The production and comparative evaluation of native and recombinant antigens for the fast serodiagnosis of cystic echinococcosis with dot immunogold filtration assay

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SUMMARY
Clinical diagnosis and post-surgery assessment of cystic echinococcosis depend on laboratory serodiagnosis and ultrasound examinations. This study aims to produce the recombinant antigen (rAgB) and compare its diagnostic effect with natural antigens (crude fluid antigen, protoscolex antigen). After rAgB, crude fluid antigen, protoscolex antigen were produced, and the diagnostic accuracy was evaluated with dot immunogold filtration assay (DIGFA) by the sera from the following groups: surgically confirmed cystic echinococcosis patients (n = 113), alveolar echinococcosis patients (n = 46), other parasitic diseases (n = 49), nonparasitic hepatic diseases (n = 63) and healthy people (n = 121). In diagnosing cystic echinococcosis, the sensitivity of recombinant AgB was 77.9% and the specificity was 98.3%. The crude fluid antigen B showed a sensitivity of 92.9% and specificity of 81.0%. The protoscolex antigen had sensitivity of 87.6% and specificity of 90.9%. The recombinant AgB indicates the advantage of no cross-reaction with other parasite diseases or nonparasite hepatic diseases. Recombinant antigen B can improve the specificity but decrease the sensitivity. The combination of native and recombinant antigens will improve the overall performance of serodiagnosis of cystic echinococcosis.

Keywords dot immunogold filtration assay, echinococcosis, fast serodiagnosis, hydatid fluid, protoscolex, recombinant antigen B

INTRODUCTION
Cystic echinococcosis is a parasite infection caused by infection with the larval stage of the tapeworm Echinococcus granulosus that may cause chronic, cyst-forming, parasitic lesion in sheep, dog and human (1). It is a serious public health problem in Europe, Australia, Africa, central Asia (2) and especially highly endemic in north-west China. At least 270 million people (58% of the total population) are at risk of cystic echinococcosis in Central Asia and western China. The annual surgical incidence rate in Uzbekistan and Tajikistan has been estimated to be as high as 25–27 cases/100 000 with the highest prevalence reaching 10% (range from 0.8 to 11.9%) in some Tibetan communities in western China (3). Hydatid disease causes not only illness but also productivity losses (3). With the quick urbanization in China, the disease has spread to nonendemic areas due to the pet raising, people migration and live stock exchanges. The diagnosis, post-surgery follow-up and field survey depend on ultrasonography and serodiagnosis (3). The fast immune test plays an important role. Our previous study has shown dot immunogold filtration assay (DIGFA) is a cheap and easy method for field screening. Our former epidemiological investigation has applied crude antigens in DIGFA in the field trial and found the main drawback is the high cross-reaction (4). Our previous clinical investigation also indicates the antigen purification is the major cause of the false immune diagnosis (5). In contrast, the genome of the Echinococcus has been tested and presents a draft genomic sequence
comprising 151.6 Mb encoding 11,325 genes. Particularly, the EgAgB family, whose products are secreted by the parasite to interact and redirect host immune responses, has been pictured in details (6). It accelerates the development of recombinant antigen and antibody and shows the advantages such as the commercial availability of reagents, standard protocol and high protein yields. This study aimed to compare the sensitivity and the specificity with native and recombinant antigens.

MATERIALS AND METHODS

Crude hydatid fluid antigen

The native antigens were prepared in Xinjiang Hydatid Institute as described before (7). In brief, hydatid fluid was collected in Urumqi abattoirs. The fluid was harvested by centrifugation at 1000 g for 60 min to remove the daughter cysts and protoscolices. The supernatant was crude hydatid fluid antigen and stored at −80°C. The protoscolices were rinsed with phosphate-buffered saline (pH 7.2) and then underwent three cycles of freeze and thaw. After the centrifugation at 10,000 g at 4°C for 30 min, the protoscolices were collected and ground by sonication. The extract was centrifugated at 10,000 g at 4°C for 30 min. The sediment was protoscolce antigen.

Recombinant antigen

The production of recombinant antigen B has been described before (4, 5). Briefly, a 191-bp DNA fragment which encodes the antigen B sequence was generated from E. granulosus protoscolex-derived cDNA by the polymerase chain reaction. The DNA fragment was expressed as a maltose-binding protein (MBP) in the vector pMalc2X after transformation into E. coli JM 109. The recombinant protein was purified by an amylose column. Then, the MBP was cut-off by factor X and the purified recombinant AgB was produced.

Immunoblotting analysis

Three antigens were analysed on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane. Blots were pre-incubated in blocking buffer (5% nonfat dry milk, 1% Tween-20, in 20 mM TBS pH 8.0) for 1 h at room temperature, incubated with sera from surgically confirmed cystic hydatid patients (1:200) in blocking buffer overnight at 4°C followed by incubation with IgG conjugated with horseradish peroxidase and detected by chemiluminescence and autoradiography using X-ray film.

Protein measurement

Protein concentrations were estimated using the Bio-Rad Bradford protein assay kit (Bio-Rad Hercules, CA, USA) and bovine plasma gamma globulin as a standard.

Patient and sera collection

The study was approved by the Institutional Ethical Committee at the Xinjiang Hydatid Institute and the First Affiliated Hospital of Xinjiang Medical University. During the study period, patients with surgical or radiological diagnosis were followed-up and the blood samples were divided into the following groups: cystic echinococcosis (n = 113), alveolar echinococcosis (n = 46) and healthy people (n = 121). To find out any possible cross-reactions, sera were also collected from patients with confirmed liver lesions: hepatic distomiasis (n = 18), cysticercosis (n = 21), visceral leishmaniasis (n = 10), hepatocellular carcinoma (n = 36) and hepatic hemangioma (n = 27).

Serodiagnosis with dot immunogold filtration assay

The protocol has been prescribed before (4, 5), in brief; antigens were coated as a 1 lL dot onto nitrocellulose (NCP) paper (pore size 0.45 μm, Millipore Co., Bedford, MA, USA). 1:10 diluted test serum was added onto the dot. After washing with 20 mM Tris-HCl (pH 8.2), colloidal gold conjugated anti-human IgG antibody. Colloidal gold was made by sodium citrate reduction of hydrogen tetrachloroaurate (HAuCl4) and conjugated with goat anti-human IgG (Sigma, St. Louis, MO, USA). In human cystic hydatid patients’ sera, the dot will become red antigen and antibody combination from the colloidal gold conjugate while in those healthy controls, the dot showed no colour change. The intensity of the red colour indicated the degree of immune reaction.

Statistical analysis

Data were analysed using spss version 19.0. SPSS, Chicago, IL, USA were used to compare the diagnostic power between different groups. P ≤ 0.05 was considered statistically significant.

RESULTS

The molecular weight identification of recombinant antigen B

After digestion by protease and affinity chromatography, the recombinant antigen B was collected on an amylose column. SDS-PAGE showed that recombinant antigen B is a 12-kDa protein (Figure 1).
The immunoblotting identification of recombinant antigen B

In Western blotting, antigen B was recognized by sera from cystic hydatid patients (Figure 2). The single blot showed that recombinant antigen B can specifically recognize by sera from patients with hydatid disease.

The comparison of antigens

The crude fluid antigen B showed a series of bands with molecular weight ranging from 8 to 100 kDa (Figure 3).

Western blot confirmed that crude hydatid fluid antigen from the proteins of E. granulosus protoscolices are immunogenic. The components of recombinant antigen and crude hydatid cyst antigen were compared. The recombinant antigen has one protein while the crude hydatid cyst antigen has multiple proteins as shown in Figure 3. HPLC confirmed the purity of the recombinant antigen B only has one peak (Figure 4).

The diagnostic performance of the native and recombinant antigen B in the serodiagnosis

In testing 113 sera from surgically confirmed cystic echinococcosis patients, 46 from alveolar echinococcosis patients and 121 sera from healthy control, the recombinant antigen B has a sensitivity of 77.9% and a specificity of 98.3%; the crude fluid antigen B showed a sensitivity of 92.9% and specificity of 81.0%; the protoscolex antigen had a sensitivity of 87.6% and a specificity of 90.9%. The crude hydatid fluid antigen also showed high positive rate in the 46 cases of alveolar hydatid patients (93.5% and 95.7%, respectively) while recombinant antigen B had 60.1% positive rate (Table 1).

The cross-reactive with other diseases

The native and recombinant antigens were used to test sera from 49 other parasitic diseases and 63 nonparasitic hepatic diseases. Among sera from 18 hepatic distomiasis,
the protoscolex antigen crude fluid antigen B had 16.7% cross-reaction, respectively. Among sera from 21 cysticercosis, the protoscolex antigen crude fluid antigen B showed cross-reaction of 28.6% and 33.3%, respectively.

Table 1 Diagnostic performances of the native and recombinant antigens in the serodiagnosis

<table>
<thead>
<tr>
<th>Sera tested</th>
<th>Positive rate</th>
<th>Positive rate</th>
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<tbody>
<tr>
<td>Protoscolex antigen</td>
<td>Crude fluid AgB</td>
<td>Recombinant AgB</td>
</tr>
<tr>
<td>Cystic echinococcosis</td>
<td>113 (99.8%)</td>
<td>105 (92.9%)</td>
</tr>
<tr>
<td>Alveolar echinococcosis</td>
<td>46 (93.5%)</td>
<td>44 (95.7%)</td>
</tr>
<tr>
<td>Healthy control</td>
<td>121 (11.1%)</td>
<td>23 (19.0%)</td>
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</table>

^aIn cystic echinococcosis patients sera, there is no significant difference on the positive rate tested by recombinant AgB vs. protoscolex antigen. (P > 0.05); ^bIn cystic echinococcosis patients sera, there is no significant difference on the positive rate tested by tested recombinant AgB vs. crude fluid AgB. (P > 0.05); ^cIn alveolar echinococcosis patients sera, there is significant difference on the positive rate tested by recombinant AgB vs. protoscolex antigen. (P < 0.05); ^dIn alveolar echinococcosis patients sera, there is significant difference on the positive rate tested by recombinant AgB vs. crude fluid AgB. (P < 0.05); ^eIn health control, there is no significant difference on the positive rate tested by recombinant AgB vs. protoscolex antigen. (P > 0.05); ^fIn health control, there is significant difference on the positive rate tested by recombinant AgB vs. crude fluid AgB. (P < 0.01).

Table 2 Cross-reaction with other diseases

<table>
<thead>
<tr>
<th>Sera tested</th>
<th>Positive rate</th>
<th>Positive rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoscolex antigen</td>
<td>Crude fluid AgB</td>
<td>Recombinant AgB</td>
</tr>
<tr>
<td>Hepatic distomiasis</td>
<td>18 (16-7%)</td>
<td>3 (16-7%)</td>
</tr>
<tr>
<td>Cysticercosis</td>
<td>21 (28-6%)</td>
<td>7 (33-3%)</td>
</tr>
<tr>
<td>Visceral leishmaniasis</td>
<td>10 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>36 (8-3%)</td>
<td>4 (11-1%)</td>
</tr>
<tr>
<td>Hepatic hemangioma</td>
<td>27 (7-4%)</td>
<td>3 (11-1%)</td>
</tr>
</tbody>
</table>

These two crude hydatid fluid antigens had no cross-reaction with sera from 10 visceral leishmaniasis. In nonparasite disease, the protoscolex antigen crude fluid antigen B had false-positive rate of 8.3% and 11.1%, respectively, in 36 cases of hepatocellular carcinoma. As for 27 cases of hepatic hemangioma, the native antigens showed 7.4% and 11.1% false-positive rate. Among all 112 sera, recombinant antigen B showed no cross-reaction (Table 2).

**DISCUSSION**

A 12-kDa antigen B from Echinococcus granulosus has been cloned, expressed and used in diagnostic DIGFA to
test human sera for evidence of cystic echinococcosis. Here, we describe the development of DIGFA using crude hydatid cyst fluid and a recombinant protein (rAgB) as antigens for the detection of E. granulosus antibodies in serum samples. The DIGFA was evaluated with serum samples from surgically confirmed cystic echinococcosis patients \((n = 113)\) and several kinds of controls including alveolar echinococcosis patients \((n = 46)\), other parasitic diseases \((n = 49)\), nonparasitic hepatic diseases \((n = 63)\) and healthy people \((n = 121)\).

No significant differences and high degrees of agreement were found between the DIGFA and an enzyme-linked immunosorbent assay. The DIGFA developed in this study is a promising tool for the simultaneous detection and discrimination of CE and other hepatocellular diseases. This test will be useful for serodiagnosis of CE in clinical settings and screening programmes.

The performance of an appropriate immunodiagnostic test depends on the exam characteristics and the exam purpose. The serodiagnosis sensitivity and specificity of the immunological tests usually vary based on antigen type, methodology, the geographical region and cross-reaction. There are many immune tests have been commonly used in diagnosing hydatid disease such as enzyme-linked immunosorbent assay, indirect hemagglutination antibody test (IHAT) and latex agglutination test (LAT) while the immunofluorescence antibody test (IFAT), immunoelectrophoresis (IEP) and some other tests are also used in laboratories but less frequently. ELISA is the most sensitive of the currently available immunodiagnostic techniques in detecting antibodies to hydatid disease \((8, 9)\). But in field trial or endemic area survey, the fast diagnostic technology is preferred \((4, 5)\). DIGFA takes 3 min to read the result by reading the dot colour.

Protoscoleces and hydatid fluid are the most frequently used for routine serodiagnosis \((8, 9)\). Antigen B is a polymeric lipoprotein and a highly immunogenic major antigen in Echinococcus infection \((6, 7)\). The antigen is comprised of a group of subunit monomers of approximately 866 kDa in molecular size. The main problems in the hydatid disease laboratory diagnosis are often associated with the antigen standardization \((8)\). Crude hydatid fluid antigen exhibits different serodiagnosis sensitivity and specificity due to the differences in antigen origination and preparation procedure. In comparison, the recombinant antigen can be qualitatively and quantitatively standardized \((9)\).

To evaluate the diagnostic sensitivity and specificity of recombinant antigen B, its efficacy of rapid serodiagnosis of human cystic echinococcosis was compared to conventional hydatid fluid antigen and protoscolex soluble antigens. According to the SDS-PAGE and Western blotting, the crude hydatid fluid antigen had multiple bands while recombinant antigen B had only 12-kDa subunit. This explains the serodiagnosis efficacy. Protoscoleces and hydatid fluid antigens showed the high sensitivity in diagnosis of cystic echinococcosis \((87.6\% \text{ and } 92.9\%)\) while recombinant antigen B was lower in sensitivity \((77.9\%)\). It is similar in alveolar echinococcosis. Protoscoleces and hydatid fluid antigens showed the sensitivity of 93.5\% and 95.7\%, and the recombinant antigen B had 60-1\% positive rate in 46 surgically confirmed alveolar echinococcosis patients’ sera.

The advantage of recombinant antigen B was in improving the diagnostic specialty \((16)\). In 121 healthy controls from nonhydatid disease epidemic area, Protoscoleces and hydatid fluid antigens showed 9.1\% and 19.0\% false-positive rate while the recombinant antigen B had only 1.7\%. Although most of the immunodiagnostic techniques in this study yielded acceptable sensitivity, the use of recombinant antigen B in serodiagnosis increases specificity notably but decreases sensitivity.

Crude fluid antigen B and protoscolex antigen exhibit the lack of specificity \((9)\). This was clearly confirmed by the cross-reactivity observed with alveolar echinococcosis sera and the high level of cysticercosis cross-reactivity. There was also a marked cross-reactivity with sera from patients with nonparasitic disease. To elucidate potential specificity, cross-reactivity, or false positivity of the serologic reactions. In comparison, the recombinant antigen B demonstrated a specific combination with 12-kDa subunit. Combining the native and recombinant antigens, immunoblotting enables discriminate between hydatid disease and cross-reactive hepatic infections or malignancies. The combination of several antigens, including native fluid antigen B and protoscolex antigen, may improve the performance of the immunosassays by reducing the number of false-negative or false-positive reactions.

Many serological tests have been applied such as indirect hemagglutination, latex agglutination, immunofluorescence, immunoelectrophoresis, Western immunoblotting and ELISA \((9, 10)\). Many antigens of hydatid have been developed such as HCF, Ag5, and AgB \((9, 11)\). The most popular one is AgB. The sensitivity of crude cyst fluid antigen for cyst hydatid cases was 92.6\%. The specificity of AgB for cyst hydatid cases was 88.1\% \((4)\).

In summary, with three different antigens, a dot immunogold filtration assay was developed for the fast serodiagnosis of cyst hydatid disease. The data suggest that recombinant antigen B be used in parallel with crude antigens to increase the overall sensitivity and specificity.
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COMPETING INTEREST DISCLOSURE

The funding has no interest confliction with study design, data collection or analysis. The authors declare no competing interests. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

REFERENCE