Humoural immune response and pathological analysis in patients with false immune diagnosis of cystic echinococcosis

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SUMMARY

The patients with false immune diagnosis of hydatid disease were investigated for the humoural immune response to analyse the possible reasons and mechanism leading to false immune diagnosis. Two hundred and thirty-nine patients with nature-unknown cysts and 30 healthy controls were detected by immunological assays (four hydatid antigen-based immunogold filtration assay and enzyme-linked immunoblot assay) and ultrasound. Sensitivity of and specificity of immunological assay and ultrasound were calculated, respectively. The serological diagnosis was compared with surgical pathology to screen the patients with false immune diagnosis for the immunoglobulin measurement and pathological analysis. The history and cyst characteristics were also reviewed. The results indicate the immunoglobulin has little influence on false immunodiagnosis. The false-negative immunodiagnosis was caused by the cysts inactive status while the false positive caused by previous rupture, antigen cross-reaction. The clinical diagnosis of cystic echinococcosis requires a combination of immunodiagnosis and ultrasonography, which is the necessary complementary confirmation.

Keywords: false negative, false positive, humoural immune response, hydatid disease, immunological assay

INTRODUCTION

Human cystic echinococcosis (CE) is a chronic infection caused by the tapeworm Echinococcus granulosus (1). It is a serious public health problem in South America, Western and Southern Europe, the Middle East and North Africa and North-west China (2). The early diagnosis of CE is difficult because of the silent progress of the infection. The clinical diagnosis often requires a combination of physical examination, ultrasonography (US) and serology (3). The invention of the fast serological kits is applied in the outpatient department and field screening. They cannot replace the radiological examinations but can provide physicians with immunodiagnosis in a few minutes. In the current study, we are extremely interested in the false serological diagnosis because of the application of fast serological diagnosis changed the traditional diagnostic procedure. Physicians collect the history and complete the physical examinations first. Then the fast eye-read serological diagnosis can be carried out in 3 min with one drop of blood from finger tip as simple as the commonly used home pregnant test. The most challenging thing is what other diseases should be carried on in physicians mind for differentiation. That is why we collect all false diagnosis subjected to serological diagnosis to help physicians schedule the further examinations for differential diagnosis. Many other immune diagnostic methods have also been developed; however, the false-positive and false-negative cases were poorly described and analysed (4). This study evaluated the false-positive and false-negative results based on two commercialized human hydatid diagnostic kits (immunogold filtration assay and enzyme-linked immunoblot assay), which are widely used in the clinical laboratories in Xinjiang, China. The patients with false immune diagnosis were selected for histopathological analysis to illuminate the possible of reasons and mechanism for differential diagnosis.

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MATERIAL AND METHODS

Patients with nature-unknown cysts (n = 239)

These patients received the surgery in the First Affiliated Hospital of Xinjiang Medical University for their nature-unknown cysts in different organs. Before the surgery and other medical intervention, their sera were collected. The clinical information and the surgical pathology were also recorded.

Healthy control (n = 30)

Healthy controls are 30 volunteer adults, 17–32 years, residents in Xinjiang since birth, no contact with animals of hydatid disease. They are classified as normal control by physical and ultrasound examinations.

It is a perspective study from 2000 to 2003. All persons included signed the informed consent for their serum to be collected and assessed in the study. Ethical permission was granted by the Xinjiang Medical University Hospital Ethical Committee.

Serum samples

Serum samples were obtained from the above patients and controls in the First Affiliated Hospital of Xinjiang Medical University. The blood was collect in untreated test tube. Then, the blood was incubated undisturbed at room temperature for 20 min, centrifuged at 1000 g for 10 min at 4°C. The supernatant was stored at –80°C.

Pathology analysis

Pathological diagnosis is the golden standard for final diagnosis to evaluate the accuracy of immunodiagnosis or US. During surgery, the cysts were removed, measured and then fixed in 10% neutral buffered formalin prior to paraffin processing. Sections were stained with H&E and assessed microscopically by pathologists.

The immunological assays

Two human hydatid diagnostic kits (immunogold filtration assay and enzyme-linked immune absorbent assay based on four different purified hydatid antigens) were purchased from Base-Ming Biotech, (Xinjiang, China). The four native antigens are crude and partially purified hydatid cyst fluid extracts from *Echinococcus granulosus* (EgCF and AgB), *E. granulosus* protoscolex extract (EgP) and *Echinococcus multilocularis* metacestode antigen (Em2). In brief, antigens were purified by affinity chromatography using a normal human serum coupled to CNBr–Sepharose 4B to remove nonspecific host reactive proteins from sheep hydatid cyst fluid. Together with a quality control (diluted normal human sera), antigens EgCF, EgP and AgB, and antigen Em2 were coated 1 μL/dot onto nitrocellulose (NCP) paper (pore size 0.45 μm; EMD Millipore, Billerica, MA, USA) fixed in a plastic frame. Twenty millilitre serum drops onto the NCP. Then, the well was rinsed with three drops of washing buffer. Finally, colloidal gold-conjugated anti-human IgG antibody solution was added. The red dot indicates positive result. The whole procedure takes 3 min and can be read by eyes. When DIG-FA test is finished, the degree of positive colour change was subjective and judged between + to ++++, according to the colour-darkness level compared with the quality control. When ELISA is finished, the ELISA plates were read at 450 nm with a Bio-Rad 550 plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Positive control sera from confirmed CE or AE patients, and negative control sera from healthy individuals, were used in each microtitration plate for quality control. Sera were tested in duplicate, and the positive–negative cut-off value was determined as the mean optical density of a panel of negative controls (n = 35) plus three standard deviations (OD cut-off for EgCF = 0.286; EgP = 0.609; AgB = 0.105; Em2 = 0.187). Sensitivity and specificity were calculated using 95% confidence intervals, and significance values were also determined at the 95% probability level.

Humoral immune response measurement

The level of immunoglobulin can help to decide whether excessive or deficient immunoglobulin was produced. The concentrations of total IgM, IgA and IgG were determined by immunoturbidimetry (Array System 360; Beckman Coulter, Inc., Brea, CA, USA).

Ultrasound examination

Each patient underwent abdomen sonography with conventional B mode ultrasound with a 7 MHz multifrequency transducer. All images were stored digitally.

Statistical analysis

Statistical analysis was performed with spss 15.0 for windows (SPSS, Chicago, IL, USA). Quantitative variables were expressed as means ± SD and analysed by one-way ANOVA followed by tukeys test. Results were considered statistically significant at P < 0.05.
RESULTS

The comparison of surgical pathology and immunological diagnosis

The surgical pathology was used as golden standard to evaluate the accuracy of the immune diagnosis (Table 1). The clinical features of the 239 patients and their clinical outcome were followed up. The cases with false immune diagnosis were summarized and shown in Tables 2 and 3. Among 12 false-negative cases, eight patients had hydatid cysts with necrosis or calcification in liver. Two hydatid cysts were in lungs, one in thyroid gland, one in brain and one in abdomen. Among 12 false-positive patients, one patient was

Table 1 Evaluation of the (a) efficacy of immunological assays (b) efficacy of ultrasound

| Surgical pathology | + | – | Total number |
|--------------------|---|---|-------------|
| (a) Immune test    | 147 | 12 | 159 |
| Total number       | 159 | 80 | 239 |
| (b) Ultrasound     | 152 | 5  | 157 |
| Total number       | 159 | 80 | 239 |

(a) Surgical pathology was used as golden standard to evaluate the accuracy of immunological assays. Sensitivity of immunological assays (commercialized DIGFA and ELISA) was 92.4% (147/159), specificity is 85% (68/80), false-negative rate is 7.5% (12/159), false-positive rate is 15% (12/80), accuracy is 89.9% [(147 + 68)/239]. (b) Surgical pathology was used as golden standard to evaluate the accuracy of ultrasound. Sensitivity of ultrasound was 95.6% (152/159), specificity is 93.7% (75/80), false-negative rate is 4.4% (7/159), false-positive rate is 6.3% (5/80), accuracy is 95% [(152 + 75)/239].

Table 2 Clinical information of the false-negative cases

| No. | Sex  | Age | Surgical pathology |
|-----|------|-----|--------------------|
| 1   | Male | 50  | Single hydatid cyst in right lobe of liver. A mass of necrosis |
| 2   | Male | 25  | Single hydatid cyst in liver, full of yellow and sticky necrosis |
| 3   | Female | 27 | Single hydatid cyst in liver. Adhere to phrenic muscle |
| 4   | Male | 69  | Single hydatid cyst in low lobe of right lung. Inner cyst had infected, broken and necroses |
| 5   | Female | 55 | Multiple hydatid cysts both in liver and lung. Inner cyst had broken and degenerated |
| 6   | Female | 15 | Hydatid cyst in abdomen, malignant tumour in ovary. Hydatid cyst had calcificated |
| 7   | Male | 68  | Single hydatid cyst in left lobe of liver full of necrosis |
| 8   | Female | 34 | Hydatid cyst in thyroid gland. Deep in thyroid gland |
| 9   | Female | 42 | Single hydatid cyst in lung |
| 10  | Female | 30 | Single hydatid cyst in right lobe of liver. Deep in liver, the wall is thick, protoscoleces were found in puncture |
| 11  | Male | 11  | Hydatid cyst in brain |
| 12  | Male | 31  | Hydatid cysts with necrosis in right lobe of liver |

Table 3 Clinical information of the false-positive cases

| No. | Sex  | Age | Results of operation and pathology |
|-----|------|-----|----------------------------------|
| 1   | Male | 41  | Cysticercosis in brain |
| 2   | Male | 41  | Malignant tumour in mediastinum |
| 3   | Female | 63 | Carcinoma of the lung |
| 4   | Male | 23  | Granuloma in socket of the left eye |
| 5   | Female | 30 | Peritonitis caused by tuberculosis |
| 6   | Female | 60 | Cholelithiasis, multiple cysts in left lobe of liver |
| 7   | Female | 7  | Metastatic tumour in mediastinum |
| 8   | Male | 66  | Multiple cysts in kidney and liver |
| 9   | Female | 38 | Carcinoma of the lung |
| 10  | Male | 32  | Lipoma in upper lobe of lung |
| 11  | Male | 26  | Metastatic tumour in mediastinum |
| 12  | Female | 30 | Pulmonary tuberculosis |

Figure 1 Immunoglobulin levels in the sera with false immune diagnosis. Humoural immune response was measured by the levels of immunoglobulin IgM, IgA and IgG by immunoturbidimetry in 30 healthy controls, 12 false-positive and 12 false-negative cases. Compared with the healthy control, the sera with false immune diagnosis showed no significant difference in IgA and IgM. The false-positive cases indicate significantly higher IgG and the false-negative cases the lower IgG (P < 0.05).
surgically confirmed the cysticercosis, two tuberculosis, two benign cysts and seven malignant tumours.

**The humoral immune response**

A quantitative immunoglobulin test was used to detect whether abnormal levels of the three major classes of Ig (IgG, IgA, and IgM) in blood caused the false immunodiagnosis. The total IgM, IgG and IgA in 30 healthy controls, 12 false-positive and 12 false-negative cases were summarized in Figure 1. Compared with the healthy control, the sera with false immune diagnosis showed no significant difference in IgA or IgM. The false-positive cases showed statistically higher IgG (17.3 ± 3.2) compared with healthy control (13.7 ± 5.2). The false-negative cases indicate lower IgG (11.4 ± 3.5) (P < 0.05). Considering IgG varies between 8 and 18 mg/mL in normal population, the difference above is of little clinical significance.

**The histopathology study of the cases with false-negative immune diagnosis**

A typical hydatid cyst demonstrates the germinal layer, which produce the active antigens. In the false-negative cases, the hydatid cyst was surrounded by a parasite-derived thick laminated layer, which separates the parasite from host and caused the negative immune result. The ruptures of cyst were found in the CE with higher IgG level (Figure 2).

**The measurement of cyst thickness and diameter**

The cyst thickness and diameter were measured to see whether the physical parameters of the cyst affect the immunodiagnosis. The measurement of 121 cysts dissected in the surgery from 82 patients indicates the mean cyst thickness was 0.2871 ± 0.0103 mm. Mean diameter was 6.2 ± 3.3 cm. A probable correlation between the diameters of cysts or the wall thickness was searched for by analysing the data, but no significant correlation was found.

**The sensitivity and specify of the fast immunodiagnosis and US**

Surgical pathology was used as golden standard to evaluate the accuracy of immunological assays. Sensitivity of immunological assays (commercialized DIGFA and ELISA) was 92.4% (147/159), specificity is 85% (68/80), false-negative rate is 7.5% (12/159), false-positive rate is 15% (12/80), accuracy is 89.9% [(147 + 68)/239].

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**The concordance analysis of the fast immunodiagnosis and US**

In the screen of ultrasound, the typical hydatid cyst manifest as a single or multiple well-defined cysts. Other diagnostic illustrations include sand sign, daughter cysts, floating membranes inside the cavity and water lily sign.

Seven cases that surgical pathology confirmed CE but ultrasound missed were reviewed. Five cases were CE in organs outside the abdomen: brain (one), lungs (two), spines (one) and skin (one). CE lesions in these cases locate outside the abdomen and so the abdominal ultrasound showed negative results. X rays, MRI and CT detected the lesion. Immunodiagnosis also detected these five cases with positive results.

There were two cases of CE with total calcification. Ultrasound showed small calcified shadowing in liver and mesentery. The radiologists cannot tell the exact pathogen and suggest further examination. Immunodiagnosis missed these two cases too because of the inactive of parasite.

Five cases that ultrasound misdiagnosed as CE were also reviewed. They were primary hepatocellular carcinoma (two), colon tumour metastasis in liver (two) and liver abscess (one). For these five cases, the immunodiagnosis was negative and accurately ruled out CE.

**DISCUSSION**

Parasite can evoke host immune response, so immunological test is one of the important methods to diagnose...
human hydatid disease (5). But false-negative and false-positive results in immunological test bring difficulties to clinical diagnosis (6). There are many studies evaluating different immune tests, while little clinical follow-up has been carried out to figure out the reasons the mechanism of false positive and false negative (7–10). In this study, 239 sera from patients with nature-unknown cysts and 30 healthy controls were tested to screen the sera with false-positive and false-negative immune diagnosis.

Most of the immunological assays for CE are set up according to the antigen–antibody reorganization. So we first test the host total immunoglobulin to see whether the individual variations in the response come from the level of immunoglobulin.

The IgA, IgG and IgM level in 30 healthy controls, 12 false-positive and 12 false-negative cases showed no significant difference in IgA and IgM. Compared with the healthy control, the false-positive cases had significant higher IgG and the false-negative cases the lower IgG (P < 0.05). It indicates the host immune function is the inner factor for the accuracy of immune tests. Exhaustion of complement, acceleration of decomposition of antibody, production of autologous antibody, disorder of endocrine system and malnutrition inhibit hosts immunological response directly or indirectly (11).

Reasons for the false-negative immune diagnosis

Host tolerance
In the history review of our 147 hydatid patients, parasite cyst growth was highly variable and ranges from 3–5 cm a year. The period between first infection and clinical manifestations is also variable and often prolonged from 1 to 20 years. Among 147 cases, there were 86 cases who acquired parasite infestations in the childhood, and the clinical symptoms appeared in their adulthood. The slowly growing hydatid cyst made the host immune system tolerant to it.

Necrosis of the hydatid cyst
In 12 cases of false negative, there were eight cases whose cysts had degeneration, necroses, consolidation or calcification. There are many causes that can lead to necrosis of cysts. The host-produced inflammation can damage laminated layer, destroy germinal layer and prevent protoscoleces from proliferation. The dead parasite cannot stimulate the host for antibody production, and CE lesion was limited inside the host-produced fibrosis (12).

Location and thickness of the cyst
In the 147 cases with accurate immune diagnosis, 70.7% had hydatid cysts in liver (104/147). Other organs involve-ments are lung (17%), brain (3-4%), bone (2%), heart (2%), spleen (1-4%), kidney (1-4%) and other organs (2.1%). In 12 false-negative cases, two cysts locate in lungs, one cyst locate in thyroid gland, one cyst locate in brain and one cyst locate in abdomen. The hydatid disease has high appetency in liver but low immune reaction when the cyst forms in the lung, brain and other organs (13). The major hydatid antigen is 60 kDa while the haematoencephalic barrier only allows molecules <40 kDa to get past. The histopathology also showed hydatid cysts with thick capsule in the cases with false-negative result. The measurement of 121 cysts from 82 patients indicates the cyst thickness is 0.2871 ± 0.0103 mm. Thick wall of cysts made a special protective fence, which can separate the parasite from hosts immune system and help the parasite escape from hosts immune surveillance (14).

Low age
Different age group showed different immune reaction intensity in immunological response (15). In 12 false-negative cases, two children suffered from single hydatid cysts that did not degenerate, the diameter is more than 3 cm, but the immune test showed negative result. Blood supply is abundant in children’s livers, so hydatid cyst can grow up easily while their immune systems are not mature enough to produce strong immunological response as adults do (16–18).

Mechanism analyses of low humoral immune response
Humoural immunity protects people from infection by producing antibodies that target foreign material such as parasite. The cellular immunity kills foreign material by releasing cytokines and toxins. When analysing the false immunodiagnosis, immunoglobulin was tested to see whether the host has the immune response that produces and secretes antibodies to a specific antigen.

The false-negative group showed a lower IgG level compared with healthy control. IgG in suppressed responses are lower than 2 mg/mL. Considering that IgG vary between 8 and 18 mg/mL in normal population, therefore the differences could not be really significant with bigger samples (whereas in this study, the different groups analysed are small samples). So in this study, the data showed the IgG, IgA and IgM were not clinically significant to explain the false immunodiagnosis. Maybe a follow-up of the IgG titration could better demonstrate such a possible difference.

Almost all kinds of parasites can stimulate host immune reaction and injury. The host immune reaction may be specific or unspecific, and it can reduce hosts ability to resist parasite so that parasite could have a possible
E. granulosus responsible for the immunomodulatory activities of major immunodominant antigens and are thought to be protein, and especially AgB, a 160-kDa lipoprotein, are the for adapting to its host. Antigen 5, a 67-kDa glycoprotein, and especially AgB, a 160-kDa lipoprotein, are the major immunodominant antigens and are thought to be responsible for the immunomodulatory activities of E. granulosus, promoting its survival within a mammalian host.

The hydatid cyst fluid of echinococcosis granulosis has strong immunogenity, but the complete hydatid cyst can grow safely in organs without causing obvious immune reaction (23). The wall of the cyst acts as the protective fence against attack from hosts immune system (24, 25).

**Reasons for the false-positive immune diagnosis**

**Previous exposure in high endemic area**

Xinjiang is the high endemic area of hydatid disease. Two hundred and thirty-nine patients and 30 healthy controls have been lived in Xinjiang for decades of years. The low-positive titres in false-positive sera may have been a result of previous exposure to parasite.

**Cross-reaction**

Echinococcosis has a high cross-reaction with cysticercosis (26). In 12 false-positive cases, there is a patient suffering cysticercosis in brain. Patients with cysticercosis have serologic cross reactions with echinococcosis. Western blot will help to differentiate. Individual sera from patients with either cysticercosis or echinococcosis were analysed using the immunoblot (26).

Cysticercosis is caused by infection with the cysticercus of the tapeworm Taenia solium. WB can detect the cross-reacting bands (120,105, 62, 54, 40, 38 and 12 kDa). These conservative proteins caused misdiagnosis of immune test between cysticercosis and hydatidosis. The 26 kDa protein band of cysticercus antigen is unique band to diagnose cysticercosis and has no cross-reaction with hydatidosis.

**Antigen purification**

Twelve cases of false-positive result occurred in patients with tumours or tuberculosis. The sensitivity and specificity of serological tests relies on the quality of antigen, the titre for positivity and possibly the strain of the parasite concerned (27).

Among 12 false-positive patients, seven were malignant tumours. The immune misdiagnosis between CE and tumours suggests there is an antigenic similarity between E. granulosus and neoplasm (28, 29). It happened also between bacteria and certain tumour cells. These false-positive reactions could cause by the autoantibodies which react with human host protein components found in hydatid fluid antigen.

As a noninvasive method, ultrasound plays the important role in diagnosing, staging and follows up of CE. Compared with the immune test, ultrasound can not only diagnose CE but also determine the cyst location, number, dimension and biological activity by imaging features. Not all patients with CE have a detectable immune response. Accuracy of immunodiagnosis depends on the activity of the echinococcal antigens inside cysts; there were some general correlations between ultrasound examinations and immunodiagnosis. The intact cysts with complete thick wall can elicit a minimally detectable response, whereas previously ruptured or leaking cysts present stronger immune responses. The presence of clear anthracitic fluid or daughter cysts with scolexs showed strong immune positive. The muddy jelly-like fluid, degenerated germinative membrane and the calcification cyst tend to show the false-negative immunodiagnosis.

The current false-positive cases did not exclude the presence of echinococcal cyst in other parts of the body (different from the part that underwent surgery). Maybe patients with abdominal tumours had also echinococcal cysts in the spleen, in the kidney or in other parts of the body. It is hard to tell when we could not do radiological examination. This could be a big limitation of the accuracy of the results.

In summary, hydatid cysts develop mainly in the liver lung and brain, but in fact, all organs and tissues such as bone, skin, spleen, et al. may be affected. The clinical diagnosis of CE requires a combination of ultrasonography and immunodiagnosis. The immunoglobulin has little influence on false immunodiagnosis. The false-negative immunodiagnosis was caused by the cysts inactive status while the false positive caused by previous rupture, antigen cross-reaction. CE must be differentiated from benign cysts, caviar tuberculosis, mycoses, abscesses, and benign or malignant neoplasm. The ultrasound is helpful for detecting and defining the extent and condition of...
vascular fluid-filled cysts in most organs. It is also valuable for pre-operative staging of the lesion; it is a complementary examination for immunodiagnosis.

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