such as the specific serological

method used, the quality and purity of the antigen preparation, the genotype of the parasite, the stage and location of the cyst, the number and size of cysts, the patient's immunoglobulins (isotypes), and the parasite genotype (10,11,13).

Antigen B (AgB) that found in cyst fluid is a multimeric protein consisting of subunits with varying molecular weights (8, 16, 24, and 32 kDa). This protein exhibits high immunogenic properties, making it valuable for the diagnosis of human CE through ELISA tests. The predominant antibody response to the main reactive subunits, EgAgB1, EgAgB2, and EgAgB4, was found previously to be of the IgG4 subclass (14). The present study aimed to produce an in-house antigen B (AgB) and compare its diagnostic efficacy with that of a domestic commercial kit (Pishtaz Teb Company, Iran), using ELISA method.

## 2. Materials and Methods

A total of 142 serum samples were classified into three groups: the first group included 36 sera from patients with CE confirmed by pathology (as positive controls), the second group included 60 sera from healthy people (as negative controls), and the third group consisted of 46 sera from individuals with other parasitic diseases including

giardiasis (n=6), toxoplasmosis (n=2), malaria (n=5), leishmaniasis (n=1), taeniasis (n=3), fascioliasis (n=6), toxocarasis (n=5), hymenolepiasis (n=6), ascariasis (n=5), trichostrongyliasis (n=1), strongyloidiasis (n=1), enterobiasis (n=1), blastocystosis (n=3), and amoebiasis (n=1).

### 2.1. Preparation of HCF

HCF was collected from fertile cystic echinococcosis cysts in sheep in the parasitology laboratory of Baqiyatallah University of Medical Sciences. To remove the protoscoleces and large particles, HCF was subjected to centrifugation at 3000×g for 15 minutes at 4 °C and kept at -20 °C until use.

### 2.2. Preparation of AgB

The purification of AgB from HCF was carried out based on the established protocol by Oriol (15). In this regard, 100 mL of HCF underwent overnight dialysis at 4°C using 5 mM acetate buffer (pH 5). The dialyzed content was centrifuged at 50,000×g for 30 min. The supernatant was discarded, and the resulting pellet was reconstituted in 0.2 M phosphate buffer (pH 8). To eliminate globulins, saturated ammonium sulfate precipitation was employed. Subsequent heat treatment

involved boiling the sample for 15 min, followed by another centrifugation step (50,000×g, 60 min) to isolate the thermostable AgB fraction in the supernatant. Protein quantification was performed via the Bradford assay, in which BSA served as the reference standard (16).

### 2.3. Evaluation of sera by ELISA

Microplate-based immunoassays were performed using 96-well flat-bottom plates. Each well was coated with 100  $\mu$ L of AgB (5  $\mu$ g/mL in 0.05 M carbonate-bicarbonate coating buffer, pH 9.6), and the plate was incubated overnight at 4°C. Unbound antigens were eliminated through successive washes with PBS-T (0.05% Tween 20 in phosphate-buffered saline, pH 7.4). Non-specific binding sites were blocked by 100  $\mu$ L of 3% skimmed milk (Merck, Germany) in PBST, incubated for 2 hours at room temperature. Following three washes with PBST, the plates were incubated with 100  $\mu$ L of serum samples (diluted 1:100 in PBST) for 90 minutes at room temperature. After five washes, 100  $\mu$ L of HRP-conjugated secondary antibody (Sigma, USA; 1:4000 dilution in PBST) was added, and the plate was incubated for 1 hour at room temperature.

Following a final wash cycle as above, 100  $\mu$ L of OPD substrate solution (0.025% o-phenylenediamine dihydrochloride with 0.1% H<sub>2</sub>O<sub>2</sub> in 0.1 M citrate buffer, pH 5.0; Sigma, USA) was added to each well, and the plate was incubated in the dark for 30 minutes. The enzymatic reaction was stopped by adding 1 M sulfuric acid. Absorbance was measured at 492 nm using a BIOTEK ELx800 microplate reader. The diagnostic cutoff was established as the mean optical density of negative controls plus two standard deviations.

### 2.4. Evaluation of sera using commercial ELISA Kit

The IgG antibody produced against *Echinococcus* antigens in human sera was measured using the *Echinococcus* IgG ELISA kit (Pishtaz Teb Co., Iran). In each flat-bottom 96-well microplate, four wells were designated as negative control (two wells), positive control, and the blank. Other wells received 100  $\mu$ L of diluted serum samples (1:100 in PBS), and the plate was incubated at room temperature for 30 minutes. After five washes, horseradish peroxidase-conjugated secondary antibody (100  $\mu$ L/well) was added to the wells (excluding blanks), and the plate was incubated at room temperature for 30 minutes. After repeating the washing procedure as above 100  $\mu$ L of chromogenic substrate (TMB or equivalent) was added to each well and developed in the dark for 15 minutes at room temperature.

The reaction was stopped with 100  $\mu$ L of 2 M sulfuric acid, and absorbance was immediately measured at 496 nm using a BIOTEK ELx800 microplate reader. The diagnostic cut-off was calculated by adding 0.25 to the average OD of negative controls in each microplate. Samples with an OD 10% higher than the cut-off were considered positive.

### 2.5. Data analysis

The validation parameters of AgB and the commercial ELISA Kit were determined using MEDCALC software (17).

## 3. Results

The AgB-based indirect ELISA demonstrated high diagnostic performance, with 86.11% sensitivity and 95.28% specificity for detecting human anti-CE antibodies. This antigen showed false-positive results in two patients with fascioliasis and three patients with taeniasis, ascariasis, and toxocarasis infections.

Evaluation revealed high diagnostic performance for AgB, showing 92.96% efficiency and 90.6% validity in CE antibody detection. The commercial kit presented a lower number of false-positive results in other parasitic sera (one serum corresponding to taeniasis) than AgB (five sera). The diagnostic efficiency and validity of AgB were determined to be 92.96% and 90.69%, respectively. For the commercial ELISA kit, these values were reported as 93.66% and 88.42%, respectively. The diagnostic performance of our AgB-based ELISA was systematically compared with a commercial CE detection kit through validation parameters (Table 1).

## 4. Discussion

CE represents a clinically and economically significant zoonosis affecting both human populations and livestock, particularly in endemic regions of the Middle East, including Iran (2,3). This disease is of special importance from medical, veterinary, and economic points of view. CE causes significant economic impacts on both livestock husbandry and human populations due to the direct and indirect costs incurred from medical treatments and losses related to livestock (5).

Imaging methods and serological testing are used together for the diagnosis of human diseases, providing a more comprehensive assessment of the patient's condition.

**Table 1.** Comparative analysis of the diagnostic performance of two ELISA tests.

Statistical index	No. of samples	No. of seropositive samples	
		AgB	Commercial kit
CE samples	36	31	28
Samples from healthy people	60	0	0
Samples from patients with other parasitic diseases	46	5	1
Sensitivity		86.11	77.78
Specificity		95.28	99.06
Positive predictive value		86.11	96.55
Negative predictive value		95.28	92.92
Diagnostic efficiency		92.96	93.66
Validity		90.69	88.42

Immunological diagnosis, as well as new methods, can be applied not only for initial diagnosis but also for follow-up of patients after treatment (18). Among these, ELISA is widely used by researchers as a standard method due to its remarkable sensitivity and specificity in the diagnosis of human CE (19). One of the main pitfalls of serological tests is cross-reactivity with other parasitic diseases. The cross-reactivity of antigens applied for the serodiagnosis of CE with parasites of the Taeniidae family (*Echinococcus multilocularis*, *Taenia solium*, etc.) is a drawback for the diagnosis of CE, as they have a close antigenic affinity with *E. granulosus*, leading to a reduction in the specificity of serological tests (20). HCF is the common component for the diagnosis of CE in humans as a crude antigen. AgB and Ag5 are the main antigens present in HCF. AgB, a 120–160 kDa oligomeric thermostable lipoprotein composed of three subunits at 8/12, 16, and 20 kDa, is widely used for the serodiagnosis of human CE (11,21).

Although recombinant proteins have received attention for the serodiagnosis of human CE, the use of recombinant antigens presents challenges such as antigen storage, advanced laboratory equipment requirements, and standardization, etc. (10). Simplicity, easy access, acceptable accuracy, and cost-effectiveness are crucial factors to consider when choosing a diagnostic method. In this study, in-house AgB was compared with the commercially available ELISA kit (Pishtaz Teb Co., Iran). One hundred forty-two serum samples (36 pathologically confirmed sera from patients with CE, 46 sera from individuals with other parasitic diseases, and 60 sera from healthy individuals) were evaluated by indirect ELISA, where a sensitivity of 86.11% and a specificity of 95.28% were obtained for the diagnosis of anti-CE antibodies in humans.

The commercial kit showed a sensitivity of 77.78% and a specificity of 99.06% in detecting anti-CE antibodies in humans. Also, AgB and the commercial kit showed diagnostic efficiencies of 92.96% and 93.66%, respectively. AgB and commercial kit showed the validity of 90.69% and 88.42%, respectively, for the detection of anti-CE antibodies in humans.

Lorenzo et al. (2005) examined a panel of 129 sera, consisting of 59 sera from patients with CE, 55 sera from patients with other diseases, and 15 sera from healthy individuals using the AgB-ELISA test. The sensitivity and specificity in this study were determined to be 80% and 77%, respectively. Sera from individuals with neurocysticercosis and alveolar echinococcosis were reported as cases of cross-reactivity (22). The antigenic diversity of hydatid cyst fluid, differences in parasite genotype, the duration of the disease, and the time after treatment are among the factors involved in negative or false-positive results in serology tests (23).

Another study conducted by Mohammadzadeh et al. (2012) examined three types of antigens including AgB (AgB taken from infected sheep in Iran and China), a recombinant AgB, and HCF, using the ELISA method for serodiagnosis of CE. The sensitivities of Iranian and Chinese AgB, recombinant antigen, and cyst fluid were 96.4%, 82.1%, 94.6%, and 91.1%, respectively, and their specificities were 84.8%, 94.9%, and 93.9% and 76.8%, respectively (11).

Reiterova et al. (2014) compared AgB-ELISA and FH-ELISA produced from sheep hydatid cysts. For this purpose, 177 sera, including 50 negative control samples, 55 positive control samples, and 72 samples from other diseases, were used. The sensitivity and specificity of the AgB-ELISA were reported as 96.4% and 97.2%, respectively, where the sensitivity of FH-ELISA was

higher than that of AgB-ELISA, while the specificity of AgB-ELISA was higher than that of FH-ELISA (24). Evaluation of four distinct antigen preparations derived from *Echinococcus granulosus sensu stricto*—including native HCF, lyophilized HCF, AgB, and lyophilized AgB—for serological diagnosis of active, transitional, and inactive hepatic CE demonstrated that the anti-LHCF IgG ELISA achieved the highest sensitivity (96.97%), while the LAgB-based ELISA showed optimal specificity (95.37%). These findings indicate that a combined diagnostic approach using both anti-LHCF IgG ELISA and anti-LAgB IgG ELISA would provide the most accurate serodiagnosis of human liver CE (25).

Despite the use of the same antigen in some studies, the efficacy of the test is not completely the same. The number and type of sera used, especially sera from individuals with other diseases, are additional reasons for these differences. In many studies, the stage or condition of the cyst in patients is not taken into account during evaluations. Also, collecting sera over a long period can affect the quality of serum antibodies. This study showed the very favorable sensitivity, specificity, and validity of AgB in the serodiagnosis of human CE. The sensitivity of AgB in the present research was higher than that of commercial kit (86.11% vs. 77.78%), and its specificity was found to be lower than that of the aforementioned kit (95.28% vs. 99.06%). Overall, the validity of in-house AgB in the present research was higher than that of the commercial kit (90.69% vs. 88.42%), and the diagnostic efficiency of both antigens showed a slight difference of less than 1%.

Sera from people suffering from other parasitic diseases that responded positively with AgB (cross-reaction) included two cases of fascioliasis, and one case each of taeniasis, ascariasis, and toxocariasis. There is a possibility of simultaneous infection with *Fasciola* spp., *Taenia* spp., and *Ascaris lumbricoides* parasites due to the transmission cycles of these parasites through eating contaminated vegetables.

The results of various studies show a wide range of sensitivity and specificity, and these differences in each study can be affected by factors such as the type and structure of the antigen used, the number of sera evaluated, the sampling method and their maintenance, cyst stage, type of diagnostic techniques, base test for comparison, and determination of validation parameters, etc. In conclusion, although both in-house-produced AgB

and the commercial kit showed considerable and relatively similar diagnostic efficiency, the study results demonstrated higher sensitivity and validity for AgB compared with the commercial ELISA kit.

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### Authors' Contribution

Study concept and design: T. M.

Acquisition of data: S.A, F.H, PS, E.D

Analysis and interpretation of data: T.M, F.H

Drafting of the manuscript: E.D, S.H

Critical revision of the manuscript for important intellectual content: S.M.S

Statistical analysis: T.M, F. H

Administrative, technical, and material support: T.M

### Ethics

Ethical code: IR. BMSU.1398.224.

### Conflict of Interest

The authors declare that there is no conflict of interest.

### Data Availability

All data generated are included in the current article.

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