Gene cloning, expression and serological evaluation of the 12-kDa antigen-B subunit from *Echinococcus granulosus*

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A 12-kDa subunit of antigen B from *Echinococcus granulosus* has recently been cloned, expressed and used in diagnostic ELISA to test human sera for evidence of cystic echinococcosis. The performance of the ELISA based on the recombinant antigen (rAgB) was compared with that of similar assays based on native antigen B (nAgB) or hydatid-cyst fluid.

For the preparation of the rAgB, total RNA was extracted from *Ec. granulosus* protoscoleces so that antigen-B complementary DNA could be synthesised, amplified by PCR, and then cloned into the pQE30 expression vector. The recombinant plasmid was transformed in *Escherichia coli* and induced using isopropyl-β-D-thiogalactopyranoside. Bacterial samples were collected, lysed and then analysed by SDS–PAGE and western blotting. The recombinant protein was purified by affinity chromatography.

Although the performance of the ELISA based on cyst fluid appeared identical to that of the assay based on the recombinant antigen (with a sensitivity, specificity, positive predictive value and negative predictive value of 96.0%, 97.0%, 97.2% and 95.5%, respectively), the corresponding results for the ELISA based on nAgB (98.6%, 100%, 100% and 98.5%) were slightly better. Despite this difference (which was not statistically significant), the comparative ease with which large quantities of the recombinant antigen could be produced make the antigen a potentially useful tool in the diagnosis of cystic echinococcosis.

Human cystic echinococcosis is one of the most important helminthic diseases throughout the world (Budke *et al.*, 2006; Torgerson and Deplazes, 2009) and regarded as endemic in Iran (Rokni, 2009; Sadjjadi, 2006). Canids act as the definitive hosts of the causative parasite (*Echinococcus granulosus*) while sheep and several other mammalian species act as the natural intermediate hosts. Humans become infected by ingesting the parasite’s eggs after the eggs have been excreted by an infected definitive host.

There is, as yet, no established ‘gold standard’ for the diagnosis of cystic echinococcosis, and attempts are still being made to optimise various immuno–serological methods. Indirect haemagglutination, standard and counter immuno–electrophoresis, latex agglutination, immunoblotting and ELISA have all been used (Irabuena *et al.*, 2000; Rokni and Aminian, 2006; Rokni *et al.*, 2006; Sari *et al.*, 2009). The antigens employed in these tests have mostly been extracted from hydatid-cyst fluid, with ‘antigen B’ (AgB) currently considered to be among the most promising in terms of both efficacy and validity. A thermostable
lipoprotein, AgB resolves as three bands, of 8/12, 16 and 24 kDa, in SDS–PAGE and immunoblotting (Lightowlers et al., 1989; Maddison et al., 1989; Leggatt and McManus, 1994; González et al., 1996; Carmenta et al., 2006). The unique immunogenicity of AgB in the diagnosis of cystic echinococcosis (in comparison with other components of hydatid fluid, such as ‘antigen 5’) has persuaded many researchers to use the antigen for various serological tests, including ELISA — currently the most widely used method of diagnosis (Lightowlers et al., 1989; Leggatt and McManus, 1994; Haniloo et al., 2005; Rokni and Aminian, 2006).

Although the literature contains some reports on the cloning of complementary-DNA (cDNA) sequences that encode an antigen-B subunit (Shepherd et al., 1991; Frosch et al., 1994; Rott et al., 2000; Monteiro et al., 2008), it is commonly believed that strain variations in the parasite may significantly affect serological responses to the recombinant antigens so produced. Replication and reappraisal of assays involving such antigens, in different countries, led to the realisation that a ‘new’ antigen, purified from a ‘new’ strain of *Ec. granulosus* (of the Iranian G1 strain) that had been collected from a naturally infected sheep. First-strand cDNA was then synthesised in a 20-μl reaction mixture containing 2 μl 10× reverse-transcriptase buffer (Fermentas, Vilnius), 0.5 mM of each deoxyribonucleotide triphosphate (dNTP), 1 μM random primers (Promega, Dübündorf, Switzerland), 10 U RNase inhibitor (Fermentas) and 4 U Omniscript™ reverse transcriptase (Fermentas). After incubation for 2 h at 37°C, the reverse transcriptase was inactivated by heating the mixture to 93°C for 5 min.

The cDNA was amplified in PCR, using the primers EgAgB F (5’-AAG CTT ATG CTT CTC GCT CTG GCTC-3’) and EgAgB R (5’-CTC GAG CTA TTT ACC TTC AGC AACG AACC-3’), which were based on a nucleotide sequence for AgB (GenBank accession Z26336.1) and contained *HindIII* and *XhoI* restriction sites to facilitate the subsequent cloning steps. The 30-μl reaction mixture for the PCR contained healthy volunteers at Tehran University of Medical Sciences, and a further 18 blood samples, from patients confirmed (by ELISA, stool examination, and/or IFAT) to have toxoplasmosis (N=4), strongyloidosis (N=4), visceral leishmaniasis (N=5) or tuberculosis (N=5), were obtained from the serum bank of the Tehran School of Public Health. Sera (separated off from the fresh blood samples or from the serum bank) were and used in the ELISA (see below).

Informed consent was obtained from each subject who provided a blood sample for the present study, and the study protocol was approved by the Human Ethics Committee of the School of Public Health at Tehran University of Medical Sciences.

**RNA Extraction, Cloning and Gene Expression**

The RNeasy Protect mini kit (QIAGEN, Las Matas, Spain) was used, according to the manufacturer’s instructions, to extract total RNA from the protoscoleces of *Ec. granulosus* (of the Iranian G1 strain) that had been collected from a naturally infected sheep. First-strand cDNA was then synthesised in a 20-μl reaction mixture containing 2 μl 10× reverse-transcriptase buffer (Fermentas, Vilnius), 0.5 mM of each deoxyribonucleotide triphosphate (dNTP), 1 μM random primers (Promega, Dübündorf, Switzerland), 10 U RNase inhibitor (Fermentas) and 4 U Omniscript™ reverse transcriptase (Fermentas). After incubation for 2 h at 37°C, the reverse transcriptase was inactivated by heating the mixture to 93°C for 5 min.

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**SUBJECTS AND METHODS**

**Clinical Samples and Ethics**

Blood samples were collected from 72 patients with surgically-confirmed cystic echinococcosis, at several hospitals in Tehran, Iran. For comparison, blood samples were collected from 48, apparently
20 pmol of each primer, 1.25 U Taq DNA polymerase (CinnaGen, Tehran) and 0.5 μl of a solution containing 10 mM of each dNTP. The thermocycler was set to give 30 cycles, each of 30 s at 98°C, 30 s at 50°C and 30 s at 72°C, followed by a final extension for 5 min at 72°C.

The 231-bp amplification product from the PCR was purified using a commercial gel extraction kit (Fermentas), sequenced in an ABI Prism® 377 sequencer (Applied Biosystems, Carlsbad, CA) and cloned into the pTZ57R/T plasmid vector (Invitrogen, Barcelona, Spain) using T4 ligase. The ligation products were transformed in competent *Escherichia coli* M15 (Stratagene, La Jolla, CA), which were then distributed on LB agar plates containing 100 μg ampicillin/ml, 20 μg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/ml, and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The white bacterial colonies that developed were screened for recombinant plasmids. Selected colonies were mass cultured before the recombinant plasmids were extracted using alkaline lysis and then digested with *Hind*III and *Xho*I (Fermentas). The released fragments (AgB) were purified by electrophoresis and then subcloned into the PQE30 expression vector with T4 DNA ligase (Roche Pharma). The ligation product was used to transform the recombinant plasmid into *Es. coli* M15. Selected transformants were grown in YT medium at 37°C for 14–16 h, with gentle shaking. The resulting cultures were each diluted 1:100 in YT medium and grown for a further 5 h under the same conditions. Protein expression was induced with 1 mM IPTG. Bacteria were recovered by centrifugation before the recombinant protein was extracted from the pelleted cells by sonication.

**Protein Purification**

The recombinant protein was purified by affinity chromatography, based on its *N*-terminal His tag, using Ni-NTA His-Bind® resin (Invitrogen). The purity and integrity of the recombinant protein were checked by electrophoresis in 15% (w/v) polyacrylamide gels and staining with Coomassie Brilliant Blue G-250. The concentration of antigen in the solution was determined in a bio-photometer (Eppendorf, Hamburg, Germany) before the solution was frozen at −80°C until use.

**Preparing Native and HCF Antigens**

The native AgB was prepared as reported earlier (Oriol et al., 1971) while hydatid-cyst fluid was extracted from cysts collected from naturally infected sheep at the local slaughterhouse (Rokni et al., 2006).

**SDS–PAGE and Immunoblotting**

Bacterial cell lysate was subjected to electrophoresis on a 15% SDS–PAGE gel and blotted onto a polyvinylidene difluoride membrane (U-CyTech Elispot; Aniara, Utrecht, The Netherlands). Membranes were probed using a 1:100 dilution of a pool of sera from five cases of cystic echinococcosis or a 1:500 dilution of a mouse anti-His monoclonal antibody (Invitrogen). Goat anti-human-IgG conjugated to horseradish peroxidase (Abcam, Freiburg, Germany), with diaminobenzidine/H₂O₂ as substrate, was used to visualize the antigen–antibody reactions.

**ELISA**

The ELISA were performed as described by Bora et al. (2002), with rAgB, nAgB or cyst fluid used as antigen. The purified recombinant protein was dialysed, for 2 h at 4°C, against 0.1 M carbonate–sodium bicarbonate buffer (pH 9.5), both to exchange the chromatography elution buffer for the ELISA coating buffer and to remove urea. The absorbance of the protein solution after dialysis was determined at 280 nm before the solution was diluted up to 20-fold with coating buffer to give an absorbance of 0.07 (equivalent to a concentration 2 μg/ml, which was previously determined, by serial antigen dilution, to give the optimum
amount of antigen for coating). The diluted rAgB or similar solutions of nAgB (at 10 μg/ml) or HCF (at 10 μg/ml) in the coating buffer were dispensed into the flat-bottomed wells of 96-well MaxiSorp™ plates (Nunc, Roskilde, Denmark) and left overnight at 4°C. After three washes with 0.1 M phosphate-buffered saline (PBS; pH 7.2) containing 0.05% (v/v) Tween-20 (Sigma), each well was filled with a 1:200 dilution of a test serum before the plates were incubated for 1 h at 37°C. After another three washes, the conjugate of goat anti-human-IgG (γ-chain) with horseradish peroxidase (Sigma) was added at a dilution of 1:10000. After another incubation for 1 h at 37°C and then six washes, the o-phenylenediamine solution (0.4 mg/ml; Sigma) used as substrate was added. After incubating for 10 min, the reaction was stopped by adding 0.5 M H₂SO₄. The absorbance of the contents of each well was then read at 492 nm, on an ELISA reader (Tecan, Männedorf, Switzerland), with a 620-nm reference filter.

### Statistical Analysis

In the ELISA, the cut-off value for positivity was calculated, for each type of antigen, as the mean absorbance plus 3.09 S.D. recorded among the sera from the healthy participants. Sensitivity, specificity and positive and negative predictive values were calculated, separately for each of the three types of antigen, as described by Galen (1980). All the statistical analyses were performed using version 11.0 of the SPSS for Windows software package (SPSS Inc., Chicago, IL).

### RESULTS

The PCR produced a 500-bp amplicon from the recombinant plasmid and a 200-bp one from the non-recombinant plasmid (Fig. 1). The recombinant plasmid was purified by affinity chromatography and confirmed by restriction analysis, with the products explored by SDS–PAGE and western blotting (Fig. 2).

### Evaluation of Antigens by ELISA

All 138 sera were tested in ELISA based on the rAgB, nAgB and HCF, with absorbances of 0.22, 0.36 and 0.31 used as the respective thresholds for positivity.

As shown in Figure 3, the ELISA based on rAgB and HCF each gave three false-negatives and two false-positives for cystic echinococcosis, giving these two types of assay identical results in terms of sensitivity (96.0%), specificity (97.0%), positive predictive value (97.2%) and negative predictive value (95.5%). The performance of the ELISA based on nAgB was slightly better, the recording of just one false-negative and no false-positives leading to a sensitivity of 98.6%, a specificity and positive predictive value of 100% and a negative predictive value of 98.5%. The differences in the performances of the three types of ELISA did not, however, reach statistical significance (P>0.05 for each comparison).
Whichever the type of antigen used, the absorbance readings for the samples from the cases of cystic echinococcosis were significantly higher than those of the samples from the patients who were infected with other parasites \( (P < 0.001) \) and those of the samples from the apparently healthy volunteers (Fig. 4).

DISCUSSION

In this study, the antigen-B subunit designated ‘EgAgB12 kDa’ in GenBank was cloned, expressed, and evaluated, in ELISA, as a potential tool for the diagnosis of human cystic echinococcosis. The purification, evaluation and development of the most efficient immunogenic antigens for diagnosis have been imperative goals for much of the research on this disease. Unlike many helminth infections of humans, human infection with *Ec. granulosus* cannot be detected by the microscopical examination of faeces or urine. Efforts to detect cystic echinococcosis have therefore been based on serological tests, imaging techniques (such as ultrasonography, computed tomography and magnetic resonance imaging) or surgery (Virginio *et al.*, 2003). Unfortunately, there is not yet a diagnostic method for cystic echinococcosis that can be considered the ‘perfect gold standard’ (Torgerson and Deplazes, 2009), although immunodiagnostic methods can help to identify 80%–100% of the cases with hepatic cysts and up to 65% of those with only pulmonary involvement (Shepherd and McManus, 1987; Biava *et al.*, 2001; Sbihi *et al.*, 2001; Rokni and Aminian, 2006).

The cloning and expression of the antigen-encoding genes of *Ec. granulosus* have unique advantages: large amounts of antigen can be prepared in less time than is needed to produce native antigen; the methods are relatively straightforward; and, as little energy is needed, the process is environmentally ‘friendly’. In the present study, the performance, in a diagnostic ELISA, of the rAgB was compared with that of nAgB and
HCF (currently the two most widely used sources of antigens in immunodiagnostic tests for cystic echinococcosis).

Although antigen 5, an important ingredient of HCF, was once regarded as unsuitable for diagnosis (Shepherd and McManus, 1987), a cloned version has recently been found to be very useful in the detection of cystic echinococcosis (Lorenzo et al., 2003, 2005). Antigen B (another component of HCF) has, however, also been found to be a highly specific antigen that appears to have great (potential) usefulness in the diagnosis of this disease (Lightowlers et al., 1989; Maddison et al., 1989; Rott et al., 2000; Rokni and Aminian, 2006). Such observations encouraged many researchers to clone, express and evaluate several AgB-encoding genes (Fernández et al., 1996; González et al., 1996; González-Sapienza et al., 2000; Ortona et al., 2000; Pazoki et al., 2006). Although the present results indicate that the ELISA based on rAgB were slightly less sensitive and specific than similar assays based on nAgB, the differences did not reach statistical significance. Curiously, Virginio et al. (2003) found that another recombinant antigen-B subunit, EgAgB8/2, offered more powerful features for the diagnosis of cystic echinococcosis than nAgB (although the recombinant antigen was based on bovine isolates of Ec. granulosus, not ovine as in the present study). Despite recent efforts to produce an antigen that performs consistently in the detection of the different strains of Ec. granulosus (Haag et al., 2006), the performance of diagnostic techniques based on recombinant antigens like that used in the present study might be affected by the strain of parasite involved. Both the camel/dog strain (G6 genotype) of Ec. granulosus and the more cosmopolitan and common sheep strain (G1 genotype) occur in Iran (Zhang et al., 1998).

Among the subunits of AgB that have been cloned and expressed to date, a small, 8-kDa subunit has evoked considerable interest (Maddison et al., 1989; Frosch et al., 1994; Fernández et al., 1996; Mamuti et al., 2006, 2007). Although this subunit has shown cross-reaction with human parasites other than Ec. granulosus (Rott et al., 2000), Maddison et al. (1989)

**FIG. 3.** The absorbances recorded in IgG-ELISA based on the recombinant antigen (a), the native antigen B (b) or hydatid-cyst fluid (c). The sera tested came from patients with cystic echinococcosis, toxoplasmosis, strongyloidosis, visceral leishmaniasis or tuberculosis or from apparently healthy subjects. In each plot, the horizontal line indicates the threshold for positivity.
still thought that it was specific enough to be useful in diagnosing cystic echinococcosis.

Hernández-González et al. (2008) compared the diagnostic performances of IgG-ELISA based on four different recombinant antigens of *Ec. granulosus* (B1t, B2t, E14t and C317) and reported that the assay based on B2t, which gave 91.2% sensitivity and 93.0% specificity, was the most promising. In the present study, however, the performance of the ELISA based on *EgAgB12 kDa* was superior to that of any of the assays described by Hernández-González et al. (2008).

When Li et al. (2003) evaluated a recombinant EpC1–glutathione–S-transferase fusion protein (rEpC1–GST) by IgG immunoblotting, they recorded a sensitivity of 92.2% and a specificity of 95.6%, and claimed that these results were promising and unprecedented in the diagnosis of cystic echinococcosis. Again, however, all of the ELISA tested in the present performed better than this. In Iran, Pazoki et al. (2006) cloned and expressed two 32– to 36-kDa subunits from *Ec. granulosus* AgB (Hyd1 and Hyd2) but did not evaluate them in serological tests. In the same country, Kalantari et al. (2010) recently produced recombinant (24-kDa) *EgAgB* using primers based on another GenBank accession (DQ835667) and found that it gave a sensitivity of 91.7% and specificity of 97.2% in ELISA when tested against a panel of sera (entirely different to the one used in the present study).

In conclusion, although the present results indicate that ELISA based on nAgB are at least as good, for diagnosis, as similar assays employing the recombinant antigen based on *EgAgB12 kDa*, the preparation of the recombinant antigen is easier (once the initial steps of cloning and expression have been completed) than the preparation of the native antigen. The recombinant antigen merits further study, to see if it can be used or adapted to produce a reliable and accurate method for the detection of *Ec. granulosus* infection (ideally of any strain) in humans.

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