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Original Article

Improved Serodiagnosis of Hydatid Cyst Disease Using Gold Nanoparticle Labeled Antigen B in Naturally Infected Sheep

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Abstract

Background: Cystic echinococcosis caused by the metacestode of *Echinococcus granulosus* is a major problem in both humans and domestic animals health. Therefore, a standardized and approachable diagnostic tool (rapid tests) for the serodiagnosis of cystic echinococcosis (CE) is still needed.

Methods: In the present work, antigen B labeled with gold nanoparticles was used to detect antibodies against hydatid cyst disease. The prepared antigen B was analyzed by SDS-PAGE. Tetra chloroauric acid (HAuCl₄) was used to produce colloidal gold and antigen B labeled by gold nanoparticles, then it was tested by using rabbits antisera and sera from naturally infected sheep. The labeled antigen B was evaluated using Dot-immunogold staining (Dot-IGS) method.

Results: Electrophoretic pattern of hydatid cyst fluid showed the quality of bands in the condensed fluid is better than crude fluid. SDS-PAGE analysis cyst fluid and antigen B revealed three specific protein bands that were detected at molecular weights of 24, 30 and 40 kDa that all are the subunits of antigen B. Evaluation of antigen B labeled by gold nanoparticles by using Dot-IGS technique showed 1/1 and 1/50 dilutions in comparison with another has the best immunoreaction. In this method, nanoparticles produced a typical purple color, when they binded to the strip at the site of immunoreaction.

Conclusion: Therefore, using gold nanoparticles is a good candidate for detection of helminthiasis, also as selective tools of early detection, simple and cost-effective, regardless of specific skills and equipment with optimal durability.

Introduction

Health importance of cystic echinococcosis is the most important reasons for the growing research on this disease in worldwide. Therefore, these researches are considered as extended and helpful tools in different features of hydatidose, especially in human populations.

Among the various diagnostic procedures, labeling techniques are always a special place where radioisotopes are perhaps first and then are fluorescence compounds and enzymes (1, 2). But in recent years, colloidal silver and gold nanoparticles have been used in the immune complex detection broadly. Using this method has been welcomed by communities and researchers from different disciplines because of a rapid response that is significantly visible.

Lack of the gold standard is one of the most important problems in diagnosis of hydatidose. Although researchers and laboratories need to naval approaches, they should consider that diagnostic tests have been individually characterized (3, 4).

One of the most common methods in detection of human hydatid cysts is imaging techniques such as ultrasonography, x-ray imaging, computed tomography and magnetic resonance imaging (MRI). These methods have also limits such as inability to detect cysts from the abscess, identification of small-sized cysts or lack of bone cysts are diagnosed by ultrasonography.

Therefore, early diagnosis should be confirmed by other tests that have to be of a high specificity. The sera patients should be tested by serological methods (5). Some techniques such as immunoelectrophores, double diffusion in agar, indirect hemagglutination are older than ELISA, immunoblotting, and direct immunofluorescence test that are new sensitive techniques (2, 6). The most common labeling methods such as radioisotopes, fluorescent compounds and enzymes can be used in various combinations, but nanoparticles have been used in the last one decade.

Nano-golds (because of high electron density) are used as markers for electron microscopy, also as the ability of binding to macromolecules such as immunoglobulins, streptoavidin, lectin and protein A in immuno-histochemistry (7, 8).

According to adsorption of macromolecules on the surface of gold particles the gold marker was prepared, biological macromolecules were fully preserved. The colloidal particles using as the nonisotope immunoassay and the metals like gold, silver, copper, selenium, has especially (9, 10). Zhang and colleagues (2006) designed a diagnostic kit (Immunochromatographic strip) for diagnosis of trichinellosis in pigs with excretory-secretory antigen labeled by gold particles (11). They considered the specificity and sensitivity of this tape same as ELISA method.

According to what was described, in the present study antigen B was prepared from ovine hydatid cyst fluid and was labeled with gold nanoparticles to detect antihydatid antibodies in rabbit's antisera and naturally infected sheep sera.

Materials and Methods

Sera sample collection

Forty-five blood samples were obtained from naturally infected sheep at local abattoir in Isfahan (Center of Iran, where is the endemic region of hydatidosis) and all the samples were labeled. After examining the organs of sheep carcasses for hydatid cyst, especially the liver and lung, positive and negative samples were subjected for ELISA and Dot-immunogold staining processes. Sera was separated by centrifugation at $2000\times g$ for 15 min, aliquotted and preserved at -20 °C until use.

Hyperimmune sera preparation

Four rabbits were divided randomly into two groups, two rabbits in each group. Two rabbits were inoculated intramuscularly with antigen B. The antigens (350 µg/ml) were

mixed with an equal volume of Freund's complete adjuvant and injected. Subsequently, two boosters injection (175 µg/ml) with Freund's incomplete adjuvant were administered at intervals of 4 weeks. Two rabbits were injected with distilled water as control. Blood samples from each rabbit in experiment and control group were obtained intracardially 10-days after last inoculation. All sera samples were monitored for antibodies against antigen B by ELISA method and then stored at -20°C until use.

Preparation of antigen B and SDS-PAGE

Naturally infected sheep Livers with hydatid cysts were collected from local slaughterhouse, and cysts were washed immediately with phosphate buffer saline, (PBS, pH 7.4). The antigen B purified hydatid cyst fluid as described by Oriol and colleagues, 1971 (12). Protein concentration was determined according to Bradford, 1976 (13). This antigen was prepared with two protein concentrations included 320 µg/ml and 350 µg/ml.

SDS-PAGE of antigens was carried out using a Mini-Protean III cell (Bio-Rad) at 70 constant voltages for 110 min according to the method of Laemmli, 1970 (14). Electrophoresis of samples was performed in different concentration of antigens. For size estimation, a protein ladder marker, 10-200 kDa (Fermentas, SM0661) was used for SDS-PAGE. The gels were stained with Coomassie blue.

ELISA method

ELISA for detection of parasite-specific Abs in serum samples was carried out as described by Benito et al., 2001 (15). Briefly, AgB was coated 100 µl (8µg/ml) on to the 96-well microtitre plates (Maxi SorpTM, Denmark) and incubated overnight at 37°C. Excess binding sites were blocked with skim milk 3% in PBS and incubated for 40 min at RT. 100 µl sheep sera were assayed in PBS-0.5% BSA in 1:500, 1:1000 and 1:5000 dilutions, and incubated for 1h at RT. One hundred µl Rabbit anti-sheep IgG peroxidase conjugate (Sigma-Aldrich) was

used as secondary antibody at 1:2000 dilution in PBS-0.5% BSA for 1h at RT. Following a final washing step Tetra Amino Benzidin substrate was added to each well and the reaction was stopped after adding 50 µl/well H2So4. The absorbance value of the samples was measured at 450 nm using an ELISA plate reader.

Labeled antigen preparation (Nano gold conjugation antigen)

Colloidal gold particles were obtained by reducing the gold chloride with sodium citrate (16). It is important to determine the optimal amount of protein coated on the gold particles. Antigen B was conjugated to 30-nm colloidal gold beads. Briefly, the colloidal gold was adjusted to pH 8.8 with 0.2 M K₂CO₃ and mixed with antigen B at 4 °C for 2 min. Bovine serum albumin (10%) was added in order to block the free space on the nano-gold particles and incubated for 10 min at 4 °C. The gold conjugate was washed three times with phosphate-buffered saline containing BSA 1% and stability was determined. Negative staining was carried out for this solution.

Dot-immunogold staining

Coating of membranes with antigen B was performed by added 2 µl of labeled antigen B (with protein concentration 350 µg/ml) on a nitrocellulose strip (1×6 cm). These strips were air dried at room temperature. Sera samples were diluted (1:1, 1:50 and 1:100) and 2 µl was dispensed upon each dot, developing purple color spot on membranes was considered as evidence of positiveness compared with negative control. Color reactions in controls were absent.

Results

SDS-PAGE analysis of hydatid cyst fluid showed six polypeptide bands were detected with molecular weights less than 70 kDa (Fig. 1).

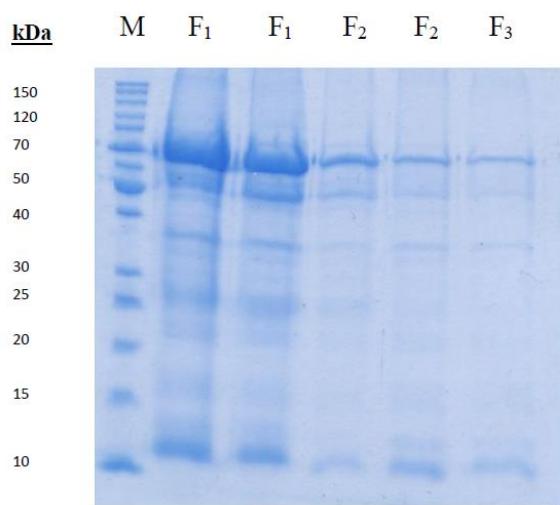


Fig. 1: Electrophoretic profile of ovine hydatid cyst fluid with protein different concentrations. Lanes F1 condensed fluid, Lanes F2 crude fluid, Lane F3 diluted fluid, M Molecular weight marker (Fermentas=SM0661)

Antigen B of hydatid cyst fluid were prepared with two protein concentrations consisting of 320 and 350 µg/ml. SDS-PAGE of antigen B in all samples were demonstrated one major protein band of approximate 42-43 kDa molecular weight (Fig. 2).

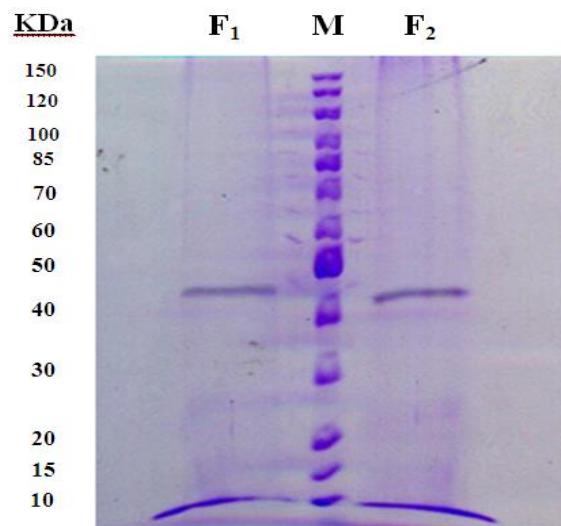


Fig. 2: Reducing SDS-PAGE of purified antigen B in a polyacrylamid gel stained with coomassie blue/F1 and F2 antigen B, M Molecular weight marker (Fermentas=SM0661)

Sera samples obtained from naturally infected sheep were analyzed by ELISA for total antibody responses. In the ELISA method for selecting positive sera, samples number 2, 4 and 7 had the higher OD than the other sera samples (Fig. 3). In the ELISA method for selecting positive sera, samples number 2, 4 and 7 had the higher OD than the other sera samples.

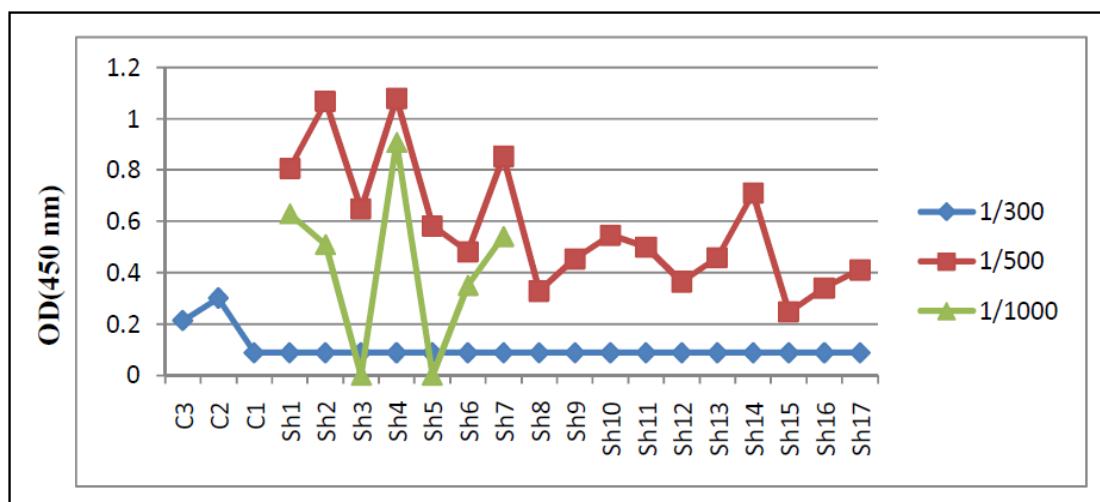


Fig. 3: The results of ELISA test, 1-17 hydatid cyst infected sera samples with different dilutions, C1-C3 negative control

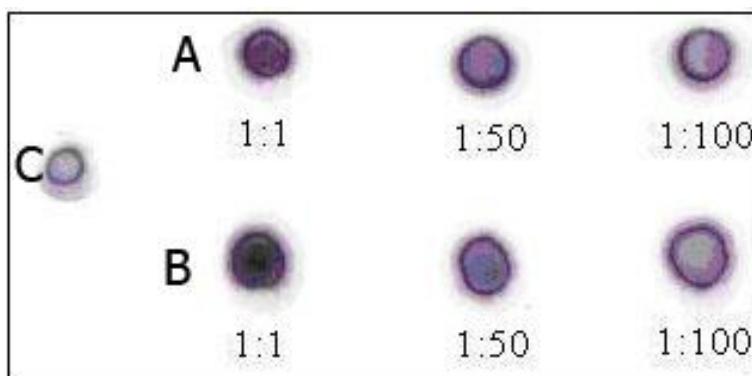


Fig. 4: Dot-Immunogold staining (Dot-IGS) of labeled antigen B with nano-gold; A and B represent hyperimmune sera reaction with different dilution (1/1, 1/50, 1/100), C represent control sera

In rapid method of gold staining the best response was detected in 1/1 diluted sera, although, acceptable results were obtained in dilution 1/50. The control sample was pale without any agglutination compared in the center of color spot with the different dilutions of sera (Fig. 4). Similar results were revealed in all sera samples.

Discussion

Cystic echinococcosis caused by metacstode of *Echinococcus granulosus*, is the most zoonotic disease with noticeable cosmopolitan disease among human and livestock health. Highlighting the importance of economic losses and clinical forms of disease, it is limited to neonatal infection with the parasite. Thousands of people are accidentally exposed to the host interface as serious complications and even death from the disease. Several methods to determine the presence of serum antibodies against hydatid cyst in the serum of infected hosts can diagnose the disease, but there are clear differences in the abilities of different immunological tests (3).

Although immunoassay methods such as ELISA are high sensitive and ideal for diagnostic purposes, but they need laboratory equipment and materials in any place and circumstances. However, the rapid tests require special equipment without personal experience.

Therefore today's methods are increasingly used in medical sciences (infectious agents, reproductive, cancer, aids and ...), agriculture (food safety and diseases of plants and grains) and environmental studies (biological pollution) and veterinary medicine (11).

Regent method does not require multiple fast measurements. This general format is often based on the three forms, agglutination (17), Flow-through and lateral-flow (18). Consequently, in these types, the last one has more benefits and in recent years immunochromatographic strips has been more concentrated.

Since 1975 the gold particles were used for labeled surface antigens of cells, bacteria, fungi and intracellular antigens. The colloidal gold needs a low amount of the macromolecules; in addition, this method is easy, fast and cheap and has a high repeatability. This has led its use in chromatographic immunoassay method appropriate and practical (19).

In this study by considering the importance and necessity of performing a method for rapid diagnosis of hydatidose, nano-gold was used. Because the hydatid cyst fluid antigens have certain characteristics in the diagnosis of patients, prepared antigen B was used. Electrophoretic pattern of crude hydatid cyst fluid in various bands was less than 70 kDa in the range but antigen B prepared in two protein types that of concentrations were slightly dif-

ferent. One with concentration of 320 µg/ml and other was 350 µg/ml. In the second type, antigen quality was better and protein bands were less than 45 kDa. The band with molecular weight of 42 kDa was more clearly, that is consistent with other researchers' studies, who describe it as one of the antigen B subunits.

Many researchers, such as Chemale and colleagues (2005) are important sources of antigen used in detection of serum antibodies and introducing antigen B as one of the main antigenic components of hydatid cyst (20).

Some researchers are intensive on antigen B and its genetic variation in different hosts and also concluded that the use of ovine antigen B in the diagnosis of human hydatidose performance is desirable (21). Crude hydatid cyst fluid antigens are low specificity which is due to the epitopes shared with other helminthic parasites that the result would be false positive and false negative reactions (22).

Briefly, speaking about the importance of considering as described before in the diagnostic potent of antigen B, in the present study, labeled antigen B was used for the detection of antibodies against hydatid cyst.

The results obtained in this study indicate that both hyperimmune sera and hydatid sheep sera showed the color difference between the serum of an uninfected and infected (positive and negative samples), were clearly in 1:1, 1:50 and 1:100 dilutions. Dilution of 1:50 the ability to identify the infected sera was more but dot staining of positive serum on 1:1 dilution showed the best reaction.

Recently, various immunometallic staining methods have been reported for different parasitic diseases, such as *Schistosoma*, *Toxoplasma* and *Clonorchis* (23- 25).

Wu and colleague (1993) in a comparative study used immunoigold-silver staining (IGSS), Dot-ELISA and Dot-IGSS for diagnosis of Clonorchiasis patients and reported these methods are highly specific and sensitive (25). Liu and colleague (2005) in a similar study by using gold particles for diagnosing Schistosomiasis due to *Schistosma japonicum*, compared

rapid-Dot-IGS and two Dot-IGSS methods (10). Their results showed that the R-Dot-IGS assay is stable, fast, simple and practical in both field and clinical study, Wang et al. (2007) also used this method with the monoclonal antibody for detection and differentiation of *Plasmodium falciparum* from *P. vivax*, and it was mentioned as a sensitive, specific, simple and rapid recognition of malaria (9). The pollution associated with tape worms, which has been the subject of this research, can be indicated to diagnosis of cysticercosis that was under investigation Liu and colleagues, 1996 (19). The results of present study were observed in sera with high antibodies titer which can be considered as a weakness on this test. But certainly, by planning of novel ideas where the sera antibody titers are normal or low, can use this method, similar methods or new strategy that in the course of this study can be considered instantaneous.

Conclusion

The above result indicates that using immunogold staining was easy, possible and enjoys high speed. After 4-5 min. the appearance of the purple colored dot shows positive reaction. This technique not only can be described as a rapid and simple method but also very stable, sensitive and specific in different terms.

Acknowledgements

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