Electronic Journal of Biotechnology 29 (2017) 57–62

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Application of a single-chain fragment variable (scFv) antibody for the confirmatory diagnosis of hydatid disease in non-endemic areas

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ARTICLE INFO

Article history:
Received 3 June 2017
Accepted 7 July 2017
Available online 13 July 2017

Keywords:
COS cells
Cysts
Echinococcosis
Hepatomegaly
Liver cyst
Novel human scFv antibody
Parasitic disease
Public health
Single-chain antibodies

ABSTRACT

Background: Hydatid disease is a serious parasitic disease threatening public health. Because of its rarity in non-endemic coastal areas, determining the nature and origin of a chronic, enlarged liver cystic mass is challenging in these regions. Under these circumstances, physicians need a confirmatory diagnostic tool beyond immunological and radiological examinations. This study investigated a novel human single-chain fragment variable (scFv) antibody for the confirmative diagnosis of 18 atypical hydatid disease cases in non-endemic coastal areas.
Results: A scFv antibody against cystic echinococcosis was produced by genetic engineering and then applied to the immunohistochemical diagnosis of 18 cases of cystic echinococcosis presented in non-endemic coastal areas. The diagnosis of these cases by ultrasound and serum-based examinations was inconclusive. The 750 bp scFv antibody gene was expressed in COS-7 cells, and the antibody localized in the cytoplasm. The scFv antibody can detect the germinal layer and protoscolices of actively growing cysts but not of the degenerating protoscolices and has a diagnostic efficiency higher than that of single serum or ultrasound testing ($P < 0.05$). The combined use of scFv antibodies with serology and ultrasound diagnostics results in a diagnostic efficiency comparable to that of surgery. The scFv antibody can be used as a confirmatory test for the diagnosis of hydatid disease in non-endemic areas, providing a beneficial supplementary diagnostic method that complements traditional immune testing and ultrasonic radiology and thus helping physicians to effectively differentiate hydatid disease.

1. Introduction

Hydatid disease, one of the world's most geographically widespread zoonoses, is caused by infection with the larval stage of the tapeworm *Echinococcus*. Hydatid disease has historically been most prevalent in rural areas or developing countries, especially in some husbandry communities in Western China [1]. Although uncommon, echinococcosis-related hospital admissions are currently more frequent in non-endemic areas such as the US and Eastern China because of infection by wild animals and transmission by immigrant populations. In fact, hydatid disease was not previously endemic in the US, but changes in immigration patterns and a marked increase in transcontinental transportation in recent decades has caused an increased incidence of this disease throughout North America. An investigation into the distribution and prevalence of *Echinococcus* in the US showed that this parasite is found in Northern and Central Indiana, Northwest Ohio and East-Central Illinois, where 15 wild canids (6.3%) were found to be infected. Further studies of gastrointestinal parasitic infections in the US showed that *Echinococcus* infection rates among all parasitic diseases were 50% and most commonly found in Afghan immigrants [2,3]. In addition to its high mortality rate, cystic echinococcosis (CE) also has a high economic burden; an analysis by the World Health Organization estimates that this disease results in the loss of at least 1 million disability-adjusted life years annually, possibly up to 3 million [4].

Diagnosis in non-endemic areas is challenging. Many cases lack typical clinical characteristics, show negative results in serology and show unfeatured single cysts by imaging approaches. Misdiagnosis and delayed treatment might be alleviated by detection using a specific antibody. Physicians must be particularly cautious when evaluating foreign-born patients from endemic areas of hydatid

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2017.07.003

disease as a specific antibody capable of confirmatory diagnosis is not yet available. Because in coastal areas it is impossible to collect natural antigen or antibodies from infected sheep or dogs, genetic engineering technology provides a feasible way to produce antibodies without parasite raising.

Selection of phage-derived engineered antibodies against target antigens has the potential to generate monoclonal antibodies with new specificities. Antibody fragments selected from synthesized human single-chain variable fragment (scFv) libraries represent a useful resource because they do not cause the harmful immune responses that can be elicited by murine monoclonal antibodies created by hybrid cells. Compared with total IgG antibodies, scFv antibodies have several advantages including a lower molecular weight and improved thermostability, solubility and diffusion. As a result, scFv antibodies are of great importance in drug delivery, in vivo therapy and immunohistochemistry-based diagnosis [5].

In this study, a specific scFv antibody was produced and used in clinical immunohistochemistry to evaluate its diagnostic efficacy. The 18 CE cases analysed here were found in non-endemic areas where physicians often have diagnostic difficulties using traditional ultrasound and serology-based testing.

2. Materials and methods

2.1. Expression and identification of scFv genes

scFv genes were cloned into the expression vector pCANTAB 5E and expressed in *Escherichia coli* HB2151 and COS-7 cells. SDS-PAGE followed by western blotting and immunohistochemistry were used to identify scFv gene expression.

2.2. Ethnic statement

All procedures performed were approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments.

2.3. Clinical characteristics of the patients

From 2004 to 2017, 18 patients underwent cyst resection surgery in Hangzhou, Shaoxing, Jingning and Shenzhen, which are non-endemic coastal cities and areas that have prevalent echinococcosis. Patients were migrants from endemic areas of hydatid disease. Half the patients presented with intermittent right upper-quadrant abdominal pain, while the others were asymptomatic. In these patients, a lesion of unknown nature was found in the liver upon physical examination. Upon clinical examination, both temperature and blood pressure were in the normal range. Further abdominal examination revealed a diffuse tenderness and mild hepatomegaly in a few cases, but no guarding, rigidity or rebound tenderness was detected. Laboratory results revealed an increased leukocyte count. A liver function panel showed increased enzyme levels and a mild increase in bilirubin, but serum amylase and lipase levels, urinalysis, chest X-ray and electrocardiogram results were within normal limits. During an exploratory laparotomy, intraoperative frozen sections were produced, and pathology slides were subsequently incubated with scFv antibodies. Informed consent was obtained from all participants.

2.4. Ultrasound

The sonography machines used in this study were portable ultrasound scanners (Echo Camera; Aloka, Tokyo, Japan). Ultrasound images were classified according to the recommendations made by the World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) (available on the WHO website (http://whqlibdoc.who.int/hq/2001/WHO_CDS_CSR_APH_2001.6.pdf).

2.5. Serum immunogold filtration assay

Sera were tested to assess the levels of a specific IgG antibody against *Echinococcus*. Serodiagnosis was performed using an immunogold filtration assay as previously described [6]. In brief, antigens were coated on nitrocellulose membranes, and diluted serum was added. After washing with Tris-HCl, membranes were incubated with colloidal gold-conjugated anti-human IgG antibody. In human cystic hydatid patients, the test band will turn red because of the antigen–antibody reaction from the colloidal gold conjugate, whereas in sera from patients without hydatid disease, the test band does not change colour. The intensity of the red colour indicates the degree of immune reaction.

2.6. Immunohistochemistry

scFv antibodies were applied to pathological slides of human hydatid cysts. Samples and slides were processed according to a manual, and the presence of scFv antibodies in tissue was revealed using an ABC Kit (Vector Laboratories, CA, USA). Briefly, tissue sections were fixed for 10 min with acetone at −20°C, and endogenous peroxidases were blocked with 0.2% HCl in ethanol for 15 min. After two washes with Tris-buffered saline, the slides were blocked with sheep serum and then incubated for 2 h in 10 μg/ml scFv antibodies. Slides were then washed and incubated for 1 h with scFv and anti-E tag Antibody (Pharmacia 1:1000 in 1% BSA in PBST). Diaminobenzidine (50 μg/ml in Tris-buffered saline) was added, and the reaction was stopped after 5 min by washing in tap water.

2.7. Statistics

Results were expressed as the mean ± SD. *P* values less than 0.05 were considered statistically significant. One way ANOVA, Student's *t*-test and chi-square test were used to compare data sets using the SPSS software (version 17.0; SPSS, USA).

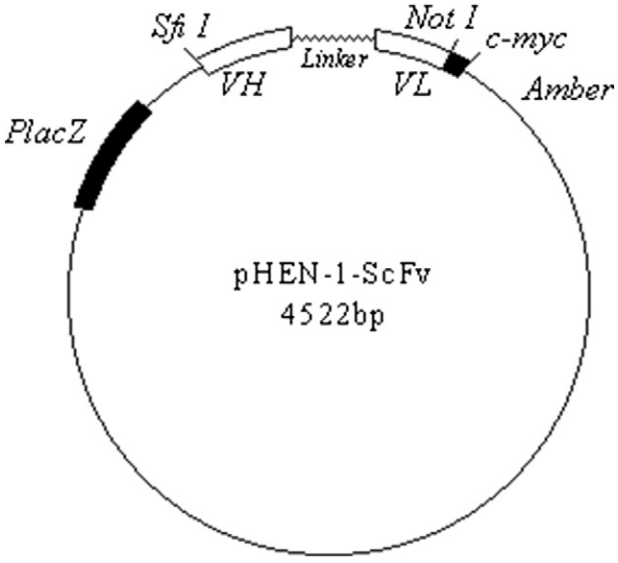

Fig. 1. Construction of the scFv antibody gene. The scFv antibody gene was constructed in the phagemid vector pHEN1. The variable regions of the human heavy- and light-chain immunoglobulin genes were arranged in a single polypeptide configuration. The VH and VL genes were randomly cloned into the phagemid, enabling the expression of scFv as a fusion protein with the minor coat protein of the filamentous phage.

3. Results

3.1. Identification of scFv antibodies

scFv antibodies were selected from a semisynthetic antibody phage display library constructed using the phagemid vector pHEN1 (Fig. 1). The gene encoding the scFv antibody was 750 bp after PCR amplification and SfiI/NOTI digestion (Fig. 2). It can be expressed in COS-7 cells (Fig. 3), and the scFv fusion protein expressed in *E. coli* HB2151 cells was stable (Fig. 3).

3.2. Inability of ultrasound, serum immunology and histology to reach a clear diagnosis

Patient clinical characteristics are shown in Table 1. These patients originated from coastal areas in Eastern and Southern China, which are non-endemic areas for hydatid disease. Ultrasound detected liver lesions but was unable to discriminate the nature of the lesions. Moreover, a serum immunological examination showed a weak positive signal that was not sufficient to confirm or exclude hydatid disease (Fig. 4).

3.3. Utility of scFv antibodies for the pathological diagnosis of human hydatid disease

scFv antibodies were used for immunohistochemical staining. Positive staining can be observed in the germinal layer and protoscolices (but not in degenerating protoscolices), indicating that scFv antibodies recognize human hydatid cysts (Fig. 5).

3.4. Diagnostic efficiency of scFv antibodies

A comparison of the diagnostic efficiency of scFv antibodies with non-invasive methods is shown in Table 2. When comparing (1) scFv antibodies and ultrasound, there was a significant difference ($X^2 = 4.433498$, $P = 0.035240$); (2) scFv antibodies and serology, there was a significant difference ($X^2 = 5.785714\ 8$, $P = 0.01615$); and (3) scFv antibodies and ultrasound + serology, there was no significant difference ($X^2 = 1.125000$, $P = 0.288844$). scFv antibodies showed higher diagnostic efficiency than the individual use of ultrasound or serology and have comparable diagnostic efficiency to the combined use of ultrasound and serology.

A comparison of scFv antibodies with surgery is shown in Table 3. When comparing scFv antibodies and surgery, there was no significant

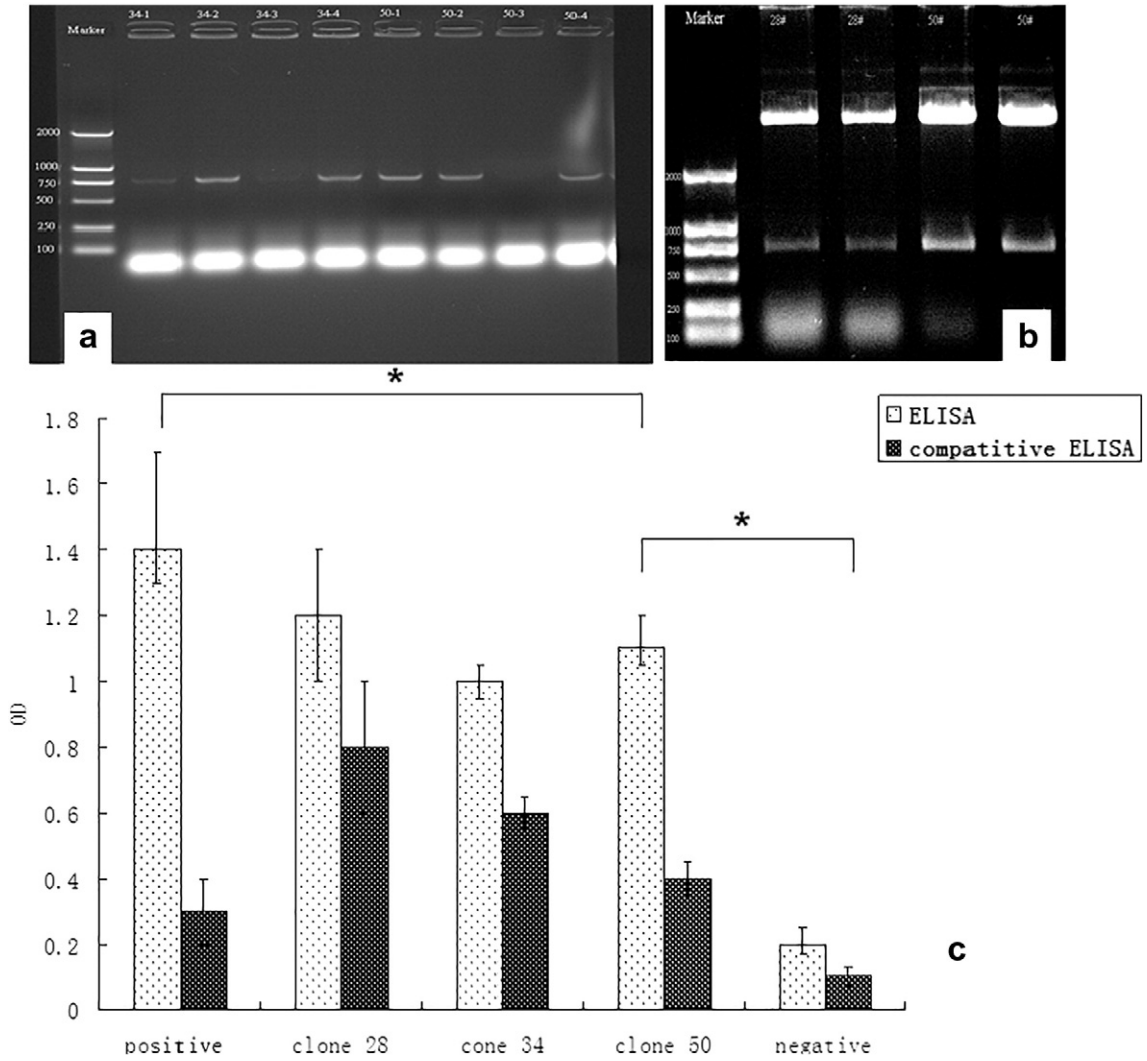

Fig. 2. Confirmation of scFv antibody identity and specificity using enzyme digestion, PCR and ELISA. The VH and VL genes encoding the scFv antibody were amplified by PCR (a). Plasmid DNA containing the scFv antibody gene was digested using the specific endonucleases SfiI and NotI (b). Three clones showed positive binding to EgAgB (c). Clone 50 had high binding and specificity and was thus selected for further DNA sequencing. Absorbance values represent the mean ± standard deviation of three independent measurements.

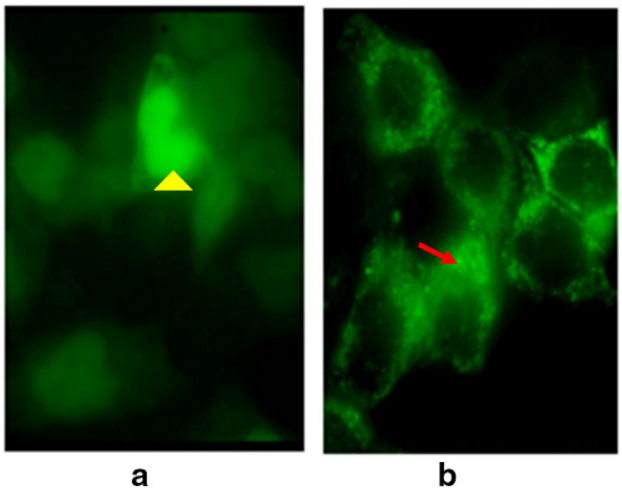

Fig. 3. Expression and localization of the scFv antibody in eukaryotic cells. The scFv antibody was inserted into the scFv-pEGFP-N3 plasmid and then transfected into COS-7 cells. The scFv proteins were synthesized in COS-7 cells and localized in the cytoplasm. COS-7 cells lacking scFv expression (indicated by a yellow triangle in Fig. 3a) and COS-7 cells expressing scFv (indicated by a red arrow in Fig. 3b) were visualized by the fluorescence intensity of the green fluorescent fusion protein. scFv proteins fused to the green fluorescent protein displayed strong and uniform green fluorescence in a ring-like pattern around the nucleus.

difference in diagnostic efficiency ($X^2 = 1.0285718$, $P = 0.310494$). Surgery has higher diagnostic efficiency than either ultrasound or serology alone and a comparable diagnostic efficiency to scFv antibodies.

Infiltration immunological kits provide a rapid blood test to detect individual parasite antigens and can replace traditional assays such as indirect hemagglutination and immunoelectrophoresis, but their sensitivity in calcified or lung lesions is low. The major concern with immunological testing is specificity. Positive reactivity can be caused by cross-reactive antigens in cysticercosis, liver cirrhosis or hepatocellular carcinoma. For this reason, immunological examinations have traditionally used radiological methods for confirmation. Often, ultrasound detects cysts of unclear origin that are defined as cystic lesion (CL) type. The CL type of liver hydatid cyst lacks typical morphological characteristics to differentiate it from other malignant or benign lesions in liver and requires further evaluation before being classified as CE.

Table 1
Clinical features of 18 patients with hydatid disease in liver.

Parameters	Grouping	n
Total cases	Cystic hydatid disease in liver	18
Age (yrs.)	<60	15
	≥60	3
Gender	male	11
	female	7
Ethnic group	Han	8
	Kazak	5
	Uighur	5
Ultrasound classification*	CL	12
	CE1	1
	CE2	1
	CE3	1
	CE4	2
	CE5	1
Location	liver	15
	Liver + lung	2
	Liver + abdomen	1
Serum immunology	positive	13
	Weak positive	3
	negative	2

* The ultrasound classification proposed by the World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) (available on WHO website (http://whqlibdoc.who.int/hq/2001/WHO_CDS_CSR_APH_2001.6.pdf).

4. Discussion

The scFv recombinant antibody of human origin used in the present study can specifically recognize human hydatid disease on the basis of the presence of parasite antigens expressed in CE. Previous reports have described the application of scFv antibodies for the diagnosis of parasitic diseases [7,8]. This study explores the feasibility of using scFv antibodies to CE in pathological diagnosis.

CE is a zoonosis that affects both humans and domestic livestock. Human, the intermediate host, carries a fluid-filled hydatid cyst that can survive for decades without being rejected by the host's immune system. The continuous growth of this parasitic cyst may cause severe pressure or rupture when it is located in the liver, lung or brain. In China, echinococcosis is mainly found in rural provinces such as Xinjiang, Inner Mongolia, Tibet, Gansu, Qinghai, Ningxia and Sichuan. One national survey revealed that the average incidence of human echinococcosis in endemic areas is 1.08%. Because of increased human movement resulting from globalization, non-endemic areas such as Eastern and Southern China are now under serious threat of echinococcosis [9,10].

Because of their human origin, scFv antibodies can be used to deliver contrast agents for parasitic localization or to enhance the dissemination of anti-hydatid disease medicine. scFv antibodies have several desirable characteristics, rendering them attractive alternatives to monoclonal antibodies for experimental and therapeutic purposes [11]. Monoclonal antibody production is typically based on hybridoma methodology that requires the regular maintenance of unstable cell cultures, a process that is time-consuming and financially costly [12].

Hydatid disease is a previously unusual disease not found in South China, but with globalization, immigration and population transfer, the occurrence is rising throughout mainland China, resulting in an urgent need for physicians to be armed with new methodologies for confirmative diagnosis. Because of biohazard concerns, storing the natural antigens/antibodies is not common. Thus, the production of recombinant antibodies from antibody libraries is a strategy to overcome this limitation. Here, a scFv based on phage display biotechnology is presented. In contrast to current contaminable natural antigens/antibodies used in pathology, our molecular bioengineering scFv can avoid contamination from infected animals or parasite. In addition to reducing the biohazard risk, it also shows other advantages: (1) large quantity production, (2) soluble single-chain antibody, (3) removal of the undesired phage particle and fusion protein and (4) conjugatable to the antigen/medicine/radiant of interest. However, the main challenge in radiological diagnosis is differentiating the target lesion from other lesions that have similar cystic appearance. Therefore, if an ELISA is combined with radiological examinations, a more accurate diagnosis can be made before the surgery.

In this study, the variable region of a human immunoglobulin gene was cloned into a phage library and expressed on the surface of the phage as a fusion protein with a coat protein. This approach enabled the in vitro isolation of human monoclonal antibodies with the desired specificity and overcomes the abovementioned problems associated with the production of monoclonal antibodies by hybridomas. Importantly, these scFv antibodies are of human origin and can thus potentially be used for both diagnostic and therapeutic purposes in humans.

5. Conclusion

Because of increased globalization, physicians are now more likely to encounter CE in non-endemic areas. This infection is poorly characterized by single serologic or ultrasound examinations, causing diagnostic difficulty in differentiating it from focal liver lesions. In this study, we developed a novel scFv antibody as a diagnostic tool to improve the diagnostic efficiency of hydatid disease. In non-endemic

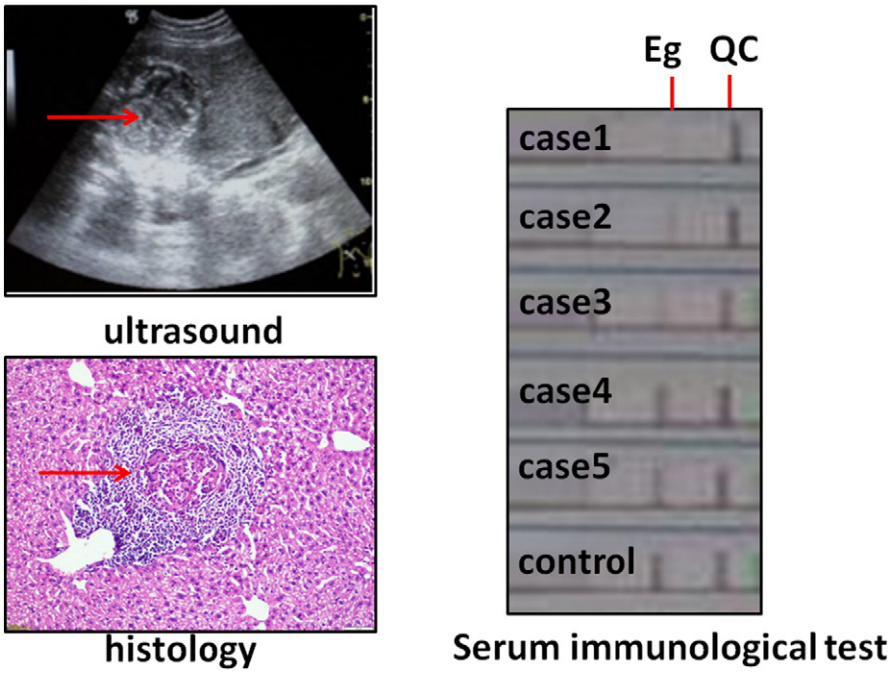

Fig. 4. Immunohistochemical staining using the scFv antibody in *E. granulosus*-infected human tissues. For 18 patients encountered in non-endemic areas, the traditional methods of ultrasound, serum immunology and histology failed to reach a conclusive diagnosis. Ultrasound analysis revealed a solid lesion but could not determine the nature. Similarly, histology indicated a cystic lesion of uncertain origin. Serology for an *E. granulosus* hydatid antigen was weakly positive and could not confirm or exclude hydatid disease. QC indicates the quality control band, which verifies that the test system was in good condition. These routine methods were unable to arrive at a definite diagnosis.

areas where the parasite antigen collection is not available, the cloned scFv antibody can be used as an additional pathological test to determine the identity of a chronic enlarged cystic mass in the liver.

Financial support

This work was supported by the Natural Science Foundation of China (81372425) and the Xinjiang Key Lab Project (2014KL002). These funding sources have no conflicts of interest regarding study design, data collection or analysis.

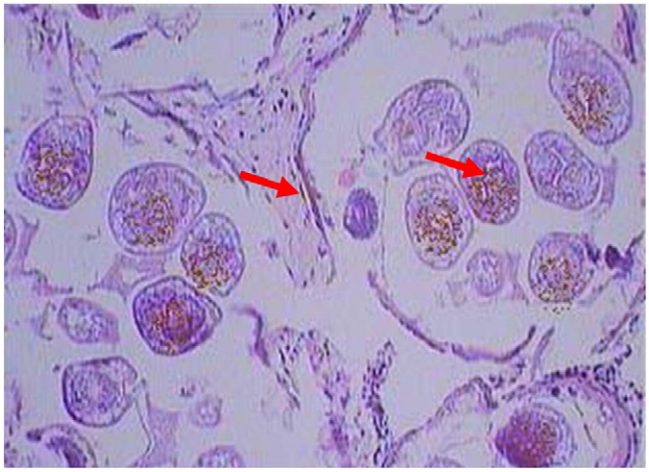

Fig. 5. Immunohistochemical staining of human hydatid disease using the scFv antibody. The scFv antibody recognizes the human hydatid cyst when used for immunofluorescence staining. Strong fluorescence intensity was detected in the germinal layer and protoscolices of actively growing cysts but not in destroyed or degenerated protoscolices.

Conflict of interest

The authors declare no conflict of interest. No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

Acknowledgments

The authors are grateful for the kind support of Dr. Hao Wen and Dr. Xinyu Duan from The State Key Laboratory Incubation Base of Xinjiang Major Diseases and Xinjiang Key Laboratory of Echinococcosis.

Table 2
Diagnostic efficiency comparison of scFv and non-invasive methods.

	Positive case	Uncertain cases	X^2	*P*
scFv	17	1		
Ultrasound	12	6	4.433498	0.035240[#]
Serology	11	7	5.785714	0.016157[#]
Ultrasound + serology	15	3	1.125000	0.288844

The comparisons: (1) scFv vs ultrasound; (2) scFv vs serology; (3) scFv vs ultrasound + serology.
[#] $P < 0.05$ when compared with scFv.

Table 3
Diagnostic efficiency comparison of scFv and surgery.

	Positive case	Uncertain cases	X^2	*P*
Surgery	18	0		
Ultrasound	12	6	7.200000	0.007290[*]
Serology	11	7	8.689655	0.003200[*]
ScFv	17	1	1.028571	0.310494
Ultrasound + serology + scFv	18	0		

The comparisons: (1) surgery vs ultrasound; (2) surgery vs serology; (3) surgery vs scFv; (4) surgery vs ultrasound + serology + scFv.
[*] $P < 0.05$ compare with Surgery.

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