

The Diagnosis of Sheep Cystic Echinococcosis by Native Antigen B - ELISA Method

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Abstract

Cystic echinococcosis (CE) is one of the most prevalent zoonotic diseases in some countries in the world. Cystic echinococcosis is considered a neglected disease. This disease increases economic damage via medical costs and loss of human and livestock productivity. The aim of the current study gains a better understanding of the prevalence of CE in sheep. Totally 250 sheep sera were collected. 25 serum samples from newborn lambs were used as negative, and 25 others were obtained from slaughterhouses as positive to infection with CE and 200 unknown sera. Antigen B Isolated from hydatid cysts fluid was used for designing ELISA methods. The first Method used anti-Sheep conjugate (SIGMA, at 1:3000 dilution).

According to the results, the seroprevalence of CE in East Azerbaijan of Iran was 5.5% and sensitivity and specificity for the diagnosis of hydatidosis in sheep by AgB-ELISA methods was 92%.

Using Antigen B in ELISA design for hydatidosis diagnosis has attracted researchers in recent years. During this study, an Iranian native B antigen was used to design the specific detection of hydatidosis in sheep using a specific ELISA technique. The results have shown that using Antigen B in ELISA design is so valuable.

Introduction

Cystic echinococcosis (CE) or Hydatidosis is a zoonotic parasitic infection of humans and domestic animals caused by larvae of the cestode *Echinococcus granulosus* (Dabaghzadeh et al. 2018). Hydatidosis is common in sheep farming regions like Australia, New Zealand, China, South America, India, some African countries, the Middle East, and Iran (Fomda et al. 2015). This disease has been reported to be endemic in various parts of Iran (Ghabouli-Mehrabani et al. 2014).

In humans, the diagnosis of hydatidosis is mainly based on a positive serological test along with an imaging finding as recommended by WHO (Eckert et al. 2002). Among different serological methods, ELISA has been reported to be a useful test for the diagnosis of human hydatidosis. Antigen B (EgAgB) is the main protein generated by the pathogenic larval stage of cystic echinococcosis, and this Ag is highly immunogenic and can be recognized in most sera of intermediate hosts (Zhang et al. 2010). WHO recommends using specific serological methods with the specific Ag specially AgB (Sajjadi et al. 2007).

In animals, CE seriously affected the production and growth of livestock, causing a loss in livestock production and bringing enormous danger to public health (Tianli et al. 2019). Slaughterhouse studies are known as useful references for evaluating the epidemiological aspects of some diseases, especially parasitic diseases (Azami et al. 2013).

Considering the presence of informal livestock slaughterhouses and unsanitary slaughter of livestock in some parts of Iran, and also the lack of systematic recording of parasitic disease information in slaughtered animals, Therefore, there is no accurate information on the prevalence of infection in

slaughter livestock in different parts of Iran. Serological investigations seem to be necessary in some cases. This study aims to develop and evaluate a Native Antigen B for serodiagnosis of CE in sheep.

Methods

Study Area

Tabriz is the [fifth-most-populous city](#) in northwestern [Iran](#) with latitude 38°04'47" N and longitude 46°17'30" E. This city is surrounded by mountains in the north, south, and east, with cold winters and temperate summers. Tabriz's elevation ranges between 1,350 and 1,600 meters above sea level (Fig 1).

Serum Samples

A total of 250 sheep sera were investigated in this study. 25 serum samples from newborn lambs were used as negative, and 25 others were obtained from slaughterhouses as positive to infection with CE and were entered the study. At last, 200 unknown sera were collected from the sheep herds. All sera were collected from Tabriz city, East Azerbaijan province of Iran. Sera were kept at -80°C until the experimental assay.

Extraction of antigen B:

To extract antigen B from the hydatid cyst fluid, the modified method was implemented [9]. Hydatid cysts fluid was extracted from livers of sheep slaughtered at the abattoir of Tabriz, then it was transferred to a dialysis bag and was placed in a container containing polyethylene glycol (PEG) 4000 for 1 hour. This stage leads to the condensation of AgB in the hydatid cyst fluid. After that, the fluid was filtered using a 0.2 microfilter, and the resulting liquid was centrifuged at 1500g for 30 minutes. The extracted fluid was dialyzed for one night, and after that, the contents of the dialysis container were centrifuged with a refrigerated ultracentrifuge with 30000g in a vacuum condition and at a temperature of 4° C for 30 minutes. The resulting precipitate was dissolved in 10 ml of 0.2M phosphate buffer with pH 8 and the solution was saturated with 40% ammonium sulfate then the solution was centrifuged for 30 minutes at 3000g. The supernatant was placed in a boil-water bath for 15 minutes. In the next step, it was centrifuged for one hour using the ultracentrifuge, and finally, the supernatant-soluble antigen B was collected. After filtration with Millipore (0.2 microns) and the addition of 2% sodium azide (NaN₃), it was stored at a temperature of -70 ° C until next use (Shirazi et al. 2013).

Finally, the Bradford protein analyzes method was used to measure the protein content of the prepared solutions. Also, the solution containing the prepared antigen B was evaluated using SDS-PAGE.

Designing the ELISA method with Antigen B:

For design, the suitable level of serum dilution and the acceptable concentration of antigen B that should be coated was determined in the first step. Thus, different levels of antigens and serum concentrations were tested.

To prepare serum dilutions, a potent positive serum and a negative serum were used. Then, dilutions of 1:100, 1:200, and 1:400 were prepared.

The used wells were polyester (Nunc, Denmark), and to achieve antigen binding, 100 µl of each antigenic concentration was added to each well and stored in the refrigerator for one night to complete the binding process of antigen to the wells.

In the next step, Skim Milk 5% was used to block the wells containing 100 µl of antigen solution, they were drained and washed three times using PBST and then, 250 µl of blocking buffer was added to the wells and they were placed in a humid condition for 75 minutes in a 37 ° C incubator.

Wells were emptied and then, 100 µl of sera were transferred into the wells. They were incubated in a humid environment for 75 minutes at 37 ° C. Wells were emptied and 100 µl of the anti-Sheep conjugate (manufactured by SIGMA USA at 1: 3000 dilution) was prepared in wells for 75 minutes in a humid environment at 37 ° C. In the next stage, 100 µl of BM Blue POD (Roche Company, Germany) was transferred to the wells and placed in a dark environment for 12 minutes. The stop solution includes sulfuric acid 1M, of which 50 µl is transferred into each well, and then, the wells should be read immediately. To measure the optical absorbance of each well, an ELISA reader with a 450 nm filter was used and the absorbance of all wells was read.

Due to the high sensitivity of the extracted antigen (AgB) in this study, a concentration of 0.5 µg / ml and a serum dilution of 1:400 were used. After determining the concentration of AgB and dilution of serum, in the last step, unknown sera were tested with the same concentration and dilution.

Table 1: Sensitivity and Specificity of AgB-ELISA in sheep sera

Case	Positive	Negative	Serum
Infected	23	2	50
Non infected	2	23	50
Total	25	25	100
Sensitivity	92%		
Specificity	92%		

Results

AgB was measured by Bradford assay and its concentration was 0.7 (mg/ml) and SDS-PAGE result shows that bands on 20 and 24 KDa subunits of AgB.

At first, 0.5 µg /ml AgB concentration and also 1:3000 the sheep's conjugate were used. To find the best serum dilution, 2 sera of sheep with hydatidosis and 2 sera without infection (newborn lamb) were used.

According to the results, the best dilution of serum was determined at a dilution of 1:400.

In the next step, for finding the cut-off, definitive positive and negative sheep sera were examined by the ELISA method. According to the results, it was equal to one. In other words, the sera with up to one, positive and below one, optical absorption was considered negative. Also according to the results of this study, the sensitivity and specificity of the ELISA designed with AgB for detecting hydatidosis in sheep determined 92% (Table 1). In the last step, unknown serum samples were evaluated and out of 200 serum samples, 11 (5.5%) were detected as positive.

Discussion, And Conclusion And Recommendations

Hydatidosis caused by *Echinococcus granulosus* is one of the most important zoonotic diseases which has a pattern of distribution for CE has including western China, Central Asia, South America such as Peru, Chile, Argentina, Uruguay, southern Brazil, Mediterranean countries such as Iran and eastern Africa (Shirazi et al. 2013; Wen et al. 2019; Agudelo Higuaita et al. 2016). World Health Assembly (WHA) noted CE as one of the neglected zoonosis diseases and recent reports indicate that there is a high rate of infection in humans in different parts of Iran and its health problem (Fasihi-Harandi et al. 2012; Mablesen et al. 2014). The prevalence of CE in sheep is very important because the highest prevalence of CE in humans is found in populations that raise sheep (Merino et al. 2017).

Hydatidosis causes severe economic damage in livestock due to the infestation of important organs such as the lung and liver and these diseases can decrease productivity, lowering the quality of meat, production of fiber and milk (Merino et al. 2017). Therefore, find reliable data for monitoring epidemiologic aspects of disease very valuable. Abattoir surveys are a simple way of gathering data on livestock parasitic disease but it does not a useful method for farmers and owners of industrial sheep herds because, during the inspection of carcasses in the slaughterhouse, many organs (whole or partial) and in some cases the whole carcass are removed (Azami et al. 2013).

In the present study, the prevalence of CE in East Azerbaijan of Iran was relatively the same reports from neighboring countries in the Middle East such as Iraq: 7.3% (Abdulhameed et al. 2018) Syria: 2.8% (Seimenis 2003) Saudi Arabia: 2.33% (Adbel-Baki et al. 2018) but differs from those reported in other studies in Turkey: 30.6, Spain: 0.1 %, France: 0.004%, Italy: 21%, Romania: 11-13%, Greece:29%, Kenya: 10.8% and Southern Africa (Miambo et al. 2020; Tamarozzi et al. 2020).

Previous studies in other regions of Iran have revealed different levels of prevalence of hydatidosis infection than the 5.5% found in this study: 74.4% in the northwest (Daryani et al. 2007), 20.6% in the northeast (Rokni 2009), 16.4-9.3% in the center of Iran (Azami et al. 2013; Fakhar & Sajjadi 2007; Fallah et al. 2002), 9.2% in the east (Sharifi 1996) and 11.1% in the west (Rokni 2009) of Iran possibly indicating different factors affecting the transmission of this disease among sheep in these regions.

The different CE prevalence in Iran might be due to the climate and cultural differences among different socio-geographic distinctions.

This difference in the prevalence rate can be due to the geographic location, climate, cultural differences among different socio-geographic distinction, method of sampling (Slaughtered sheep or Random sampling), the season in which the studies were conducted, and another important issue is the low proportion of the sheep population be slaughtered as well as the method of sheep breeding and the relationship of sheep with infected dogs (Fasihi-Haramdi et al. 2012; Jawad et al. 2018).

There are a clear need and demand for research into the development of diagnostics programs. World Organization for Animal Health recommendations includes sequential testing based on a screening test model such as enzyme-linked immunosorbent assays (ELISA) (Agudelo Higueta et al. 2016). But in Iran, there is no specialized commercial ELISA kit for diagnosing CE in sheep. Using the ELISA method can be very efficient because of the precision and rapid results it provides (Asghari et al. 2013). The antigen used for testing is so important and is likely a significant source of variability in test performance (Agudelo Higueta et al. 2016). Diagnosis of human hydatid cyst by a serological method usually use hydatid cyst fluid of sheep (as the intermediate host) and Compared among the all hydatid cyst antigens showed that the AgB has higher antigenicity, sensitivity, and specificity (Jiao et al. 2014).

AgB is the major antigen in hydatid cyst fluid and has been implicated in immunomodulation processes, as well as in lipid uptake and transport. AgB has been studied for a long time and it is known to generate a strong humoral response (da Silva et al. 2018). Rahimi et al. (2011) show that AgB isolated from the liver had a better performance in the diagnosis of CE patients in comparison with AgB from other sources.

In Iran, no valuable study has been found using native antigen B to design an ELISA method to diagnose seroepidemiology of hydatidosis in sheep. In the current study, an Iranian native B antigen was used to design the detection of CE in sheep using an ELISA technique and the sensitivity and specificity of this ELISA method for detecting hydatidosis in sheep was 92%.

In previous studies on human by native AgB-ELISA method, the average of sensitivity and specificity was 92.9% and 91.2% respectively (Sajjadi et al. 2007; Rahimi et al. 2011; Mohammadzadeh et al. 2012; Savardashtaki et al. 2017).

Awareness of veterinarians and medical researchers of the public health importance of echinococcosis, especially in areas where it is endemic such as Iran is crucial. In most countries, one single genotype complex within a relatively large spectrum of intermediate host species indicates the presence of a dominant transmission dog–sheep cycle involving additional host species which may act as a disease reservoir for human infections (Chaligiannis et al. 2015). Knowledge of the prevalence of echinococcosis in sheep and dogs is essential for diagnosis and a prerequisite for multidisciplinary decisions on treatment strategy of public health (Wen et al. 2019; Youssefi et al. 2018). Hence, the rancher of industrial herds on the farm must be aware of the infestation status of herds to important parasitic diseases such as hydatidosis by conducting screening tests so that he can take preventive measures if needed. Seroepidemiological methods such as the AgB-ELISA technique seem to be suitable in these cases.

Declarations

Acknowledgment:

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Ethical approval:

Authors were not involved with animal handling and the sera samples were obtained directly through the veterinary laboratories.

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Figures

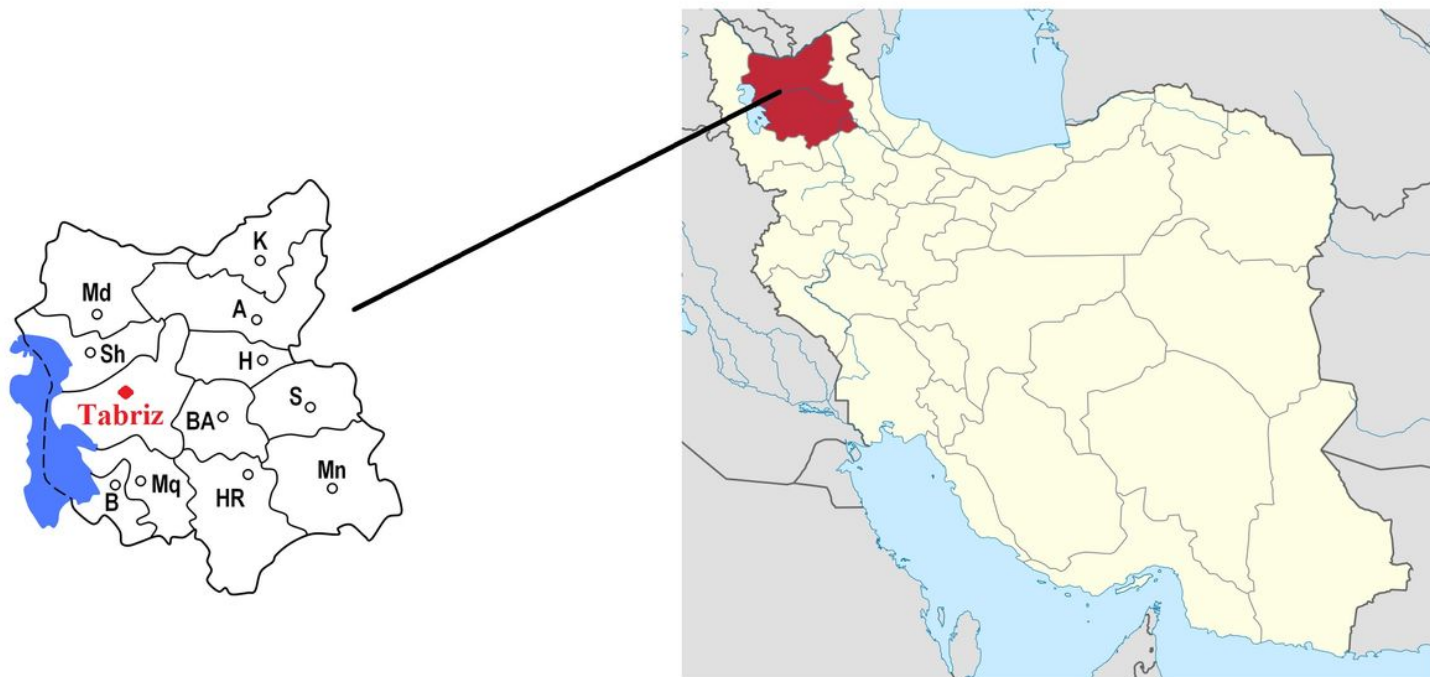


Figure 1

Map of Iran and Tabriz city as the studies area. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.