Pakistan J. Zool., vol. 46(5), pp. 1249-1254, 2014

Establishment and Optimization of Two-dimensional Electrophoresis Technique in Hydatid Fluid Proteome of *Echinococcus granulosus*

Juyi Li[1], Xiufang Wang[2], Yi Wang[1*], Zhaoqing Zhang[2], Ling Chen[3] and Jinhu Wu[3]
[1]*Department of Pharmacy, The Central Hospital of Wuhan, Wuhan, Hubei Province, China.*
[2]*Department of Rehabilitation, The Third Hospital of Wuhan, Wuhan, Hubei Province, China.*
[3]*Department of Pharmacy, The Third Hospital of Wuhan, Wuhan, Hubei Province, China.*

Abstract - The aim of this study was to establish and optimize the two-dimensional electrophoresis (2-DE) technology for hydatid fluid proteome of the *Echinococcus granulosus*, and obtain 2-DE map of hydatid fluid proteome. Total proteins of hydatid fluid were extracted by lyophilization. A series of important factors, such as sample preparation, protein quantities, pH range of immobilized pH gradient (IPG) strip and extraction methods, were optimized to improve the resolution and repeatability. Two-dimensional electrophoresis maps were analyzed after staining. The 2-DE profiles with high resolution and good repeatability were obtained, when the hydatid fluid dealt with ReadyPrep™ 2-D Cleanup Kit were analyzed with the established 2-DE using 400μg of quantitative loading and IPG strips pH7-10. We identified 30 protein spots using PDQuest 8.0 2D analysis software. The molecular weight of most of these proteins ranged from 43 to 97kDa and the isoelectric points of these proteins ranged from 5 to 9. An optimized 2-DE system is set up successfully in this study, electrophoresis pattern of which shows a high resolution and good repeatability, and can be used for the study of *E. granulosus* proteomics effectively.

Key words: Proteomics, cestode, immobilized pH gradient, cystic echinococcosis

INTRODUCTION

Cystic echinococcosis (CE) is a zoonosis caused by the larval form of the cestode *Echinococcus granulosus* (Li and Zhao, 2012). The domestic life cycle of *E. granulosus* is maintained through definitive host and intermediate hosts. The adult form lives in the small intestine of a carnivore (definitive host). A wide range of mammal species (intermediate host), including humans, may acquire the infection through accidental ingestion of eggs containing infective oncospheres of *E. granulosus*. The egg hatches in the intestinal tract and the oncosphere is freed, migrates through the intestinal wall, and gains access via the circulatory system to the muscles, brain and other tissues of the host, where it transforms into the metacestode form or cysticercus. The cyst of cysticercus forms a relatively stable internal environment to avoid damage to the larvae from the host immune system (Li *et al*., 2013). The disease has a worldwide distribution, with a considerable impact on both human and animal health and causes important socio-economic consequences in endemic areas (Shi *et al*., 2009).

Hydatid-cyst fluid (HCF) is a clear or clear yellow liquid with antigenic properties and an important component of the internal environment and fills the entire cyst. HCF provides needed nutrition for larval growth, playing an important role in their life cycle of *E. granulosus*. Previous studies focused on livestock such as sheep and cattle. Shepherd and McManus (1987) discovered five major subunit antigens of relative molecular mass (Mr) 12,000, 16,000, two at 20,000, and 38,000 in HCF through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from *E. granulosus*. Zhu *et al.* (1989) used improved two-dimensional polyacrylamide electrophoresis to identify 111 proteins in the liver HCF of infected sheep , and 130 HCF proteins were identified from fertile cysts of sheep and human origin and infertile cysts from cattle (Aziz *et al*., 2011). Chemale *et al.* (2003) attempted to analyze proteins in liver HCF of cattle but failed to establish

* Corresponding authors: Yi Wang 694531611@qq.com

0030-9923/2014/0005-1249 $ 8.00/0

a 2-DE database because of the effect of highly abundant albumin and immunoglobulin.

HCF is a complex biological mixture containing a wide range of proteins of both parasite and host origin, playing an important role in the life cycle of *E. granulosus*, however, the research on HCF proteins is limited, because of the effect of highly abundant albumin and immunoglobulin (Chemale *et al*., 2003). Therefore, it is very necessary to find effective methods to dissociate and identify the proteins of HCF, and it may help reveal new candidates for immunodiagnosis and vaccines.

Proteomics offers a set of tools for investigating proteins from HCF. Thus, a series of important factors, such as sample preparation, protein quantities, pH range of IPG strip and extraction methods, were optimized to improve the resolution and repeatability, to establish and optimize the 2-DE technology for hydatid fluid proteome of the *E. granulosus*, and obtain satisfied 2-DE map of hydatid fluid proteome, which stands as a valuable resource for further hydatid fluid proteomics research of *E. granulosus*.

MATERIALS AND METHODS

Purification of hydatid-cyst fluid (Li et al.*, 2013)*

Human HCF was collected after the surgical removal of fertile cysts from patients with cystic hydatid disease in The General Hospital of Ningxia Medical University. In total, 21 cysts of different sizes were isolated in a germ-free environment, and 55ml of cyst fluid was aspirated from these cysts using sterile needles under aseptic conditions and centrifuged at 10,000g for 15min at 4°C to remove particles. The supernatant fluid was stored at -80°C until use. This study had ethical approval and all subjects gave informed consent to participate.

2D Sample preparation

First, 20 mL of cyst liquid was pre-frozen at -85°C for 5 h and then place in the freeze-dryer (-60°C vacuum) overnight. After 24h, the cyst liquid was freeze-dried into a powder. The powder was lysed using lysis buffer [9mol/L urea, 4% 3-(3-cholamidopropyl) dimethyl-ammonio-1-propane sulfonate (CHAPS), 1% dithiothreitol (DTT), and 0.5% protease-inhibitor cocktail], fully oscillated and blended at 4°C for 1h, and centrifuged at 12,000g at 4°C for 30min. The final supernatant fluid was stored at -85°C until use. TCA-Acetone Extraction: the above supernatant was added directly to an equal volume of 20% TCA in acetone. Proteins were precipitated overnight for at least at -20°C. Precipitated protein was centrifuged at 12,000g at 4°C for 30min, washed three times in 1mL in ice-cold acetone, centrifuged at 12,000g at 4°C for 15min, the precipitation were re-solubilized in immobilized pH gradient (IPG) rehydration buffer.

To remove salt, lipids, and undesired detergents, cleanup was performed with the ReadyPrep™ 2-D Cleanup Kit (BIO-RAD).

The Aurum™ Serum Protein Mini Kit (BIO-RAD) was used to remove the most abundant proteins.

The protein pellets were re-solubilized in IPG rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 50mM DTT, 0.2% Bio-Lyte ampholyte, 0.001% bromophenol blue) and then centrifuged. The supernatant was held at 4°C. The total protein concentration for each sample was determined using the Bradford assay. Bovine serum albumin was used at 9 different concentrations (0.25, 0.5, 1, 1.5, 2, 3, 4, 5 and 6 mg/mL) to prepare a protein standard. After diluting the protein standards, the stock dye reagent was prepared [500 mg Coomassie Blue was dissolved in 500 mL methanol and was added to 100 mL phosphoric acid and 50 mL double-distilled water H_2O (ddH_2O)] that was diluted in 8 mL ddH_2O. A total of 2 mL of dye reagent was added to each tube of protein standard and was incubated at room temperature for at least 5 min. Absorbance of the protein standards and experimental samples were carried out by the spectrophotometry (Bausch and Lomb, Germany) at 595 nm and finally a standard curve was plotted (Assady *et al*., 2011).

Electrophoresis (Li et al.*, 2013)*

Areas of the 17cm IPG strips (BIO-RAD) were wetted with the above sample in a rehydration tray, and mineral oil was added to prevent evaporation. The isoelectric focusing (IEF) program was set as follows: step 1: 250V (linear) for 30min; step 2: 500V (rapid) for 1h; step 3: 4,000V (linear) for 4h; step 4: 4,000V (rapid) for 30,000Vh; and step 5: 500V (rapid) for 20h (holding step). A total

of 2mL of equilibration buffer I [6mol/L urea, 0.375mol/L Tris-HCL (pH 8.8), 20% glycerol, 2% SDS, 2% DTT] was added to the top of the strip in an 11-cm equilibration tray, followed by gentle rocking for 15min. Equilibration buffer was discarded and replaced with equilibration buffer II [6mol/L urea, 0.375mol/L Tris-HCL (pH 8.8), 20% glycerol, 2% SDS, 2.5% iodoacetamide], followed by gentle rocking for 15min. IPG was loaded onto an SDS-PAGE gel composed of a 12% separation gel.

Gel staining, imaging, and image analysis

The proteins were stained using Coomassie Brilliant Blue. The image was scanned using Bio-Rad GM800 Calibrated Densitometer scanner, Spot numbers were measured analyzed using Bio-Rad PDQuest software, version 6.2.1.

RESULTS

Comparison of protein extraction methods

In the untreated sample (Fig. 1A), the host serum proteins remain relatively abundant, there was no boundaries between protein spots, the gel showed obvious streaking and high background. To the whole protein extract, that was precipitated using ReadyPrep™ 2-D Cleanup Kit (Fig. 1B), compared to Figure 1A, the protein spots were more clear, and the striations were less. To the whole protein extract, that was precipitated using Aurum™ Serum Protein Mini Kit (Fig. 1C), there was almost no protein spots in the map. And to the whole protein extract, that was precipitated using TCA-Acetone (Fig. 1D), there were more single protein spots.

Comparison of pH range of IPG strip

The molecular weight of most of these proteins ranged from 43 to 97kDa and the isoelectric points of these proteins ranged from 5 to 9 (Fig. 1). Compared with pH3-10 IPG strip, the proteins separation was more effective with pH7-10 IPG strip (Fig. 2).

Comparison of protein loading amount

With 400μg of quantitative loading and IPG strips pH 7-10 (Fig. 3D), the proteins spots were more evident than with 200μg of quantitative loading and IPG strips pH7-10 (Fig. 3C), but the gel showed obvious streaking and high background. With 400μg of quantitative loading and IPG strips pH 3-10 (Fig. 3B), the proteins spots were more evident and larger than with 200μg of quantitative loading and IPG strips pH 3-10 (Fig. 3A), but the proteins spots were less than with 800μg of quantitative loading and IPG strips pH3-10 (Fig. 3E). Lastly, with 200μg of quantitative loading and IPG strips pH 3-10 (Fig. 3E) was observed obvious streaking and high background.

DISCUSSION

Two-dimensional electrophoresis technology is the most effective method to isolate the proteins of organizations with complex components and is one of the main technologies for proteomics research. The 2-DE profiles with high resolution and good repeatability are prerequisite to analyze and identify the proteins of *E. granulosus*. In this study, a series of important factors, such as sample preparation, protein quantities, pH range of IPG strip and extraction methods, were optimized to improve the resolution and repeatability to obtain satisfied 2-DE map of hydatid fluid proteome for further hydatid fluid proteomics research of *E. granulosus*.

HCF proteins are composed of 44% albumin, 39% α-globulin and β-globulin, and 17% γ-globulin (Zhao, 1987), therefore, if the highly abundant proteins were not removed, it could interfere the detection and analysis for the low abundant proteins. When the whole protein extract was precipitated using Aurum™ Serum Protein Mini Kit, there was almost no protein spots in the map, it suggested that the low abundant proteins were removed along with the highly abundant proteins, so the Aurum™ Serum Protein Mini Kit was not suitable for establish the 2-DE map. Using the TCA Acetone Extraction methods, the 2-DE map gets better resolution and repetition, indicating that TCA-Acetone Extraction method is suitable for the 2-DE map. Compared with pH3-10 IPG strip, the proteins separation was more effective with pH7-10 IPG strip and it obtained more single proteins spots. With more proteins quantitative loading, more

proteins spots were obtained, however, with highly

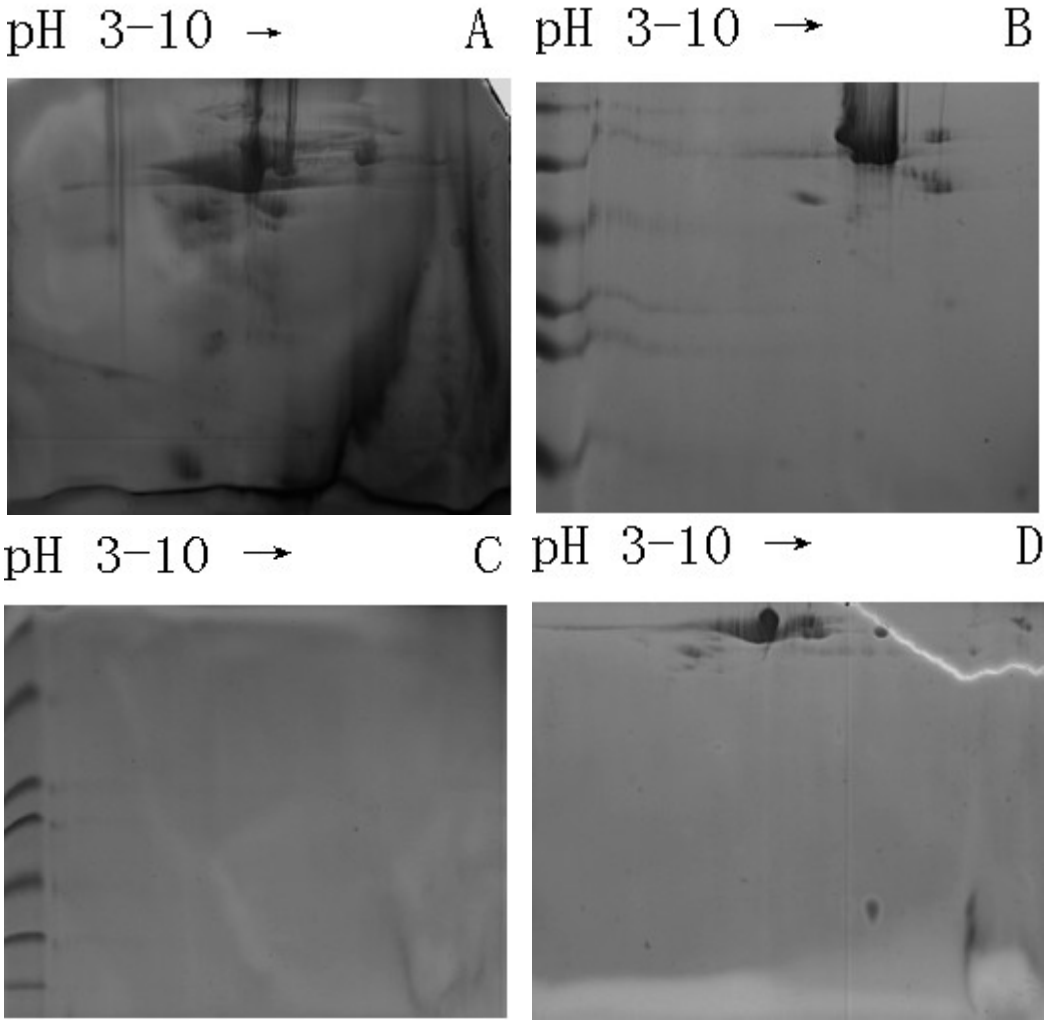

Fig. 1. A comparison of hydatid-cyst fluid protein extracted by different extraction methods using two-dimensional electrophoresis from *Echinococcus granulosus*
A, Untreated sample; B, Sample was treated using ReadyPrep™ 2-D Cleanup Kit; C, Sample was treated using Aurum™ Serum Protein Mini Kit; D, Sample was treated using TCA-acetone.

proteins quantitative loading, the gel showed obvious streaking and high background and the highly abundant proteins were too large to cover other important proteins with similar molecular weight and isoelectric point, and with lower proteins quantitative loading, it was not suitable for detect the low abundant proteins which might play an important role in the life.

In this study, we employed different loading amount proteins with 200μg, 400μg, 800μg, and the results showed that 2-DE map was more beautiful with loading amount proteins with 400μg than others. The study showed that the 2-DE profiles with high resolution and good repeatability were obtained, when the hydatid fluid dealt with ReadyPrep™ 2-D Cleanup Kit were analyzed with the established 2-DE using 400μg of quantitative loading and IPG strips pH7-10.

Antigen 5 (Ag5) and antigen B (AgB) were identified from *E. granulosus* hydatid cyst fluid, these two antigens are considered the most important antigens for the serodiagnosis of human

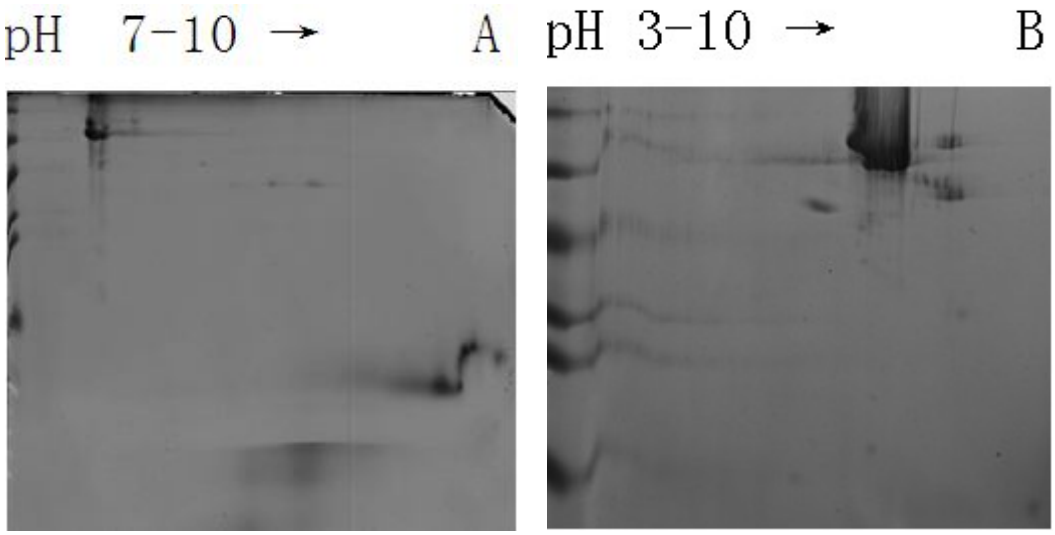

Fig. 2. A comparison of hydatid-cyst fluid protein with different pH range of IPG strip using two-dimensional electrophoresis from *Echinococcus granulosus*
Am pH7-10 IPG strip; B, pH3-10 IPG strip

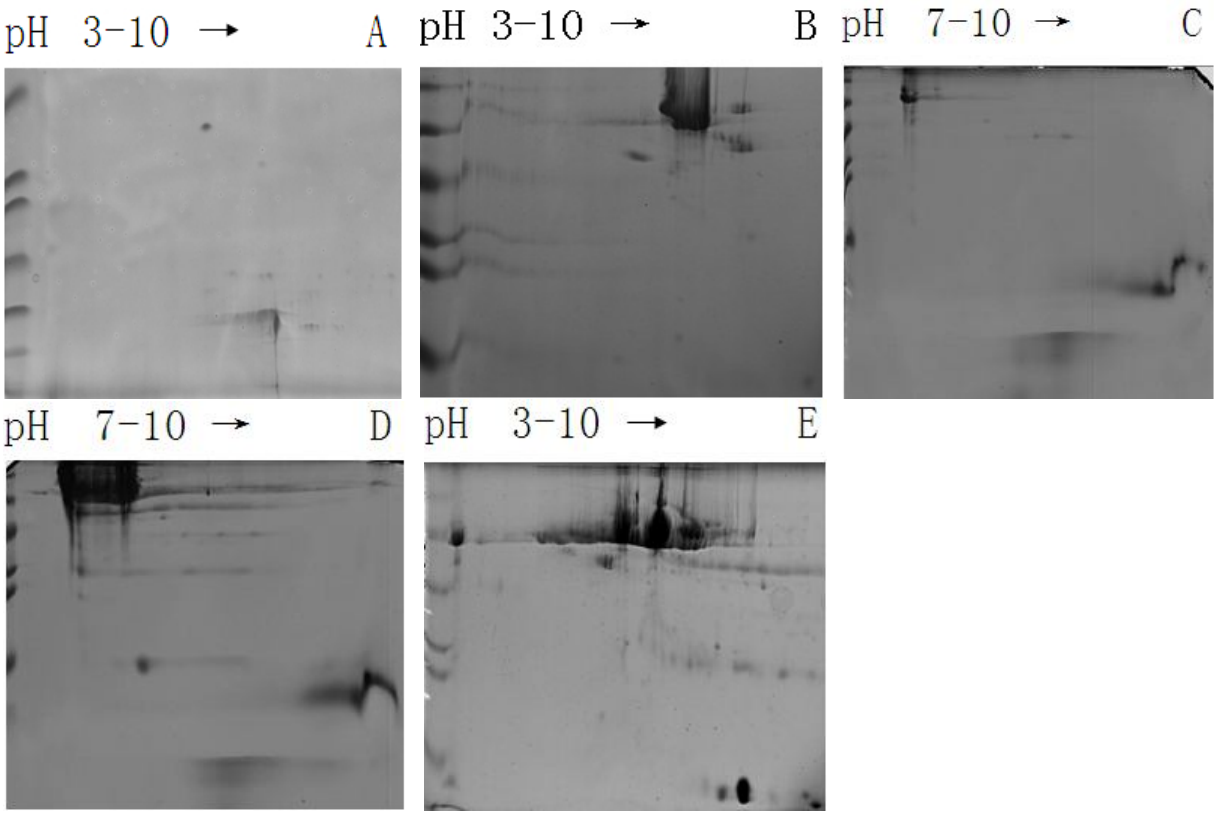

Fig. 3. A comparison of hydatid-cyst fluid protein with difference loading amount using two-dimensional electrophoresis from *Echinococcus granulosus*
A/C, 200μg of quantitative loading; B/D, 400μg of quantitative loading; E, 800μg of quantitative loading.

CE (Verastegui *et al*., 1992; Rott *et al*., 2000; Mamuti *et al*., 2002). AgB is strongly immunogenic in patients with echinococcal infections, about 80 to 90% of serum samples from CE patients and 40% of serum samples from AE patients exhibit specific antibodies against this antigen (Maddison *et al*., 1989; Siles-Lucas and Gottstein, 2001; Mamuti *et al*., 2002). AgB possesses the highest diagnostic value among these antigens (Gonzalez-Sapienza *et al*., 2000).

The 2-DE in hydatid fluid proteome of *E. granulosus* was successfully established with high quality map of separation under the optimization of the experimental conditions, which stands as a valuable resource for further hydatid fluid proteomics research of *E. granulosus*.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (Nº 30960360).

Conflict of interest declaration

The authors declare that there is no conflict of interest.

REFERENCES

ASSADY, M., FARAHNAK, A., GOLESTANI, A. AND ESHARGHIAN, M., 2011. Superoxide dismutase (SOD) enzyme activity assay in *Fasciola* spp. parasites and liver tissue extract. *Iran J. Parasitol*., **6**: 17-22.

AZIZ, A., ZHANG, W., LI, J., LOUKAS, A., MCMANUS, D.P. AND MULVENNA, J., 2011. Proteomic characterisation of *Echinococcus granulosus* hydatid cyst fluid from sheep, cattle and humans. *Proteomics*, **74**: 1560-1572.

CHEMALE, G., VAN ROSSUM, A.J., JEFFERIES, J.R., BARRETT, J., BROPHY, P.M., FERREIRA, H.B. AND ZAHA, A., 2003. Proteomic analysis of the larval stage of the parasite *Echinococcus granulosus*: Causative agent of cystic hydatid disease. *Proteomics*, **3**: 1633-1636.

GONZALEZ-SAPIENZA, G., LORENZO, C. AND NIETO, A., 2000. Improved immunodiagnosis of cystic hydatid disease by using a synthetic peptide with higher diagnostic value than that of its parent protein, *Echinococcus granulosus* antigen B. *J. Clin. Microbiol*., **38**: 3979-3983.

LI, J.Y., JU, Y., WANG, X.F., ZHANG, Z.Q., LI, J.L., ZHU, M.X. AND ZHAO, W., 2013. Analysis of the chemical components in hydatid fluid from *Echinococcus granulosus*. *Rev. Soc. Bras. Med. Trop.*, **46**:605-610.

LI, Z.J. AND ZHAO, W., 2012. Analysis of protoscoleces-specific antigens from *Echinococcus granulosus* with proteomics combined with Western Blot. *Biomed. Environ. Sci*., **25**: 718-723.

MADDISON, S.E., SLEMENDA, S.B., SCHANTZ, P.M., FRIED, J.A., WILSON, M. AND TSANG, V.C., 1989. A specific diagnostic antigen of *Echinococcus granulosus* with an apparent molecular weight of 8 kDA. *Am. J. trop. Med. Hyg*., **40**: 377-383.

MAMUTI, W., YAMASAKI, H., SAKO, Y., NAKAYA, K., NAKAO, M., LIGHTOWLERS, M.W. AND ITO, A., 2002. Usefulness of hydatid cyst fluid of *Echinococcus granulosus* developed in mice with secondary infection for serodiagnosis of cystic Echinococcosis in humans. *Clin. Diagn. Lab. Immunol*., **9**: 573-576.

ROTT, M.B., FERNANDEZ, V., FARIAS, S., CENI, J., FERREIRA, H.B., HAAG, K.L. AND ZAHA, A., 2000. Comparative analysis of two different subunits of antigen B from *Echinococcus granulosus*: gene in *Escherichia coli* and serological evaluation. *Acta Trop*., **75**: 331-340.

SHEPHERD, J.C. AND MCMANUS, D.P., 1987. Specific and cross-reactive antigens of *Echinococcus granulosus* hydatid cyst fluid. *Mol. Biochem. Parasitol*., **25**: 143-154.

SHI, Z.Y., WANG, Y.N., LI, Z.J., LI, Z.Y., BO, Y., MA, R. AND ZHAO, W., 2009. Cloning, expression, and protective immunity in mice of a gene encoding the diagnostic antigen P-29 of *Echinococcus granulosus*. *Acta biochim. biophys. Sin.*, **41**: 79-85.

SILES-LUCAS, M.M. AND GOTTSTEIN, B.B., 2001. Molecular tools for the diagnosis of cystic and alveolar echinococcosis. *Trop. Med. Int. Hlth*., **6**: 463-475.

VERASTEGUI, M., MORO, P., GUEVARA, A., RODRIGUEZ, T., MIRANDA, E. AND GILMAN, R.H., 1992. Enzyme linked immunoelectrotransfer blot test for diagnosis of human hydatid disease. *J. clin. Microbiol*., **30**: 1557-1561.

ZHU, C.L., YE, B.H., ZHU, X.L., ZHAO, X.Z., HUANG, J. AND ZHANG, J.Y., 1989. 2-D electrophoresis preliminary research of hydatid cyst fluid, scolex and cyst wall. *Chinese J. Zoon.*., **5**: 27-29.

ZHAO, W.X., 1987. *Human parasitology*. People's Medical Publishing House, Beijing, China.

(Received 20 February 2014, revised 18 June 2014)