

Multi-Epitope Fusion Protein Eg mefAg-1 as a Serodiagnostic Candidate for Cystic Echinococcosis in Sheep

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Abstract: Cystic echinococcosis (CE) in sheep is a hazardous zoonotic parasitic disease that is caused by *Echinococcus granulosus* (Eg). At present, serological test is an important diagnostic method for Eg infection in domestic animals. Here, a fusion protein Eg mefAg-1 harboring 8 dominant B-cell epitopes of Eg such as antigen B, tetraspanin 1, tetraspanin 6, reticulon and Eg95 was produced in *E. coli* and evaluated for CE in sheep by indirect ELISA. Eg mefAg-1 showed in ELISA a high sensitivity (93.41%) and specificity (99.31%), with a coincidence rate of 97.02%. Overall, it is suggested that the Eg mefAg-1 could be a potential antigen candidate for CE serodiagnosis in sheep.

Key words: multi-epitope, fusion protein, serodiagnostic, antigen, cystic echinococcosis, indirect ELISA, sheep

Echinococcosis, also known as cystic echinococcosis (CE), is a zoonotic parasitic disease that is caused by the growth of larvae of *Echinococcus granulosus* (Eg) in the liver, lung and other organs of the host such as sheep and human beings [1-3]. This disease is distributed worldwide, across the 5 continents [4,5]. It has been found in 21 provinces or autonomous regions of China, especially serious in livestock of Xinjiang, Gansu, Ningxia, Qinghai, Tibet, Sichuan and Inner Mongolia [6-8]. It has been reported that the average infection rate of sheep CE in Xinjiang area is 30-50% [9,10]. Animal CE seriously affected the production and development of livestock, causing great loss in livestock production and bringing enormous danger to the health of pastoralists [11,12].

Screening and preparation of highly specific and sensitive Eg antigen is the premise of the development of CE serological diagnosis [13-15]. Over the past years, diagnosis of CE mainly depends on serological detection of the intermediate host (especially in sheep) using cyst fluid, protoscolex protein or re-

combinant proteins as coating antigens [16-23]. Purified cyst fluid antigen is highly sensitive. However, it is affected by the developmental stage of the worm, resulting in differences in sensitivity and specificity in serological diagnosis [24]. Moreover, the purification process has not been standardized thus purified cyst fluid cannot be prepared in large scale, which limits the application of cyst fluid antigen [16,20,23]. By contrast, the use of recombinant protein for antibody detection displays good specificity [13,25], however, sensitivity is lower than that of fluid antigen. Therefore, development and preparation of CE recombinant antigens with high reactionogenicity is the key to enhancing the diagnostic performance of CE serodiagnosis.

The aim of this study is to develop and evaluate a multi-epitope fusion protein Eg mefAg-1 for serodiagnosis of CE in sheep. Here, we constructed and expressed multi-epitope fusion cDNA Eg mefAg-1 in *E. coli*, purified and evaluated the specificity and reactionogenicity of the recombinant protein Eg multi-epitope fusion Ag-1 (reEg mefAg-1) via indirect ELISA for CE serodiagnosis in sheep.

A total of 235 slaughtered sheep were collected from 6 different pastures of Shawan County in Xinjiang China, where is the endemic region of CE in sheep.

The blood samples were collected and marked with the animal's identification number before slaughter. Then sera of

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these sheep were respectively prepared, numbered and stored at 4°C. The internal organs (especially lung and liver) of carcasses of sheep were examined for parasites. Those parasites were confirmed by PCR amplification of mitochondrial COX1, nad1, and nad5 genes and further verified by sequencing. The corresponding sera from infected sheep were labelled as positive or negative for CE according to the post-mortem examination result. Of 235 sera, 91 were positive for CE, 5 were positive for *Echinococcus multilocularis*, 8 were positive for *Cysticercus tenuicollis* (Ct) and 8 were positive for *Fasciola hepatica*. These sera were separated by centrifugation at 2,000×g for 15 min, aliquotted and preserved at -20°C until used.

The linear antigenic epitopes of antigen B (AgB), tetraspanin 1 (TSP1), tetraspanin 6 (TSP6), reticulon (RTN4), and Eg95 proteins were predicted using ABCpred (<http://www.imtech.res.in/raghava/abcpred/>), BepiPred 1.0 Server (<http://www.cbs.dtu.dk/services/BepiPred/>), Immunomedicine Group (<http://imed.med.ucm.es/Tools/antigenic.pl>) and DNASTar-Protean software [25]. Eight dominant antigenic epitopes were selected based on the prediction results (Table 1). The multi-epitope fusion cDNA Eg mefAg-1 was obtained by connecting the nucleotide sequences that were corresponding to the amino acid sequences of dominant epitopes by a flexible peptide coding sequence in certain order. The multi-epitope fusion cDNA Eg mefAg-1 was analyzed by codon prediction software Racc (<http://nihserver.mbi.ucla.edu/RACC/>) and subsequently optimized to preferred codons of *E. coli* by synonymous mutations, and sent to Beijing Genomics Institute (BGI, Shenzhen, China) for cDNA synthesis.

Primer 5.0 software was used to design specific primers for synthesized multi-epitope fusion cDNA Eg mefAg-1. Forward primer P1: 5'-CCGGAATTCATGGTGGTAAAAAACGT-3' (underline indicates *Eco*R I restriction site), reverse primer P2: 5'-CCGCTCGAGTTACATTACAGTGCTTTCC-3' (underline *Xho*I restriction site). Amplification conditions: pre-denaturation

at 95°C for 5 min, 30 cycles of denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec, extension at 72°C for 50 sec and final extension at 72°C for 10 min. The PCR product was recovered and double digested by the restriction endonuclease *Eco*R I and *Xho* I (TaKaRa, Tokyo, Japan) together with pET-28a (+) plasmid (Invitrogen, Carlsbad, California, USA). The target fragment and the vector fragment were recovered individually and ligated using T4 DNA ligase (TaKaRa) at 4°C overnight, and then the ligation product was then transformed into competent *E. coli* BL21(DE3) cells (TaKaRa). The recombinant plasmid pET-meAg-1 extracted from *E. coli* was verified by PCR and double nuclease digestion.

The positive clone was inoculated into liquid LB medium (Difco, Detroit, Michigan, USA) and cultured at 37°C until OD_{600 nm} reached 0.6-0.8. IPTG (TaKaRa) was then added at a final concentration of 1.0 mmol/L to induce the cDNA expression. The bacterium was harvested at 4 hr, 6 hr, and 8 hr following IPTG addition. The expressed recombinant Eg multi-epitope fusion Ag-1 (Eg mefAg-1) was analyzed by SDS-PAGE. Meanwhile, Western blot analysis was performed using sheep Eg positive serum as the primary antibody, and horseradish peroxidase (HRP) labeled rabbit anti-sheep IgG (EarthOx, San Francisco, California, USA) was used as the secondary antibody.

Briefly, 50 ml of culture medium was centrifuged to collect the bacteria. The bacteria were resuspended in 25 ml of binding buffer and ultra-sonicated until the solution was clear. The solution was centrifuged, and the supernatant was collected and filtered through a 0.22 µm filter. The processed product was purified by passing through Ni-NTA affinity column (Invitrogen). After protein concentration determination, the Eg mefAg-1 was aliquotted and stored at -80°C until used.

Indirect ELISA was established according to Baoumi et al.'s method [20] with appropriate adjustments. Briefly, the Eg mefAg-1 was diluted to 4.0 µg/ml with 0.05 mol/L pH 9.6 car-

Table 1. Epitope sequences selected from different antigen proteins of *Echinococcus granulosus*

Protein	Epitope sequences	Position
AgB1 (HM357141)	DDGLTSTSR SVMKMFGEV KYFFERDPLGQKVVDLLEEL	1-38
AgB2 (HM357142)	VVKKRWGELRDFFRND	11-26
AgB4 (HM357144)	RDFFRSDPLGQK	19-30
AgB4 (HM357144)	LEEEDEDDLK	61-70
Eg95 (AF465599)	IETPRAGKKESTVM	120-133
TSP1 (KM459009)	TGNCDQGKAQSANVTG	195-210
TSP6 (XP_024355367)	TQAITAPDLTTPYPSS	150-165
RTN4 (KM459011)	KFTEKPKREWR	185-195

bonate buffer, aliquoted to the 96-well microtiter plate (Costar, Cambridge, Massachusetts, USA) at 100 µl/well, and coated at 4°C overnight. On the next day, the coated plate was washed with 0.05 mol/L PBST (pH 7.4, containing 0.5 ml/L Tween-20) 3 times, blocked with 10 g/L BSA (100 µl/well) at 37°C for 2 hr and washed 3 times with PBST. The serum sample was 1:100 diluted with dilution buffer, added to each well (100 µl/well) and incubated at 37°C for 1 hr, followed by washing 3 times with PBST. Then, plates were treated with horseradish peroxidase-conjugated rabbit-anti-sheep IgG (Abcam, Cambridge, England, UK) in optimal dilution 1:10,000 for 1 hr. After repeated washing, 50 µl/well chromogenic substrate solution was added to the plate and incubated at 37°C in dark for 15 min. The reaction was terminated by 50 µl/well 2M H₂SO₄. The OD_{450 nm} value of each well was determined by an enzyme-linked detector (Biotek, Winooski, Vermont, USA), with the blank well as the negative control.

A total of 30 negative serum samples from sheep were subjected to Eg meAg-1-ELISA. The OD_{450 nm} value was determined and the data were analyzed using Excel 2000 and SPSS 17.0 software for Windows (Release 17.0 standard version, SPSS Inc.,

Chicago, Illinois, USA). The average (X) and standard deviation (SD) were calculated and X+3SD was considered as cut-off value to discriminate positive and negative samples.

The established indirect ELISA based on Eg meAg-1 was used to evaluate the specificity by detecting 30 anti-Eg positive sera, 5 anti-*Echinococcus multilocularis* positive sera, 8 anti-*Cysticercus tenuicollis* (Ct) positive sera, 8 anti-*Fasciola hepatica* positive sera and 30 anti-Eg negative sera to determine whether they have cross-reaction with Eg meAg-1. A total of 235 sheep serum samples were subjected to the detection of Eg antibodies by Eg meAg-1 and the commercial anti-*Echinococcus* IgG ELISA Kit (ab108733, Abcam, Cambridge, Massachusetts, USA). Thereinto, HRP labeled rabbit anti-sheep IgG (EarthOx) was used as the secondary antibody in the test. The sensitivity, specificity and agreement of the Eg meAg-1-ELISA were calculated according to the true-positive and true-negative in 2×2 tables [17,20]. Sensitivity (%)=[true positive/(true positive+false negative)]×100; Specificity (%)=[true negative/(true negative+false positive)]×100; Agreement (%)=[(true positive+true negative)/total number of samples]×100.

A 468 bp fragment of Eg meAg-1 cDNA was successfully

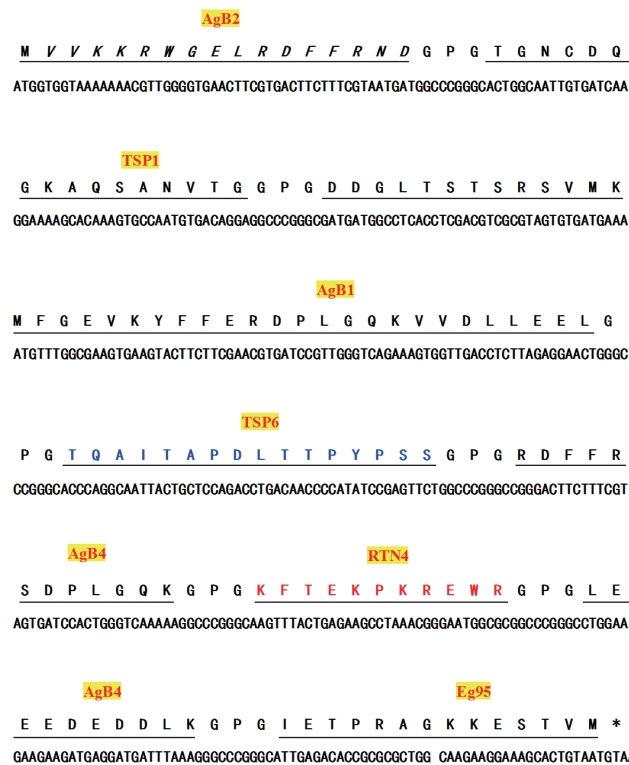


Fig. 1. Amino acid sequence of Eg meAg-1 and its *E. coli*-codon optimized nucleotide sequence. A boxed GPG is linker.

synthesized according to optimized nucleotide sequence of Eg mefAg-1 (Fig. 1). Then the cDNA was amplified using P1-P2 specific primers and cloned into pET28a (+) to generate recombinant plasmid pET-meAg-1. The pET-meAg-1 was subsequently verified by PCR amplification and double enzyme digestion, respectively, which produced target cDNA fragment with expected size, indicating the successful construction of the recombinant pET-meAg-1.

The results of SDS-PAGE revealed that positive clone expressed a 23.5 kDa protein, consistent with expected size of Eg mefAg-1. The analysis of Western blot confirmed that recombinant protein Eg mefAg-1 purified by Ni-NAT affinity chromatography could be specifically recognized by anti-Eg positive serum (Fig. 2).

Indirect ELISA based on Eg mefAg-1 antigen indicated that the optimum coating amount of the antigen was 4.0 µg/ml and the best serum dilution was 1:100. The OD_{450 nm} values of the 30 Eg negative serum samples were mainly distributed at a range of 0.15-0.20. Statistical analysis showed that the average (X) OD_{450 nm} value of the negative serum samples was 0.159 and the standard deviation (SD) was 0.037. Accordingly, the confidence interval upper cut-off value was 0.270. Samples with OD_{450 nm} val-

ue greater than 0.270 were considered as positive, whereas less than or equal to 0.270 were considered as negative.

Specificity tests of Eg mefAg-1-ELISA showed that the 30 sheep Eg positive sera were positive and those positive sera against other parasites were negative, indicating that the Eg mefAg-1 was highly specific and had no cross-reactivity with positive sera against other parasites (Fig. 3).

A total of 235 sheep sera were detected for Eg antibody by Eg mefAg-1-ELISA. Of 235 sera, 86 were CE positive for Eg mefAg-1-ELISA method (86/235, 36.60%), whereas 89 were CE positive for commercial anti-Echinococcus IgG ELISA Kit (89/235, 37.87%). Out of 91 positive sera confirmed by necropsy, however, 6 sera were judged as negative by developed Eg mefAg-1-ELISA method (Table 2). Compared with the results of necropsy, Eg mefAg-1-ELISA presents high sensitivity (93.41%, 85/91) and specificity (99.31%, 143/144). Accordingly, the agreement of between Eg mefAg-1-ELISA and the necropsy was 97.02% (228/235) (Table 2). Moreover, when compared with the commercial anti-Echinococcus IgG ELISA Kit (ab108733, Abcam, USA), there were little discrepancy between the commercial anti-Echinococcus IgG ELISA Kit and the developed ELISA among individual sera.

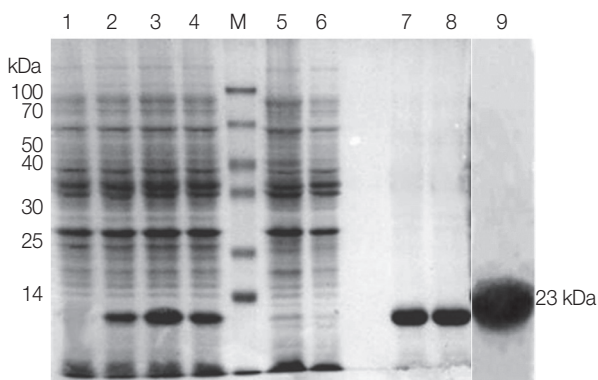


Fig. 2. Induction and purification of reEg mefAg-1 on SDS-PAGE gel and Western blotting. Mr, protein molecular weight marker; lane 1, culture supernatant of *E. coli* BL21(DE3); lanes 2-4, IPTG-induced for 4, 6, and 8 hr; lanes 5-6, pET-28a (+) vector (control); lanes 7-8, purified reEg mefAg-1 protein; lane 9, western blot with anti-Eg positive serum.

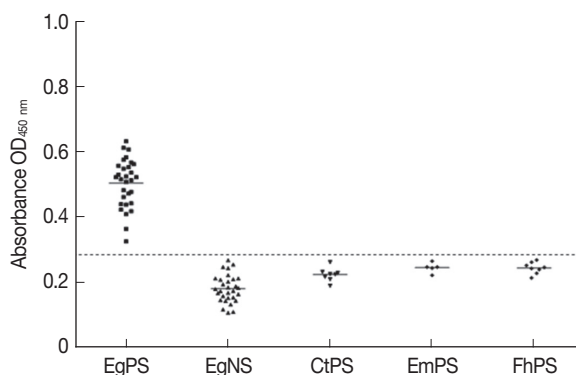


Fig. 3. Antigenicity of Eg mefAg-1 toward sheep hydatidosis sera in ELISA. A cut-off line was set at OD 0.270. Eg PS, *E. granulosus* hydatid cyst positive; Eg NS, *E. granulosus* hydatid cyst negative; Ct PS, *Cysticercus tenuicollis* positive; Em PS, *E. multilocularis* cyst positive; Fh PS, *F. hepatica* positive.

Table 2. Comparison among the results of necropsy, commercial anti-Echinococcus IgG ELISA Kit and Eg mefAg-1-ELISA in sheep sera

		Necropsy		Commercial anti-Echinococcus IgG ELISA Kit		Total
		Positive	Negative	Positive	Negative	
Eg mefAg-1-ELISA	Positive	85	1	84	2	86
	Negative	6	143	5	144	149
	Total	91	144	89	146	235

The cyst fluid (CF) and protoscolex proteins of Eg had been evaluated and proved to be valuable antigens for the CE serological diagnosis [16,22,24]. However, sensitivities and specificities of these antigen proteins differ greatly, and some of the cyst fluid antigen components such as metabolites and host tissue components can cross-react with nonspecific antibodies that target other worms, resulting in poor specificity [15,22]. Golassa et al. [17] evaluated the diagnostic value of crude cyst fluid in bovine CE *via* indirect hemagglutination test (IHA) and found that the false positive and false negative rates were high, and the accuracy is relatively low. Jin et al. [20] established ELISA using cyst fluid as coated antigen to detect the Eg antibody in Uzbekistan sheep, confirming that the sensitivity and specificity of the method were relatively high, but it had cross-reaction with other *cysticerci* and *Clonorchis sinensis* infected serum.

Some researchers extracted natural antigen from Eg cyst fluid as the diagnostic antigen and established serological test to detect Eg antibodies [18,24]. Feng et al. [18] used dot-immunogold filtration assay (DIGFA) to evaluate purified Eg cyst fluid antigen AgB for its diagnostic effect for Eg and found the sensitivity and specificity of this method were 90.7% and 94.6%, respectively. Furthermore, an 8 kDa purified cyst fluid protein AgB for its diagnostic value for sheep CE was evaluated. The results showed that the diagnostic sensitivity and specificity of purified 8 kDa protein was beyond 90% [27]. However, so far, there is no standard purification procedure that can be used to effectively prepare large scale standard 8 kDa AgB protein, which limits the application of AgB antigen in CE serological diagnosis [28].

Recently, serological tests based on recombinant protein such as Eg AgB and Eg95 has been developed in CE serological diagnosis [13,29]. DIGFA based on recombinant protein AgB (reAgB) for the detection of Eg antibody confirmed that reAgB had higher specificity but lower sensitivity than the crude purified AgB [25]. To compensate for weak reactionogenicity of single antigen, multi-epitope antigens based on dominant epitopes were explored in recent years. As a new strategy, the dominant epitopes of different antigens can be combined to generate multi-epitope antigens, which has been utilized to improve the antigenicity of proteins. Jiang et al. [14] predicted the structures of Eg AgB1, AgB2, and AgB4 subunits using the online software and constructed recombinant multi-epitope antigens. The results showed that the reactionogenicity of recombinant multi-epitope proteins is stronger than that of single AgB subunit. In this study, the developed ELISA based on

the Eg meAg-1 displayed high agreement with the commercial anti-Echinococcus IgG ELISA Kit, which also confirmed that constructing multiple epitope antigens was useful strategy to improve reactionogenicity of diagnostic antigens.

With the accomplishment of complete genome sequence of some tapeworms [30,31], many important antigens of Eg were analyzed and predicted with serological diagnosis potential, such as antigen B (AgB, EGR_06805), tetraspanin (TSP1, EGR_10196; TSP6, EGR_00715), reticulon (RTN, EGR_04657), protease inhibitors (PI, EGR_03566) and Eg95 (EGR_05614) [32]. On the basis of these studies, we developed multi-epitope fusion cDNA Eg meAg-1 and prepared Eg meAg-1. The recombinant antigen Eg meAg-1 had no cross-reaction with sera against other parasites in sheep in the same sites. The sensitivity of 94.38% and specificity of 98.63% were observed in the indirect ELISA based on Eg meAg-1, which shared high agreement with necropsy, the gold standard recommended by OIE Manual. Our results demonstrated that Eg meAg-1 had high serodiagnostic performance, which may be a potentially valuable candidate for CE serodiagnosis in sheep.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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