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Original Article

Prokaryotic expression and identification of B- and T-cell combined epitopes of Em95 antigen of *Echinococcus multilocularis*

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Abstract: Objective: This study is to clone and identify B- and T-cell combined epitopes from Em95 antigen. Methods: The B- and T-cell combined epitopes were predicted using bioinformatic software. Two DNA sequences of Em95-1 (which contained the coding region of one B- and T-cell combined epitope) and Em95-2 (which contained the coding regions of two B- and T-cell combined epitopes) were amplified by PCR. Em95-1 and Em95-2 were cloned into pET32a vector for protein expression. Rabbit was immunized with the expressed proteins of rEm95-1 and rEm95-2 to produce polyclonal antibodies. The immunogenicity and antigenicity of rEm95-1 and rEm95-2 were examined by Western blot analysis. Results: The three B- and T-cell combined epitopes were successfully cloned and expressed in PET32a vector. The recombinant antigens of rEm95-1 and rEm95-2 could specifically bind the human serum from patients with alveolar echinococcosis and specifically bind the prepared polyclonal antibodies. Conclusion: Three B- and T-cell combined epitopes were successfully cloned with good immunogenicity and antigenicity. Our data suggest that B- and T-cell combined epitopes predicted from the Em95 antigen may be used for the construction of high-valence vaccines and as targets for prevention of echinococcosis.

Keywords: Em95 antigen, bioinformatics, B- and T-cell combined epitopes, prokaryotic expression

Introduction

Alveolar echinococcosis (AE), caused by infection with larval stage of *Echinococcus multilocularis* (*E. multilocularis*), is a parasitic zoonosis which severely impairs human health and the development of livestock industry [1, 2]. Currently AE is mainly treated by surgery and drugs. However, treatment with surgery could cause severe trauma and treatment with drugs could cause severe side effects. At the early stage of infection, AE is latent and without obvious clinical symptoms, which is hard to be diagnosed. When there are obvious clinical symptoms, AE is usually at the late stage of infection and treatment at this stage is less effective. Therefore, early prevention is vital to decrease the mortality rate of AE.

It is widely proposed that antigens from the eggs or protoscolex of *E. multilocularis* can be used as targets to develop effective vaccines against *E. multilocularis*. Kouguchi et al [3] found that the recombinant emY162 antigen could induce immune protection in the host rats at a level of 74.3%. Jia et al [4] constructed the recombinant plasmid of pET28a-Eg95 and found that recombinant Eg95 showed a high immunogenicity in cystic echinococcosis patients.

In this study bioinformatic methods were used to predict the secondary and tertiary structures of the Em95 antigen and the B- and T-cell combined epitopes of the Em95 antigen. After prokaryotic expression, the immunogenicity and antigenicity of the B- and T-cell combined epitopes were then examined using Western blot analysis. Our results provide experimental evidence for the development of polyvalent vaccines against AE.

Materials and methods

Reagents and animals

The pET-32a(+) vectors were kept by our group. The protoscolex of *E. multilocularis* were obtained from abdominal cavities of the mice infected with AE. AE infected mice were provided by the Laboratory Animal Center, Xinjiang Medical University. *E. coli* DH5 and *E. coli* BL21 (DE3) were purchased from Tiangen Biotech Co., LTD (Beijing, China). New Zealand white rabbits were provided by Xinjiang Medical University. All animal experiments were conducted according to the ethical guidelines of Xinjiang Medical University.

The SV Total RNA Isolation System was purchased from Promega (Madison, Wisconsin, USA). The reverse transcription kit was purchased from Invitrogen (Carlsbad, California, USA). Freund's complete and incomplete adjuvants and His-Binding-resin columns were purchased from Sigma (St. Louis, Missouri, USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 3, 3'-Diaminobenzidine (DAB) were purchased from Sangon (Shanghai, China). The horseradish peroxidase conjugated goat anti-rabbit IgG (IgG-HRP) and the goat anti-human IgG-HRP were purchased from Sigma (St. Louis, Missouri, USA).

The prediction of signal peptides, secondary structure, B-cell epitopes, and T-cell epitopes of Em95 antigen

The Signal P 4.0 Server software was used to predict the signal peptides of Em95 antigen. The SOPMA Sever software was used to predict the secondary structure of Em95 antigen. The DNA Star software and IEDB website were used to predict the B-cell epitopes of Em95 antigen. The SYFPEITHI software and Propred website were used to predict the T-cell epitopes of Em95 antigen. The common epitopes shared by both B-cell epitopes and T-cell epitopes were defined as B- and T-cell combined epitopes.

PCR

Total RNAs were extracted from the protoscolex as per the instructions of the SV Total RNA Isolation System kit. Then RNA was reverse transcribed into cDNA according to the instructions of the reverse transcription kit. PCR reactions in 20 μl volumes were performed using the protoscolex cDNAs as templates. The primers for epitopes of Em95 antigen were designed using DNAman. The sequences of forward and reverse primers for Em95-1 were 5'-CGG AAT TCC AGG AAT ACA GAG GA-3' and 5'-CGC AAG CTT ATC CG A GAA CTG TGC-3', respectively. The sequences of forward and reverse primers for Em95-2 were 5'-CGG AAT TCG GAC AAC TCG CCA TC-3' and 5'-CGC AAG CTT GAC AAT TAC TAT GCA GCT-3', respectively. Em95-1 contained one predicted epitope and Em95-2 contained two predicted epitopes. The primers were synthesized by BGI (Beijing, China).

Construction of prokaryotic expression plasmids

After sequencing, Em95-1 and Em95-2 were cloned into pET32a vector through T-A cloning. Briefly, the ligation products were transferred into competent *E. coli* BL21 (DE3) cells. Single colonies were selected and identified via PCR or restriction enzyme digestion. The recombinant pET32a/Em95-1 and pET32a/Em95-2 plasmids were then sequenced by BGI (Beijing, China).

Expression and purification of His-Em95-1 and His-Em95-2

Expression of recombinant pET32a/Em95-1 and pET32a/Em95-2 plasmids were induced by 0.5 M IPTG at 35°C for 3 h. After induction, the fusion proteins of rEm95-1 and rEm95-2 were purified through His-binding-resin column using different concentrations of imidazole buffer (300 mM and 500 mM). The purified proteins were then detected by SDS-PAGE electrophoresis.

Immunization with proteins of rEm95-1 and rEm95-2

The purified rEm95-1 and rEm95-2 were condensed and injected subcutaneously into New Zealand white rabbits. Briefly, 1 ml of protein and 1 ml of Freund's complete adjuvant was mixed together and injected subcutaneously to the neck and back of rabbits (0.5 mg per rabbit). After 2-3 weeks, rabbits were immunized with incomplete Freund's adjuvant to boost immunization. After 7-10 days, blood was collected and serum was isolated, divided into aliquots and stored at -20°C for later use.

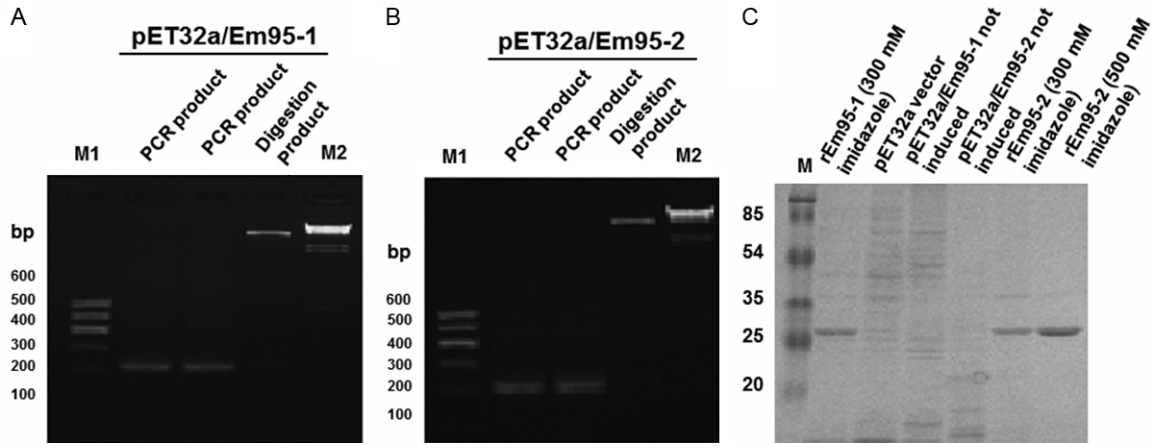

Figure 1. Construction and expression of pET32a/Em95-1 and pET32a/Em95-2 recombinant plasmids. A: Agarose gel electrophoresis results of PCR products and restriction enzyme digestion products of pET32a/Em95-1. M1 and M2 were DNA makers. B: Agarose gel electrophoresis results of PCR products and restriction enzyme digestion products of pET32a/Em95-2. M1 and M2 were DNA makers. C: Expression and purification of rEm95-1 and rEm95-2. Proteins of rEm95-1 were eluted by 300 mM imidazole and rEm95-2 proteins were eluted by 300 mM imidazole and 500 mM imidazole.

Western blot analysis

The proteins were separated by SDS-PAGE and transferred onto membrane. After blocking, the membrane was blotted with antibodies. The polyclonal antibody containing serum prepared from the immunized rabbits and the human serum from AE patients were used as primary antibodies (dilution 1:1000). Serum samples from normal rabbits and healthy individuals were used as negative controls. The alkaline phosphatase, goat anti-rabbit IgG-HRP and the goat anti-human IgG-HRP were used as secondary antibodies (dilution 1:2000). Then the membrane was developed by DAB reagent.

Results

Em95-1 and Em95-2 antigens are successfully predicted and expressed

To analyze the B- and T-cell combined epitopes of Em95 antigen, we first predicted the amino acid sequence and the secondary structure of Em95. The Signal P4.0 Server software was used to predict the amino acid sequence of Em95. And it was found that amino acids of 1-16 were the signal peptide (data not shown). The SOPMA Sever software was used to predict the secondary structure of Em95 antigen. And the results showed that 55% of the amino acids were in flexible domains, including 47.86% in random coils and 7.14% in β-turns (data not shown).

In our previous study [5], we predicted the B-cell epitopes and T-cell epitopes of Em95. Using DNA Star and the IEDB website, six B-cell epitopes were predicted, including amino acid regions of 10-19, 43-48, 52-59, 78-82, 92-100 and 110-115 in Em95. Meanwhile, using SYFPEITHI and the Propred website, five T-cell epitopes were predicted, including amino acid regions of 32-40, 51-60, 82-98, 108-116 and 126-138 in Em95. The B-cell epitopes of 52-59, 92-100 and 110-115 were found to be partially overlapping with the T-cell epitopes of 51-60, 82-98 and 108-116. Thus the three combined regions of 52-59, 92-98 and 110-115 were predicted to be B- and T-cell combined epitopes. Em95-1 contained the predicted epitope of 52-59 and Em95-2 contained predicted epitopes of 92-98 and 110-115.

To express Em95-1 and Em95-2 antigens in vitro, we cloned Em95-1 and Em95-2 genes and constructed recombinant plasmids of pET32a/Em95-1 and pET32a/Em95-2. The total protoscolex RNA was extracted using RNA kit. The cDNA was used as template for PCR. Then the amplified fragments of Em95-1 and Em95-2 were cloned into pET32a vector through T-A cloning. The correct constructed plasmids were verified by restriction enzyme digestion and sequencing. The representative results of PCR amplication and restriction enzyme digestion for Em95-1 and Em95-2 were shown in **Figure 1A**, **1B**, respectively. The

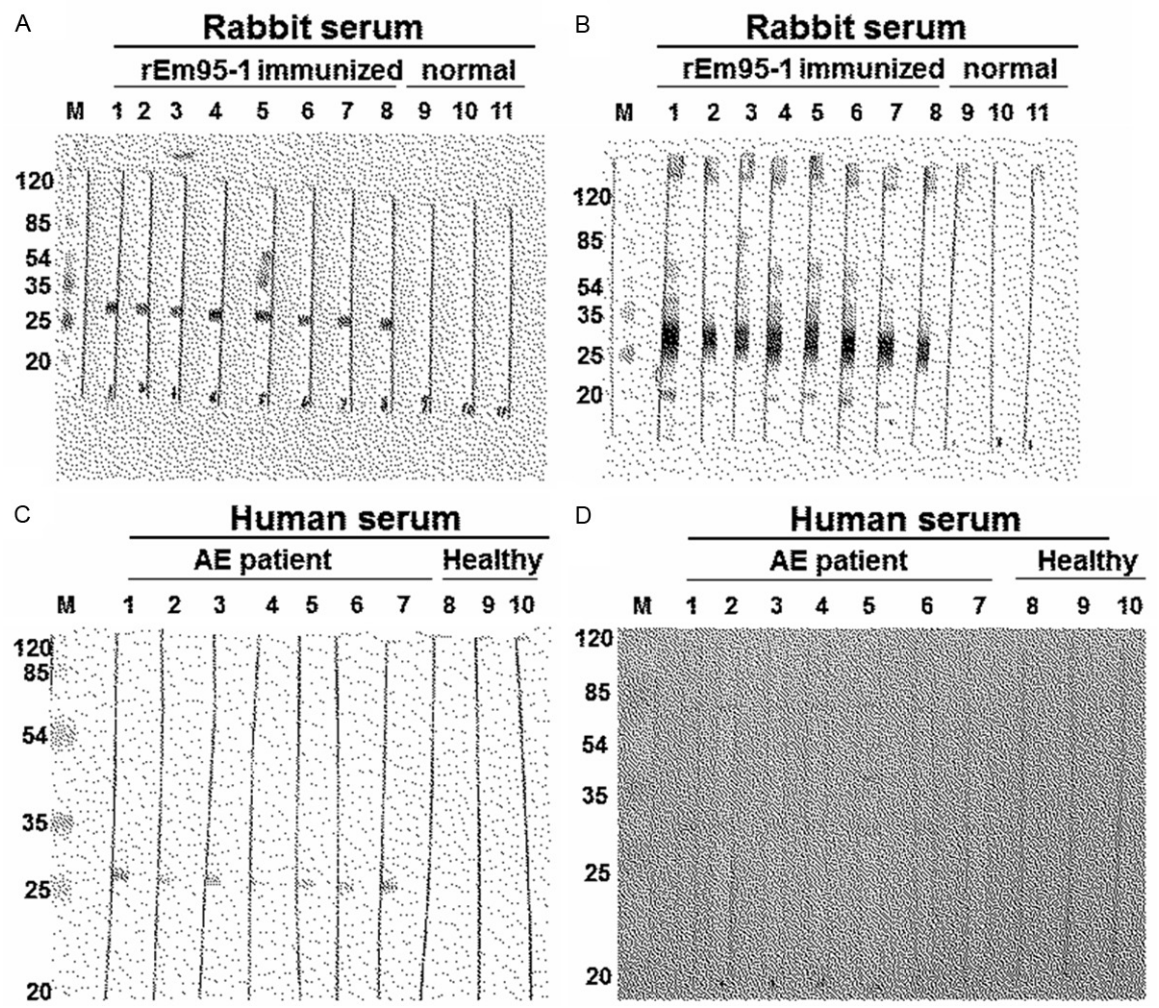

Figure 2. Immunogenicity analysis of rEm95-1 and rEm95-2. Immunogenicity of rEm95-1 and rEm95-2 was analyzed by Western blot analysis. Representative Western blot results were shown. A: Western blot results of rEm95-1 blotting with rabbit serum. M: marker. Lane 1-8: serum samples from rEm95-1 immunized rabbits. Lane 9-11: serum samples from normal rabbits without immunization. B: Western blot results of rEm95-2 blotting with rabbit serum. M: marker. Lane 1-8: serum samples from rEm95-1 immunized rabbits. Lane 9-11: serum samples from normal rabbits without immunization. C: Western blot results of rEm95-1 blotting with human serum. M: marker. Lane 1-7: serum samples from AE patients. Lane 9-10: serum samples from healthy individuals. D: Western blot results of rEm95-2 blotting with human serum. M: marker. Lane 1-7: serum samples from AE patients. Lane 9-10: serum samples from healthy individuals.

length of Em95-1 and Em95-2 was about 200 bp. After sequencing, the correct recombinant plasmids were induced at 35°C with 0.5 M IPTG. Proteins were purified by His-binding-resin column and analyzed by SDS-PAGE electrophoresis. The theoretical molecular weight of Em95-1 and Em95-2 was 28 kD and 27 kD, respectively. As shown in **Figure 1C**, two proteins with a molecular weight of about 30 kD were identified, which indicated that the recombinant proteins (rEm95-1 and rEm95-2) were successfully expressed (**Figure 1C**).

Recombinant proteins of rEm95-1 and rEm95-2 are identified by immunized rabbit serum

To determine the immunogenicity and antigenicity of rEm95-1 and rEm95-2, rabbits were immunized with proteins of rEm95-1 and rEm95-2. After immunization, rabbit serum was collected and blotted with rEm95-1 and rEm95-2. Representative Western blot results of rEm95-1 and rEm95-2 were shown in **Figure 2A**, **2B**, respectively. Western blot results showed clear specific target bands with immu-

nized rabbit serum as the primary antibody. However, there was no specific band with the normal rabbit serum without immunization. The result indicates the antibodies in immunized rabbit serum had good specificity, and that rEm95-2 and rEm95-1 had good immunogenicity and antigenicity.

Recombinant proteins of rEm95-1 and rEm95-2 are identified by serum from AE patient

The immunogenicity and antigenicity of rEm95-1 and rEm95-2 was further evaluated with serum from AE patients. Serum samples were collected from 10 AE patients and 16 healthy individuals, and were used as primary antibodies in Western blot analysis. Representative Western blot results of rEm95-1 were shown in **Figure 2A** and those of rEm95-2 were shown in **Figure 2B**. Similarly, there were clear specific bands in lanes blotted with patient serum whereas there were no clear bands in lanes with serum from healthy individuals. These results further indicate that rEm95-2 and rEm95-1 had good immunogenicity and antigenicity.

Discussion

In recent years, bioinformatic methods have been successfully used in the prediction of antigen epitopes. Bioinformatic methods can predict epitopes with high immunogenicity. Thus the coupling of antigens with other foreign proteins to improve immunogenicity is no longer needed. Scorza et al. [6] constructed a 30 k polyvalent DNA vaccine against malaria using genomic libraries which contain 30,000 sequence entries. The constructed DNA vaccine could induce protective immunity in mice against multiple types of plasmodia. With the help of DNA prime/poxvirus boost strategy, researchers used multi-stage DNA vaccines and poxvirus vector vaccines containing multiple epitopes to induce antibodies and cellular immune response against varieties of antigens. And with the help of new adjuvant Vaxfectin, the immunoreaction level induced by low dosages of the DNA vaccines containing the epitopes of five types of falciparum malaria was enhanced [7, 8].

The aim of the present study is to identify immunodominant epitopes of Em95 antigen. In our previous study [5], three B- and T-cell combined epitopes of Em95 were predicted through bioinformatic methods. In the present study, we constructed two recombinant plasmids of pET32a/Em95-1 and pET32a/Em95-2 through molecular cloning techniques. Em95-1 gene contained sequences of one B- and T-cell combined epitope and Em95-2 gene contained sequences of two B- and T-cell combined epitopes. The recombinant proteins, rEm95-1 and rEm95-2, were then expressed, purified and used to immunize rabbits, thus producing polyclonal antibodies. The recombinant proteins were found to have good immunogenicity and antigenicity by Western blot analysis, which indicates that the predicted B- and T-cell combined epitopes were included in the recombinant proteins. Our data also confirmed the effectiveness of bioinformatic methods in predicting candidate antigen epitopes via amino acid/nucleotide sequence analysis. Moreover, our results provide experiment data for the construction of highly efficient and immunodominant vaccines against AE.

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Disclosure of conflict of interest

None.

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