IgG4 detection of *Echinococcus granulosus* paramyosin: a useful diagnostic test for human hydatidosis

Running title: *E. granulosus* paramyosin for diagnosis of hydatidosis

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ABSTRACT

Hydatidosis is a public health problem in many parts of the world, and improvement in diagnosis of the disease is still being pursued. Protoscolecites of *Echinococcus granulosus* were isolated from hydatid cysts collected from naturally infected sheep slaughtered in abattoirs in Iran. Sonicated extract of protoscolex was subjected to two-dimensional gel electrophoresis and western blot analysis. Primary antibodies were from serum samples from 130 hydatidosis patients, 38 individuals infected with other parasitic infections and 30 healthy people; while peroxidase (HRP)-conjugated anti-human IgG and IgG4 were used as secondary antibodies. The recombinant form of the identified protein was produced and tested for its sensitivity and specificity for the detection of human hydatidosis. An antigenic band of ~ 60 kDa was found to be sensitive (82%) and specific (100%) for the detection of hydatidosis when probed with anti-human IgG4-HRP, while the sensitivity and specificity were 33% and 100%, respectively, with anti-human IgG-HRP. By mass-spectrometry, the band was identified as protoscolex tegument paramyosin. The sensitivity and specificity of full-length paramyosin-recombinant protein in IgG4 blots were found to be 86% and 98%, respectively. In conclusion, IgG4 detection of *Echinococcus granulosus* paramyosin was found to be useful for the diagnosis of human hydatidosis.
Hydatid cyst, or hydatidosis, is caused by the larval stage (metacestode) of the tapeworm *Echinococcus granulosus* in human and livestock. As the intermediate host, humans acquire the disease by accidental ingestion of vegetables or water contaminated with the ova of adult worms that live in the small intestine of canids, the definitive host. After exposure to gastrointestinal enzymes, the infective ovum hatches into an oncosphere, which is able to reach organs such as the liver and lungs through the vascular and lymphatic systems. The larva then develops into a hydatid cyst, which is gradually filled with fluid and protoscoleces. Hydatid cysts not only cause severe illness, but also cause economic losses due to costs related to diagnosis and surgery (3). This disease is scattered throughout the world, with an emerging or re-emerging status in several countries (11) and is also highly prevalent in Iran (26). It is estimated that 2-3 million people are infected with this neglected disease worldwide (10). Early diagnosis is based on clinical signs, which is followed by imaging of suspected organs. Clinical signs in humans are not specific, and the imaging methods cannot differentiate between hydatid cysts, tumors and other lesions (1, 14). Therefore, immunodiagnosis remains an important tool in the diagnosis of the disease. Chordi and Kagan were the first to use immunoelectrophoresis to identify the antigenic components of sheep hydatid cyst fluid, and subsequently determined which antigenic components were active in detecting antibodies in the sera of patients with hydatid cysts (8). A successful immunodiagnostic test depends on the use of highly specific and sensitive antigens, as well as the detection of the appropriate antibody class or subclass (18, 27). Detection of circulating antigens in serum was reported to be less sensitive than detection of *Echinococcus*-specific antibodies (31). In this study, two-dimensional electrophoresis (2-DE) and western blots using IgG and IgG4 as secondary antibodies were performed to identify antigen of *E. granulosus*.
protoscolex with good diagnostic potential.

MATERIALS AND METHODS

Serum samples. Group I serum samples were collected from 81 patients with cysts in their liver or lungs who were diagnosed based on clinical symptoms and serodiagnosis and/or with magnetic resonance imaging (MRI). The samples were obtained from sera banks of pathology laboratories in Tehran, Iran. Nine pools of sera, containing 9 samples per pool, were created. Group II serum samples (obtained prior to surgery) were individual samples from 49 confirmed hydatidosis patients from Imam Hospital, Tehran, Iran who had cysts in their liver or lungs, and who underwent surgical removal of the cysts. Consent was obtained from the patients for collection of the serum samples, and the Tarbiat Modarres University research ethics committee approved the study. Group III control samples (68) were from sera from healthy people (n=30) and patients infected with other parasites (n=38) [Ascaris lumbricoides (5), Fasciola hepatica (2), Taenia saginata (1), Toxocara canis (2), Toxoplasma gondii (4), Trichuris trichiura (5), Strongyloides stercoralis (2), Brugia malayi (11), Gnathostoma spinigerum (1), Taenia solium (cysticercosis) (1), Necator americanus (2) and Ancylostoma duodenale (2)].

Preparation of the protoscolex antigens. Protoscoleces were aseptically isolated from hydatid cysts collected from infected sheep slaughtered in abattoirs in Iran. The protoscoleces were washed three times with sterile phosphate-buffered saline (PBS, pH 7.2) by centrifugation at 3000 ×g for 10 min. The final pellet was suspended in an equal volume of sterile PBS containing a cocktail of protease inhibitors (Roche Diagnostics, Germany) at 40 µ/l/ml. Three cycles of freeze–thaw (water bath and liquid nitrogen) were performed, followed by sonication on ice.
(Liquid Pressor: Model XL2020, USA). The homogenate was centrifuged (10,000 ×g, 1 hour), and the pellet was discarded. The supernatant was washed twice with 10 mM Tris and concentrated by centrifugation at 3,000 ×g for 30 min using a spin filter with a 5 kDa MW cut-off (Vivaspin™, Sartorius, Germany). The protein concentration of the supernatant was determined using a Bio-Rad RCDC protein assay kit (Bio Rad, USA), and then the supernatant was stored in aliquots at -80 °C.

One-dimensional electrophoresis. One-dimensional electrophoresis was performed using an OFFGEL Fractionator (Agilent, USA) to separate the protein according to isoelectric point. Ready-made 13 cm IPG dry strip gels with a pH range of 3-10 were used. In total, 1000 µg of desalted protein was fractionated into 12 wells.

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The second dimension of electrophoresis was performed on a 12% SDS-PAGE gel Mini-Protean 3 cell apparatus (Bio-Rad, USA). The amount of protein loaded was optimized at 20 µg per well. Standard molecular weight markers were loaded in the first lane. Samples were run at 100 V for 110 min.

Western blot analysis of native antigens. Protein was electro-transferred from gel to a nitrocellulose paper (NCP, 0.45) using a Trans-Blot Cell (Bio-Rad, USA) at 12 A for 30 min. The NCP was stained with Ponceau S solution (Sigma, USA) and washed with TBS-T (Tris-HCl buffered saline-0.05% Tween 20) washing buffer. It was blocked with blocking buffer (Roche Diagnostics, Germany) for one hour at room temperature. The NCP was then washed four times.
at 1, 5, 10 and 15-min intervals consecutively and cut into 3 mm strips. The strips were then incubated with human serum diluted 1:100 in blocking buffer for 2 hours at room temperature and then overnight at 4 °C. The next day, after a washing step, anti-human IgG4 or IgG conjugated to horseradish peroxidase at dilutions of 1:2000 or 1:1000, respectively, (Invitrogen, USA) were incubated with the strips for 1 hour at room temperature. The strips were then washed and developed with chemiluminescence substrate (Roche Diagnostics, Germany).

Initially, the potential diagnostic band was identified from results of western blots performed with each of the nine pools from the Group I sera and pooled healthy sera, using anti-human IgG4-HRP as the secondary antibody. Subsequently, individual sera from Group II and Group III samples were tested to determine the sensitivity and specificity of the identified band, using both HRP-conjugated anti-human IgG and IgG4 as secondary antibodies.

**Staining of gels.** The gels were stained with Coomassie blue or with MS-compatible silver stain. The latter was performed according to the method described by Shevchenko (29). The identified band for MS-MS analysis was manually excised and transferred to a microfuge tube.

**In-gel digestion of a silver stained gel.** Fifty microliters of acetonitrile was added into each microfuge tube containing the sliced band and incubated for 15 min at room temperature. The supernatant was removed and 25 µl of ammonium bicarbonate (25 mM) was added and incubated for 10 min. The supernatant was again removed and the gel pieces were air dried for one hour. Resuspension solution containing 10 ng/µl trypsin was added and the gel pieces were incubated on ice for 5 min. Subsequently, the gel pieces were immersed in 25 µl of ammonium bicarbonate (25 mM) and incubated at 30 °C overnight. Two volumes of acetonitrile were added
for digestion, and then the samples were vortexed and incubated for 20 min at room temperature. Finally, the supernatant was transferred into a fresh 1.5 ml microfuge tube. Sample cleanup for MALDI –TOF mass spectrometry analysis was performed using Zip-Tip pipette tips (Zip-Tip U-C18, Millipore, USA).

**Mass spectrometry analysis.** In-gel digested protein from a silver-stained gel was sent for MS/MS analysis using a MALDI TOF-TOF 4800 (ABSCIEX, USA) at the Protein and Proteomics Centre, National University of Singapore. Meanwhile, a Coomassie blue-stained, sliced gel band was sent for processing and analysis using a MALDI TOF-TOF 5800 (ABSCIEX, USA) at Proteomics International, Australia.

**Preparation of recombinant antigens.** The gene sequence that corresponded to the MS/MS-identified protein was custom-cloned into a pET28 expression vector by Epoch Life Science (USA). The recombinant plasmid received from the company was transformed into competent *E. coli* BL21 (DES) expression host cells. A single colony containing the recombinant plasmid was inoculated in 100 ml terrific broth containing 100 μg/ml of kanamycin and incubated overnight at 37 °C with agitation at 180 rpm. Fifty microliters of the overnight culture was inoculated into 500 ml terrific broth with kanamycin and incubated at 37 °C with agitation at 200 rpm until the OD at 600 nm reached 0.4-0.5. Protein expression was induced by incubation in 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 hours at 37 °C. The cells were harvested by centrifugation at 10,000 ×g, 4 °C for 10 min. The cell pellet was resuspended in 1.5 v/w lysis buffer containing protease inhibitors and 0.5 mg/ml lysozyme and incubated on ice for 30 min. The cell suspension was then lysed using a sonicator, followed by centrifugation at 10,000 ×g, 4
°C for 30 min. The supernatant was transferred to a new tube, DNase (2500 ug/ml) was added and incubated on ice for 10-15 min, followed by centrifugation of the tube at 10,000 ×g, 4 °C for 30 min. The resultant supernatant was filtered using a syringe filter (0.45 µm). The filtered supernatant was mixed with Ni-NTA resin (Qiagen, USA), incubated at 4 °C (rotating) for 30 min, and then the mixture was loaded into a chromatography column. Gradient washings of the column were performed using 10 ml each of four kinds of washing buffers containing 500 mM NaCl (10mM, 20mM, 30mM and 40mM imidazole respectively). The protein fractions were eluted using elution buffer containing 250 mM imidazole.

**Western blot of recombinant antigens.** Protein-containing fractions from the affinity column were pooled and buffer exchanged into PBS with 1 M urea (pH=7). The fractions were concentrated by centrifugation at 3000 ×g for 30 min with a spin filter as described above. After the protein was transferred onto the NCP, it was blocked with 5% alkali-soluble casein (Novagen, USA) for 1 hour at room temperature. After blocking, the NCP was washed with TBS-T washing buffer and incubated with His-tag antibody-HRP at a 1:1000 dilution for 1 hour at room temperature. Western blotting was also performed using human sera as described above. The same set of serum samples was used, but due to the limited volumes of some samples a reduced number of control sera were used.

**RESULTS**

In the initial set of experiments using pooled serum samples, a band of ~ 60 kDa was observed in IgG4 blots using Group I sera from patients with hydatidosis. This band was not observed with pooled healthy serum (Figure 1). Figure 2 shows representative IgG4 blots with individual serum
samples; the ~ 60 kDa band was found to be present when blotted with sera of hydatidosis patients but not when blotted with control sera.

As shown in Table 1, the 60 kDa band in IgG4 blots of the native protoscolex antigen showed 82% sensitivity (40/49) with Group II serum samples. Out of the 40 serum samples which reacted with the 60 kDa band, 18 were from patients with liver cysts, 19 with lung cysts and 3 with mixed liver and lung cysts. Out of the 9 false negative results, 5 were from patients with liver cysts and 4 from patients with lung cysts. A 100% specificity (67/67) was seen with Group III sera. In IgG blots of the native protoscolex antigen, the 60 kDa band showed 33% sensitivity of (10/30) and 100% specificity with the same set of sera.

Database search was performed using Platyhelminthes database from UniProtKB. Both proteomic service centers identified the band as *Echinococcus* tegument paramyosin (accession number gi|547974) with protein scores (> 200) and peptide scores well above the cut-off values. No protein other than paramyosin was identified. Two tryptic peptides were reported which covered 3% of the paramyosin protein sequence. It showed 100% homology to *E. granulosus* and 97% to *Taenia solium*.

Custom cloning into the pET28 expression vector was made based on the coding sequence (cds) of the previously submitted GenBank sequence, accession Z21787.1. The recombinant paramyosin showed 86% sensitivity (42/49) with Group II serum samples from hydatid cyst
DISCUSSION

Clinical signs of human hydatid cysts are variable and non-specific. Primary diagnosis is based on imaging methods such as ultrasound, computed tomography and magnetic resonance imaging (17). To complement the radiological findings, immunological tests such as enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence, immunoelectrophoresis (IEP) and immunoblotting are used in diagnostic laboratories. However, false negative (up to 25%) and false positive results remain a problem with the available immunodiagnostic assays (21). To date there is no standardized, highly sensitive and specific test available for immunodiagnosis of human cystic echinococcosis (16). Therefore, during the last three decades, extensive efforts have been made to characterize antigenic components of protoscolex, germinal layer and hydatid cyst fluid of *E. granulosus* for use in serodiagnostic assays. Hydatid cyst fluid (HCF) is one of the main antigen sources for serodiagnosis of hydatid disease. Two major components of HCF, thermostable antigen B (AgB) and thermolabile antigen 5 (Ag5), are the most widely used antigens in reported assays for the disease (4). However, there are difficulties related to their lack of sensitivity and to their cross-reactivity with antigens from other parasites, notably other *taeniid* cestodes, as well as problems with the standardization of the antigens. Thus far, the use of these antigens is predominantly restricted to scientific applications and neither is available for general use (1).
The number of reports on the use of protoscolex as an antigen for detecting antibodies in human hydatidosis is limited (5, 6, 22, 2). Chemale et al. (2003) described the analysis of *E. granulosus* metacestode protein extract by 2-DE and the identification of prominent proteins by peptide mass fingerprinting (PMF). A total of 100 prominent protein spots from three 2-DE gels were analyzed by MALDI-TOF-MS; 15 of them were identified by their PMFs, which include protoscoleces tegument paramyosin (7). Monteiro et al. (2010) performed a proteomic analysis of *E. granulosus* metacestodes in a bovine host. They used two complementary proteomic approaches, 2-DE/MALDI-TOF-MS/MS and LC-MS/MS, to analyze proteins expressed in *E. granulosus* protoscoleces. The 2-DE IgG immunoblots using a pool of six sera from hydatid disease patients showed 14 protoscolex proteins recognized by hydatid cyst patients’ sera. These proteins were reported to contribute to immunoregulatory events at the host-parasite interface during infection. According to the investigators, some of them, such as paramyosin and tetraspanin, may be potentially useful for vaccine development (19).

In this study, protoscoleces isolated from hydatid cysts from the livers of sheep were analyzed by 2-DE, using OFFGEL for the first dimension of separation followed by mini SDS-PAGE. This approach has the advantage of using less volume of the serum samples in the subsequent western blots (25). The clear band on the western blots allowed the band on the corresponding SDS-PAGE gels to be readily identified and excised for mass-spectrometry analysis. The high protein and peptide scores obtained in MS-MS analysis of the in-gel digested protein provided a confident identification of the protein as paramyosin. Paramyosin is a 97 kDa muscle protein present in the tegument of protoscoleces. It is an α-
helical protein and was first noted in invertebrate muscle as a structural component (9). The report of paramyosin-based vaccination against schistosomes led to the characterization of the protein in other organisms (12). In the platyhelminths *Taenia, Echinococcus* and *Schistosoma*, an extra muscular localization of paramyosin has been reported (28). Muhlschlegel et al. (1993) described the cloning of a paramyosin-homologous protein of *E. granulosus*; and by immunofluorescence they demonstrated its presence in the tegument, subtegument of the body wall and muscles of the four oral suckers of the *E. granulosus* larvae (20). Paramyosin has also been identified as an immunogenic protein in several parasitic infections, such as *Schistosoma japonicum, Schistosoma mansoni, Taenia solium* and *Taenia saginata* (12, 15).

In this study, the identified *E. granulosus* paramyosin amino acid sequence was 97% homologous to the paramyosin sequence of *T. solium*. Thus it is not surprising that the recombinant paramyosin protein showed cross-reactivity with serum from cysticercosis patient. Interestingly the same serum was not cross-reactive with the 60kDa protein of the native protoscolex antigen. One possible reason for this difference is that the recombinant paramyosin comprised the full length paramyosin (~97 kDa), while the native paramyosin band on SDS-PAGE was not the full-length protein since its molecular weight was ~60 kD. The paramyosin antigenic epitope may reside in the non-homologous part of the sequence. However, before performing further studies to identify the epitope fragment, several more serum samples from cysticercosis patients should be tested to confirm the western blot results.
The levels of serum IgG4 antibodies were reported to have increased and remained at high levels in patients with active cysts or in patients with relapsing disease (23, 24). Patients with relapsing disease were reported to maintain high IgG4 titers in ELISA, whereas the levels of IgG4 decreased in patients with infiltration or calcified hydatid cysts and became negative in patients after removal of cyst(s) by surgery or pharmacological treatment (13, 31). These results suggest that the IgG4 subclass is a good marker for follow-up of hydatidosis cases. Previous studies related to paramyosin used IgG in the seroanalysis; however, our study showed that the use of IgG4 as secondary antibody led to much higher sensitivity and similar specificity compared with the use of IgG. Both native and recombinant antigens demonstrated similar sensitivities and specificities; however, the latter would be more useful for patient diagnosis because it can be produced as a standardized and reproducible diagnostic reagent (30).

One limitation of this study is the lack of samples from patients post-treatment; thus, further studies should be performed on this type of samples. In addition, a lateral flow format of the IgG4 test using the paramyosin recombinant antigen would be useful in facilitating a multicenter evaluation of the test. In conclusion, this study showed that sensitive and specific diagnosis of human hydatidosis could be achieved by performing an IgG4 assay using either the native or recombinant form of *E. granulosus* paramyosin.

**ACKNOWLEDGEMENTS**

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assistance.

REFERENCES


TABLE 1  Reactivities of the 60 kDa band in IgG and IgG4 blots of native protoscolex antigen, probed with hydatid cyst patients and control sera

<table>
<thead>
<tr>
<th>Peroxidase conjugated secondary antibodies</th>
<th>Group II (Sensitivity)*</th>
<th>Group III (Specificity)**</th>
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<tbody>
<tr>
<td>IgG4</td>
<td>42/49 (86%)</td>
<td>68/68 (100%)</td>
</tr>
<tr>
<td>IgG</td>
<td>10/30 (33%)</td>
<td>68/68 (100%)</td>
</tr>
</tbody>
</table>

* Sensitivity: Number of sera reactive with the band out of the total number of sera tested.

** Specificity: Number of sera non-reactive with the band out of the total number of sera tested.

Group II: Sera from patients with hydatid cysts confirmed by surgery

Group III: Sera of healthy people and from patients with other parasitic infections (controls)