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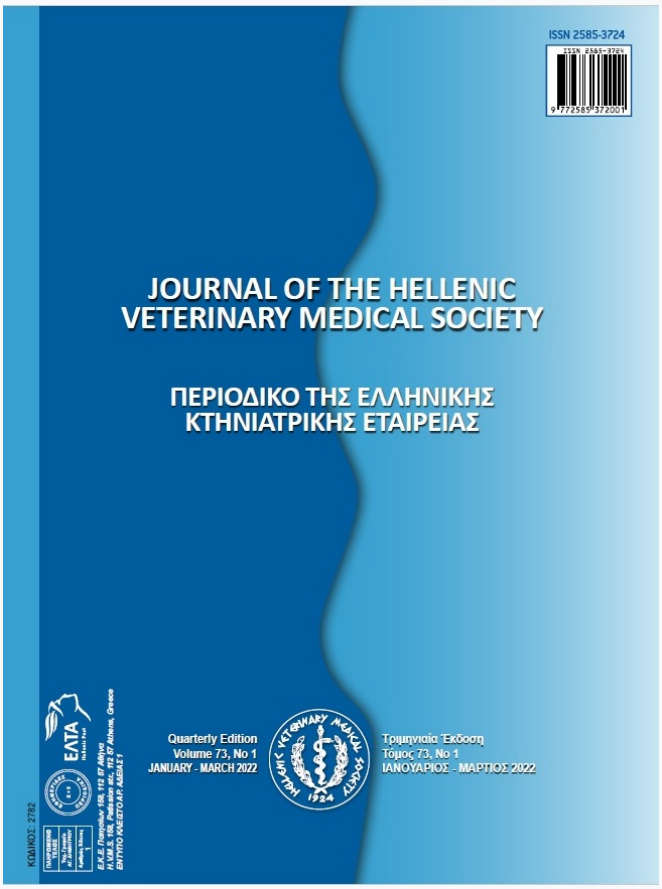

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Research article
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The comparison between AgB-ELISA and a new method of Nano-ELISA for diagnosis of hydatidosis in sheep

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ABSTRACT: Hydatidosis is one of the most important zoonotic diseases, and it is transmitted via dogs to intermediate hosts such as humans and domestic animals including sheep and cattle. Epidemiological studies and genetic investigations indicate that the sheep strain is the most common species causing hydatid cysts in humans. The prevalence and incidence of this disease are increasing. According to surveys, economic losses due to this parasite in intermediate hosts are significant. In this survey, 25 serum samples were obtained from newborn lambs as negative control and obtained 25 serum samples from infected sheep to hydatidosis as the positive control. Antigen B isolated from hydatid cysts fluid was used for designing ELISA methods. 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 first method used the anti-Sheep conjugate (SIGMA, at 1:3000 dilution), and the second method used gold nanoparticles in combination with anti-sheep conjugate. According to the results, sensitivity and specificity in sheep of the AgB-ELISA method were both 92% and for the Nano-ELISA with Gold Nanoparticles 100% and 96%, respectively. Moreover, results indicated that using Antigen B in ELISA design is valuable but specificity and sensitivity will increase significantly, especially in lower titer, when gold nanoparticles with anti-sheep conjugate are used.

***Keywords*:** Nano-ELISA, Antigen B, hydatid cysts.

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INTRODUCTION

Hydatidosis (infection with the larval stage of the *Echinococcus granulosus* parasite) is transmitted via dogs to intermediate hosts such as humans and other domestic animals including sheep and cattle. The parasite develops to hydatid cyst in the body of the intermediate hosts (Bhutani and Kajal, 2018). According to the latest reports of WHO, *Echinococcus granulosus* is endemic in South America, Eastern Europe, Russia, the Middle East and China with a relatively high incidence rate (Brunetti et al., 2010).

Genetically, distinct species of *Echinococcus granulosus* parasites are identified in the globe, of which, two strains are specified for sheep (G1 and G2), two strains for cattle (G3 and G5), a horse species (AG4), a camel strain (G6), a pig strain (G7), two cervid strains (G8 and G10), and G9 strain which was reported in a swine in Poland. However, it should be noted that some of these species require further investigation to determine more details on the hosts, geographic region, and genetic characteristics (Grosso et al., 2012).

Epidemiological studies and genetic investigations indicate that the sheep strain is the most common species causing hydatid cysts in humans. Moreover, G1 strain is common in sheep, goats, cattle, and camels of Iran (Sharafi et al., 2014).

In Iran, the prevalence of hydatidosis in Human is 5% (Mahmoudi et al., 2019) and in domestic animals in different parts of Iran is reported to be between 3.1 to 16.4 percent (Azami et al., 2013). In Ilam province of Iran, the rate of infection was reported to be 23% in sheep. In addition, the infection rate is reported to be between 0.8% and 57.7% in Iran buffaloes (Paykari et al., 2007). Recent reports indicate that there is a severe rate of infection in humans in different parts of Iran and its health complications (Fasihi-Harandi et al., 2012). Infection in the intermediate host depends on several factors including the average number and activity of the parasite eggs (Jawad et al., 2018).

Slaughterhouse studies are known as useful references for evaluating the epidemiological aspects of some diseases, especially parasitic diseases. Considering the presence of unsanitated livestock slaughterhouses in many parts of Iran, as well as the lack of accurate and systematic recording of data on the infected livestock, seroepidemiological investigations seems to be necessary in some cases. Using the ELISA method can be very efficient because of the precision and rapid results it provides. During this study, an Iranian native B antigen was used to design the specific detection of hydatidosis in sheep using a specific ELISA technique.

MATERIALS AND METHODS

Sera

A total of 50 sheep sera samples were investigated in this study. 25 samples from new-born lambs were used as negative, and 25 others were obtained from slaughterhouses as positive to infection with hydatid cysts and were entered the study. All sera were collected from the sheep in the East Azerbaijan province of Iran.

Extraction of antigen B

In order to extract the antigen B from the hydatid cyst fluid, the modified method was implemented (Shirazi et al., 2016). In the first stage, 300 ml of hydatid cysts' fluid were extracted from the sheep, then 100 ml of the obtained hydatid fluid were transferred to a dialysis bag and were 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. The solution was centrifuged for 30 minutes at 3000g afterward. The supernatant resulting from salting out was placed in a boil-water bath for 15 minutes. The mixture 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), the mixture was stored at a temperature of -70 ° C until next use.

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

Designing the ELISA method with Antigen B

In order to design this method, the most suitable level of serum dilution and the most desirable con-

centration of antigen B that should be attached to the wells (Coate) should be determined in the first step. To achieve this purpose, different levels of antigens and serum concentrations were tested.

In order to prepare serum dilutions, a robust positive serum and a negative serum were used. Then, dilutions of 1:100, 1:200, and 1:400 were prepared.

Steps for designing ELISA method with antigen B

• Binding of antigens to the wells (Coating)

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

• Sealing the gaps and empty areas of the wells (Blocking)

To this purpose, a neutral protein such as 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 after drying the wells (by tapping onto a filter paper), 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.

• Transferring serums

Wells were emptied and then, 100 µl of positive and negative serums were transferred into the wells with pre-prepared dilutions after three to four times of washing and drying. Then, they were incubated in a humid environment for 75 minutes at 37 ° C.

• Conjugation of solution

Wells were emptied and 100 µl of the anti-Sheep conjugate (manufactured by SIGMA USA at 1: 3000 dilution) was prepared in wells after five to six times of washing and drying and incubated for 75 minutes in a humid environment at 37 ° C.

• Addition of substrate (Chromogen - Substrate)

Wells were emptied and washed five to six times and were dried. 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.

• Stop Solution

The stop solution includes sulfuric acid 1M, of which 50 µl is transferred into each well, and then, the wells should be immediately read.

In order 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. The results for positive and negative samples are presented in the results section.

Due to the high sensitivity of the extracted antigen (AgB) in this study, a dilution of 0.5 µg / ml and the serum dilution of 1:400 were used.

Designing the Nano-ELISA method using antigen B

In order to design the method, the most suitable concentration of gold nanoparticles conjugate should be determined initially. The concentration of antigen B is fixed, and 0.5 µg/ml of antigen is bound to the wells.

In order to prepare serum dilutions, a robust positive and a negative serum were used. According to the hypothesis of this study, nanoparticles increase the sensitivity of the test. In this stage, serum diluted of 1:500 was used instead of 1:400 dilution.

Steps for designing Nano-ELISA with antigen B

• Binding of antigens to the wells (Coating)

The wells were polyester made (Nunc, Denmark). 100 µl of antigen B with a concentration of 0.5 µg/ml was added to each well and stored in the refrigerator for completing the antigen binding process.

• Sealing the gaps and empty areas of the wells (Blocking)

A neutral protein such as Skim Milk 5% was used for this purpose. In order to block the wells containing 100 µl of antigen solution, they were emptied and washed three times using PBST, and after drying the wells (by tapping onto the filter paper), 250 µl of blocking buffer was added to the wells and incubated in humidity for 75 minutes at 37 ° C.

• Transferring serums

Wells were emptied and then, 100 µl of positive and negative serums were transferred into the wells with a dilution of 1:500 after three to four times of washing and drying. Then, they were incubated in a humid environment for 75 minutes at 37 ° C.

• Conjugation with gold nanoparticles (Conjugation of Solution)

Preparing gold conjugate

Ready gold nanoparticle colloid (Plasma Chem, Germany) was used in this study. In order to find the

most suitable dilution for conjugate five different concentrations of conjugate were prepared with four different dilutions (1, 1:2, 1:4, 1:8). Five containers were labeled and 1 ml of gold nanoparticles were transferred to them. In the next step, anti-sheep conjugates (SIGMA, USA) were combined with these particles. In order to achieve this step, containers with gold nanoparticles were combined with different amounts of anti-sheep conjugates on the shaker and inside ice for 30 minutes (Fig 1).

Fig 1: vials contain sheep conjugate and gold nanoparticles

In addition, 100 µl of the gold conjugate (Gold nanoparticle combined with anti-sheep conjugate) with dilutions of 1, 1:2, 1:4, and 1:8 were transferred to wells and stored in a humid environment for 75 minutes in a 37 ° C incubator.

• Addition of substrate (Chromogen - Substrate)

Wells were emptied and washed five to six times and were dried. 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 9 minutes.

• Stop Solution

The stop solution includes sulfuric acid 1M, of which 50 µl is transferred into each well, and then, the wells should be immediately read.

In order 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. Results of implementing this step showed that gold conjugate Number 4 with a dilution of 1:8 was very suitable.

In the next step, all positive and negative serums were prepared with a dilution of 1:500 and tested with the designed Nano-ELISA method with antigen B concentration of 0.5 µg/ml and a concentration of 1:8 for gold conjugate Number 4 (Table 1). Then, the Cut off was calculated.

Table 1: Dilutions of Gold nanoparticles conjugated

No.	1	2	3	4	5
Anti-Sheep (µL)	2	4	6	8	10
DDW (µL)	98	96	94	92	90
GNP (ml)	1	1	1	1	1

RESULTS

AgB was measured by Bradford assay and it concentrations was 0.7 (mg/ml) and SDS-PAGE result shows that bands on 20 and 24 KDa subunits of AgB (Fig 2).

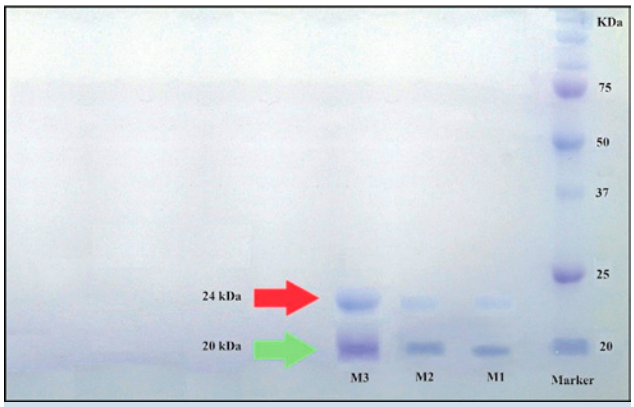

Fig 2: SDS-PAGE bands of Antigen B (M3)

At first, 0.5 µg /ml AgB concentration and also 1:3000 the sheep's conjugate were used. In order to find the best serum dilution, 2 sera of sheep with hydatidiosis and 2 sera without infection (new-born lamb) were used. According to the results of Table 2, 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 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 2: Result of sheep sera dilatation with AgB (450 nm)

Serum	**Positive Serum**		**Negative Serum**	
Serum Dilution	1	2	1	2
1/100	2.455	2.048	1.383	1.583
1/200	2.201	1.839	1.032	1.185
1/400	1.946	1.457	0.445	0.578

AgB concentration was determined to 0.5 µg / ml for binding to the end of the wells, in AgB-ELISA method. In order to minimize the interfering variables in this study although we didn't want to change the

amount of coated antigen, but it was assumed that gold nanoparticles would increase the optical absorption and increase the quality of detection so serum dilution 1:500, was selected.

According to the results of Table 3, the suitable dilution for gold nanoparticles conjugate was conjugate No.4 with dilution of 1:8 that made the best difference between positive and negative sera optical density (OD).

In the next step, for finding the cut off, definitive positive and negative sera were tested by a nano-ELISA method. Results showed that, cut off was equal to 0.8. In the other words, the sera with up to 0.8, positive and below 0.8, optical absorption was considered as negative. Accordingly, all sera of sheep with positive hydatidosis, and one of the non-infected sheep serum, showed absorbance higher than 0.8.

According to the results the sensitivity and specificity of the gold nano particles ELISA designed with AgB for detecting hydatidosis in sheep determined 100% and 96%, respectively (Table 4).

DISCUSSION

Hydatid cyst is a common zoonotic international disease that is recognized as an important disease in endemic areas, especially in North Africa, South America, China and the Middle East (Mandal, 2011). Recently, the World Health Organization (WHO) recognized *Echinococcus granulosus* in a subgroup of selected Neglected Tropical Diseases (Fasihi-Harandi et al., 2012).

Epidemiologic studies across Iran indicate that the disease has an increasing trend (Fasihi-Harandi et al., 2012). The common serological methods for diagnosing the infection to hydatid cyst are as follows according to sensitivity and specificity: ELISA, indirect immunofluorescence, indirect hemagglutination, Counter immunoelectrophoresis, and Complement fixation test (Weinberg test) (Barnes et al., 2012).

In humans, serologic methods are generally used to confirm radiological findings. Therefore, the ELISA method is an initial screening method to test the serum (Rokni, 2009). Consequently, antigens used to prepare the ELISA kit must be specific, and one of these antigens is antigen B which is recommended by WHO (Shirazi et al., 2016).

Antigen B is used in several hydatidosis seroepidemiological studies in Iran. In 2013, the researchers developed an ELISA kit using antigen B and investigated the contamination with hydatidosis in different provinces of the country. Moreover, hydatidosis has been confirmed with this antigen (AgB-ELISA) in some cities of Iran such as Shiraz and Ark. (Asghari et al., 2013).

Due to the high sensitivity and specificity of AgB-ELISA method and the increasing trend in utilizing this method in seroepidemiological investigations, and regarding the fact that this method is still used only in research facilities, using correct methods for extracting this antigen is very important.

In contrast to humans, a few types of research have been conducted to improve the immunological methods for diagnosing hydatidosis in domestic animals such as sheep and cattle. The diagnosis of hydatidosis in natural hosts is basically done during autopsy (Beard, 1973). The accurate serologic diagnosis of nfection in the livestock is difficult due to cross-reactivity

Table 3: Results of different dilutions of gold nanoparticles with 1:500 sera dilution (450 nm)

No	1		2		3		4		5	
	Pos. Serum	Neg. Serum	Pos. Serum	Neg. Serum	Pos. Serum	Neg. Serum	Pos. Serum	Neg. Serum	Pos. Serum	Neg. Serum
Crude	2.745	0.832	3.124	1.103	3.123	1.154	3.789	1.002	3.321	1.003
1/2	2.129	0.612	2.451	0.596	2.703	0.879	3.310	0.701	3.001	1.234
1/4	1.746	0.451	1.989	0.435	2.012	0.568	2.981	0.671	2.987	0.820
1/8	0.814	0.320	0.879	0.376	1.412	0.381	2.209	0.399	3.110	0.509

Table 4: The comparison between AgB-ELISA and Nano-ELISA in sheep sera

		ELISA		Nano-ELISA	
Case	Serum	No. of Positive	No. of Negative	No. of Positive	No. of Negative
Infected	25	23	2	25	0
Non infected	25	2	23	1	24
Total	50	25	25	26	24
Sensitivity		92%		100%	
Specificity		92%		96%	

with other cestodes such as *Taenia hydatigena* and *Taeniaovis*. In addition, animals produce a very weak antibody response in contrast to the high level of specific antibody produced in human. In order to detect the infection in intermediate hosts, using diagnostic methods such as CT scan and radiology requires expensive equipment, which is not available everywhere. However, using serological methods including ELISA is very simple, beneficial, and cost-effective (Rokni, 2009).

Recently, Nano-biotechnology is being used to improve the existing common methods for diagnosis of various diseases (Ambrosi et al., 2010; Cho et al., 2013). However, these studies are limited in parasitology and only a few studies are available on the subject. During a study, gold nanoparticles and antigen B were used to design a Dot-Immuno-gold Staining (Dot-IGS) method, which showed that this method could be a rapid and reliable method. However, it cannot express sensitivity and specificity (Jahani et al., 2014). During another study implemented in 2015, gold nanoparticles and EPC1 recombinant antigen were used to isolate anti-*Echinococcus granulosus* Ig Gantibodies in dogs contaminated with *E. granulosus*. ELISA and Dot-Immuno-gold Filtration Assay (DIGFA) were used in this study, and the results showed that the ELISA method had a higher sensitivity and specificity (Kord Afshari et al., 2015).

It should be noted that the follow-up on sheep showed that there was no specific ELISA kit available for detecting hydatidosis in sheep in Iran. Therefore, the comparison was conducted between the ELISA designed with antigen B and the ELISA design with antigen B along with a conjugate combined with gold nanoparticles. The results showed that Nano-ELISA method was more sensitive and more specific compared to the method without gold nanoparticles. The reason for increased sensitivity and specificity in the Nano-ELISA method in this study is probably the high-level surface to volume ratio in gold nanoparticles, which causes more antibodies to enter the antigen-antibody complex to aid nanoparticles and provide better pigmentation.

CONCLUSIONS

Using an effective and accurate method can be very helpful in seroepidemiological studies. Regarding the fact that there is no commercially ELISA Kit available for sheep, using Nano-ELISA designed with native antigen B can be very effective in evaluating the immune responses of intermediate hosts such as sheep, as well as epidemiological studies according to high sensitivity and specificity. Future studies can make progress in improving diagnosis of hydatidosis in humans using nanoparticles, as well as improving the diagnosis of other diseases.

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CONFLICT OF INTEREST

None declared by the authors.

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