Serodiagnosis of Sheep Hydatidosis with Hydatid Fluid, Protoscoleces and Whole Body of Echinococcus granulosus Antigens

G.R. Hashemi Tabar, A. Haghparast and H. Borji

Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

Abstract: The objective of the present study was to develop a specific and simple antigen-based ELISA method for diagnosis of hydatidosis in sheep with different (hydatid fluid, protoscoleces and whole body of Echinococcus granulosus) antigens. A total of 100 sera were collected from sheep with hydatidosis proven by inspection of hydatid-infested livers and lungs of the sheep slaughtered in Mashhad abattoir. Hydatid fluid and protoscoleces were isolated from livers or lungs of sheep with hydatid cyst in sterile conditions. Whole body of Echinococcus granulosus was isolated from intestine of infected dogs. Sera samples were examined by ELISA with different antigens. Statistical analysis for differences in antibody level against different antigens was determined by One-Way variance analysis method (ANOVA). The results of antibody detection by indirect ELISA, using different antigens, showed that the hydatid fluid was the most effective antigen of those assessed for detection of infection with hydatidosis in sheep. Findings of this study indicated that antibody detection assay is a sensitive approach for diagnosis of hydatid cyst in sheep.

Key words: Antibody • ELISA • Hydatid fluid • Protoscoleces • Whole body of Echinococcus granulosus antigen • Sheep

INTRODUCTION

Cystic echinococcosis (CE) is a silent zoonotic infection of humans and domestic animals caused by larvae of the cestode Echinococcus granulosus (E. granulosus). This parasite has a worldwide distribution and is one of the most important zoonotic diseases prevalent in different parts of the world including the Middle East [1]. Early diagnosis of CE can provide significant improvements in the quality of the management and treatment of disease. In most cases, the early stages of infection are asymptomatic, so methods that are relatively easy to use and that are cheap are required for large-scale epidemiological surveillance of populations at high risk [2].

As diagnosis of this disease by clinical symptoms and scanning alone is often difficult and confusing, some reliable and sensitive serological tests are required to corroborate the evidence reached [3]. The detection of circulating E. granulosus antigens in sera is less sensitive than antibody detection, which remains the method of choice. CE serology has a very long history and almost all serological tests that have been developed have been used in the diagnosis of disease. Serology is the method generally used for prevalence surveys in developing countries [4-5]. Complement fixation test (CFT) was the first immunological test used for serodiagnosis of CE. Since then, a wide number of immunological tests have been developed for the detection of hydatid antibodies and of late hydatid antigens in the serum [6]. The hydatid anti-based serological tests include indirect haemagglutination (IHA), indirect immunofluorescence (IFA), immunoelectrophoresis, counter-current immunoelectrophoresis (CIEP), radio-immunoassay (RIA) and ELISA. Development include enzyme-linked immunoelectrotransfer blot (EITB) [7], enzyme-linked immunoelectrodiffusion assay (ELIEDA), time-resolved fluorometry assay (TR-FLA) and immunoblot [8-9]. The hydatid antigen-based serological tests include mainly the ELISA [10]. Many of these assays with higher sensitivity and specificity require sophisticated equipment and trained technicians. Therefore, there is a need for an immunoassay to be simple and inexpensive.

Corresponding Author: Hashemi Tabar, Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran, Tel: +0511 8763851; E-mail: hashemit@um.ac.ir.
Also, its reagents or chemicals to be available and not requiring any heat-labile perishable reagents or chemicals [11]. There are considerable differences between the various tests, both in specificity and sensitivity. As the sensitivity of a test increases, so generally does the demand for improved antigens in order that sufficient specificity can be achieved to take advantage of the greater sensitivity. An optimum test should be specific with high sensitivity. Insensitive tests have been replaced by the ELISA and immunoblotting (IB) in routine laboratory applications [12], although the choice of diagnostic antigens exhibiting the requisite specificity and sensitivity is ever critical [2].

So, the objective of the present study was to develop a specific and simple antigen-based ELISA method for diagnosis of hydatidosis in sheep with different (hydatid fluid, protoscolices and whole body of *E. granulosus*) antigens.

**MATERIALS AND METHODS**

**Collecting of Sheep Sera:** A total of 100 blood samples were collected from sheep with hydatidosis proven by inspection of hydatid-infested livers and lungs of the sheep slaughtered in Mashhad abattoir. Blood samples were also collected from the newborn lambs (uninfected 2 weeks old) with no history of hydatidosis as negative control. Blood samples were centrifuged at 3000 x g at 20°C for 15 minutes and sera were stored at -20°C until used.

**Preparation of Antigens**

**Hydatid Cyst Antigen:** Hydatid fluid was isolated from livers or lungs of sheep with hydatid cysts in sterile conditions. Hydatid fluid was centrifuged at 5000 g for 30 min (4°C) to remove protoscolices.

**Protoscolices Antigen:** Isolated protoscolices were washed with Hank's solution for three times. The suspension was subjected to 3 cycles of freeze/thaw as follows: the tube was frozen in liquid nitrogen and thawed at 42°C. The suspension then mixed with four volumes of PBS (pH 7.4) containing sodium azide at 0.1 mg/ml. The sample was then sonicated in a 170 W ultrasonic for 3x15 sec pulses on ice at a high output setting until no intact protoscolices were visible. The preparation centrifuged for 30 min at 10,000 g and then filtered with 0.22 μm filter.

**Whole Body of *E. granulosus* Antigen:** Two hundred of mature *E. granulosus* were obtained from Parastology Department, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad. These parasites were kept in 10% formalin for 8 months. They were washed with Hank's solution for three times and after washing with phosphate buffer saline (PBS) [pH = 7.3], were kept in PBS at -20°C. Protein soluble of mature *E. granulosus* was prepared by freeze-thawing in liquid nitrogen and 42°C for three times. The sample homogenized in a blender and was sonicated at 110 V, 170 W ultrasonic disintegrator (Hielscher, Germany), for 3x15 sec on ice and then centrifuged for 15 min at 10000 g. Finally, the sample filtered with 0.22 μm filter. Protein concentration of these antigens were measured by Bradford [13] method and kept at -20°C until used.

**Indirect ELISA:** ELISA was performed in a 96-well polystyrene micro titer plates. The plates were coated with different (hydatid fluid, protoscolices and whole body of *E. granulosus*) antigens (diluted in 10 mM carbonate buffer (pH 9.6) in order to give protein concentrations of 2.5 μg/ml for detection of *E. granulosus*-specific antibody. Plates were incubated overnight at 4°C. Then, the wells were washed by ELISA washer 5 times for 10 minutes, with 300 μl PBS-0.05% Tween 20. Three hundreds μl of BSA %1 in PBS-T was added to each well as blocking and incubated for 1 hour at room temperature. Serum samples were diluted at 1:10, 1:20, 1:50 and 1:100 with PBS-T. After optimizing the ELISA kit, 100 μl of sera samples at a 1:20 dilution in PBS-T containing 1% v/v of Bovine serum albumin (pH=7.4) was loaded into duplicate wells and incubated for 1 h at room temperature (RT). Duplicate positive and negative control sera were used. The wells were washed as above. Then 100 μl of HRP-labeled polyclonal antibodies against sheep IgG at a 1:2000 and 1:5000 dilutions in PBS-T with 1% BSA was loaded into all the wells and incubated for 1 h RT. The plate was washed as described above to remove the excess conjugate. For color development, 100 μl of TMB was added to each well as a substrate and the reaction was terminated after 15 min by addition of 100 μl of 1M Sulfuric acid to each well. The absorbance at 490 nm was monitored with a microplate reader (Bio-TEK ELX-800).

**Statistical Analysis:** Statistical significance for differences in antibody level against different antigens was determined by One-Way variance analysis method (ANOVA). Also differences in the level of antibody between individual antigens in each group were determined by Tukey’s HSD and Duncan statistical method.
RESULTS

Sera samples were examined by ELISA with different antigens of *E. granulosus*. The best results were obtained by ELISA with 1/20 of antibody dilution and 1/2000 of anti-sheep IgG peroxidase conjugate. The results of antibody detection by indirect ELISA, using different antigens, showed that the hydatid fluid was the most effective antigen of those assessed for detection of infection with hydatidosis in sheep (Figure 1). According to our results, hydatid fluid antigen has significant differences with protoscolices and whole body of *E. granulosus* antigens and negative control (P<0.05). No significant difference was observed between hydatid fluid or protoscolices antigens with positive control. (Table 1). However, whole body of *E. granulosus* antigen showed significant difference with positive control.

DISCUSSION

The main problems in the immunodiagnosis of hydatidosis are the often disappointing performances of the available tests and the difficulties associated with the standardization of antigen preparations and techniques [14]. Most of the serological tests such as ELISA performed on patients' sera for detection of specific antibodies gave rise to variable results of sensitivity and specificity [15]. Immune response in hydatidosis, the basis of laboratory diagnosis, is quantitatively small and frequently insufficiently intense to be detected serologically. This has caused a constant search for increasingly sensitive techniques to detect very low antibody levels. To achieve this, several immunological methods have been evaluated in recent years [7, 9, 16]. In all of these studies, the cross-reactivity of hydatidosis was with fascioliasis [17] which can be important in
regions where both cystic hydatid disease (CHD) and fasciolosis are endemic diseases, but in Iran, most fasciolosis cases are reported from Gilan province (north of Iran) where CHD is not a prevalent disease [11]. Researchers carried out a very extensive study aimed at developing an immunological method for the identification of sheep infected with *E. granulosus* which would allow the monitoring of animals imported into countries free from hydatidosis and as an aid to countries where control schemes for the disease are in operation [18].

In our study, the results of antibody detection by indirect ELISA, using different antigens, showed that the hydatid fluid was the most effective antigen of those assessed for detection of infection with hydatidosis in sheep. In contrast to our results, three ELISAs were developed and validated, using as antigen purified 8 kDa AgB3 hydatid cyst fluid protein (8kDaELISA), recombinant EG95 oncosphere protein (OneELISA) or a crude protoscolex preparation (ProtELISA). Sera used for the assay validations were obtained from 249 sheep that were infected either naturally or experimentally with *E. granulosus* and from 1012 non-infected sheep. The highest diagnostic sensitivity was obtained using the ProtELISA at 62.7 and 51.4%, depending on the cut-off. Assay sensitivities were lower for the 8kDaELISA and the OneELISA. Diagnostic specificities were high, ranging from 95.8 to 99.5%, depending on the ELISA type and cut-off level chosen. A few sera from 39 sheep infected with *T. hydatigena* and from 19 sheep infected with *T. ovis* were recorded as positive. Western immunoblot analysis revealed that the dominant antigenic components in the crude protoscolex antigen preparation were macromolecules of about 70-150 kDa, most likely representing polysaccharides. This study demonstrated that the ProtELISA was the most effective immunological method of those assessed for detection of infection with *E. granulosus* in sheep. Because of its limited diagnostic sensitivity of about 50-60%, the assay would be useful for the detection of the presence of infected sheep on a flock basis but not for reliable identification of individual animals infected with *E. granulosus* [18].

In another study, AgB3 has been used, partially purified from hydatid cyst fluid from camels or sheep and a recombinant form of AgB (r-AgB) in an ELISA, to screen panels of serum samples from slaughtered camels and sheep naturally infected with CE [19]. Seroreactivity, however, was variable. Native AgB gave the highest sensitivity (97%) in ELISA for camel CE. In contrast, r-AgB gave lower sensitivity for camel (84%) and sheep (28%) CE. The r-AgB ELISA was, however, highly specific, yielding 90% and 95% specificity, respectively, for natural camel and sheep CE infections.

Taken together, findings of this study indicated that antibody detection assay is a sensitive approach for diagnosis of hydatid cyst in sheep.

ACKNOWLEDGMENTS

The study was financially supported by Ferdowsi University of Mashhad. The authors thanks to Mr. Eshratie and Mr. Azadi for their time and help. Statistical analysis by A. Malvandi and Ms. M. Heidari Kharaji is acknowledged.

REFERENCES


791


