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Construction of Larval cDNA Expression Library and Immunological Identification of Positive Clones in Tick *Haemaphysalis qinghaiensis*

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Abstract

A primary cDNA expression library with a titer of 5.0×10^5 PFU mL^{-1} was constructed from mRNA extracted from larval *Haemaphysalis qinghaiensis* ticks in order to identify certain genes, which would then be used as candidate molecules for development of effective vaccines to control this parasite. Totally 11 positive clones, which designated as HqL01-11, were obtained by immunoscreening of the library using a polyclonal antibody generated in rabbit with larval tick protein extract. Results of sequence analysis from BLASTN searching revealed that 6 of them had no significant homology with the adult *H. qinghaiensis* ticks' known genes, 4 of them had no significant homology with all genes deposited in GenBank database. HqL07, HqL08, HqL09, and HqL11 were deposited to GenBank database, and accession numbers were EF605263, EF605264, EF605265, and EF605266, respectively. Subsequently, HqL07 and HqL09 were expressed *in vitro* and the molecular weights of the corresponding expressed products were 60 and 70 kDa, respectively. Western blot analyses showed that HqL07 and HqL09 had immunogenicity. This study laid the foundation for future production of genetically engineered vaccines for the immunological control of *H. qinghaiensis*.

Key words: larval tick, *Haemaphysalis qinghaiensis*, cDNA expression library, immunoscreening, immunological identification

INTRODUCTION

Haemaphysalis qinghaiensis is a newly discovered tick species, which is widely distributed throughout Qinghai, Gansu, Ningxia, Sichuan, Yunnan, Tibet, and some other neighboring provinces (autonomous regions) in China. It is now known that this type of parasite causes serious damage to animal husbandry by both blood sucking and disease transmission (Guan *et al*. 2002; Yin *et al*. 2002), and thus results in severe economic losses. The current approach for the control of *H. qinghaiensis* in these regions is mainly relying on the administration of chemical acaricides. However, due to the problems of drug resistance and environmental pollution, this approach has become less and less popular. To develop more effective and environment-friendly methods for controlling ticks is of paramount important if the huge economic loss is to be curbed. Among these methods, immunological control, i.e., using vaccines, is considered to be the most promising. In the 1990s, genetically-engineered vaccines, TickGARD™ (Willadsen *et al*. 1995) and Gavac™ (Rodríguez *et al*. 1995b), became commercially available and were successfully

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used to control the tick *Boophilus microplus*. Production of these vaccines was based on the expression of Bm86 protein (as an antigen) from the *B. microplus*. According to the reports, application of these vaccines in Australia, Central and South America (Cuba and Brazil, etc.) have obtained satisfactory results in controlling *B. microplus* (Penichet *et al*. 1994; Rodríguez *et al*. 1995a; de la Fuente *et al*. 1998). However, it was also found that those vaccines were only effective with a narrow spectrum of known tick species (Sossai *et al*. 2005). In order to find out whether they could be effectively used to control *H. qinghaiensis*, we took a molecular biological approach to try to confirm whether *H. qinghaiensis* also expressing Bm86 gene, but failed (unpublished data). Later, Liao and her colleagues successfully isolated a Bm86 homologue gene, named Hl86 from partially engorged female *Haemaphysalis longicornis*. The predicted amino acid sequence shared 37% identities with that of Bm86 gene (Liao *et al*. 2007). This work demonstrated a successful example for isolating Bm86 homologue genes from those tick species that do not express Bm86 gene, including *H. qinghaiensis*, for the subsequent production of effective vaccines.

The critical step in producing genetically engineered vaccines for controlling ticks is to identify functional tick genes which display good immunogenicity. There are a number of ways currently available to obtain those functional genes and then to express corresponding proteins *in vitro*, among which construction of a cDNA expression library is still an effective and preferred method. A cDNA expression library of adult *H. qinghaiensis* has recently been constructed and a few functional genes were identified in our lab (Gao *et al*. 2006, 2007). Development of effective vaccines based on some of these genes is now under way.

H. qinghaiensis is a three-host-tick, and its life cycle consists of 4 stages: larvae, nymph, adult, and egg. Larval tick is the most vulnerable period of tick with low survival ability. Hence theoretically, they should be more prone to be attacked by host defense system. In addition, it is easy that comprehensive coverage of RNA from fresh larval tick materials could be extracted. Consequently, the larval ticks should be the best stage for construction of a cDNA expression library.

The aim of this study was to lay the foundation for the production of vaccines for effectively controlling *H. qinghaiensis* via the following steps: (1) Construction of a cDNA expression library from the larval *H. qinghaiensis*; (2) identification of functional genes from the library through immunoscreening; (3) expression of the identified candidate genes *in vitro*; and (4) evaluation of the immunogenicity of some of the expressed proteins.

MATERIALS AND METHODS

Larval *H. qinghaiensis* and rabbits

Engorged female *H. qinghaiensis* ticks collected from sheep in the field of Lintan County, Gansu Province, China, were incubated at 27°C with 70% relative humidity for oviposition. Larvae developed from the eggs laid by the female ticks were used for total RNA isolation and protein extraction. Rabbits were provided by the Animal House of Lanzhou Veterinary Research Institute, China.

Generation of rabbit anti-larval *H. qinghaiensis* protein serum

Anti-tick immune serum was generated by immunization of rabbits using larval tick extracts. Briefly, about 300 000 larval ticks (from 3 adult ticks) were ground and sonicated in PBS. After being centrifuged at 12 000 × g for 30 min at 4°C, supernatant of the tick extract was collected as antigens. Rabbits were inoculated with the prepared antigens emulsified with complete Freund's adjuvant for the initial injection, and incomplete Freund's adjuvant for 2 booster injections. Two weeks after the last booster, rabbit serum was collected from the ear vein and used to screen the cDNA library here.

Construction of a cDNA expression library

Total RNA and subsequently mRNA were isolated from larval *H. qinghaiensis* ticks by using TRIZOL® reagent (Invitrogen, USA) and an mRNA Purification Kit (Amersham, Sweden) according to the manufacture's instructions. Purity of the RNA was determined by measuring the absorbance at 260 and 280 nm

(OD_{260}/OD_{280}) in a spectrophotometer. A cDNA library was then constructed from the extracted mRNA using the Orient Express cDNA Library Construction Systems Kit (Novagen, USA) following the manufacturer's instructions. To estimate the size of inserts of the cDNA library, 8 plaques were randomly picked and soaked in SM buffer. 5 mL aliquots of the eluted phage were used in a PCR reaction with SP6 promoter primer (5′-ATTTAGGTGACACTATAG-3′) and T7 terminator primer (5′-GCTAGTTATTGCTCAGCGG-3′) which exist in λscreen-1 vector.

Immunoscreening of cDNA expression library

The amplified library was subjected to immunoscreening according to instructions provided in the λscreen vector manual (Novagen, USA) using the rabbit anti-larval *H. qinghaiensis* protein immune serum. Prior to screening of the library, the immune serum was preabsorbed with a lysate of *Escherichia coli* BL21(DE3)pLysE. Pure phage stocks were converted to plasmid subclones using the *in vivo* autosubcloning capabilities of *loxP-cre* system of λscreen vector in host strain BM25.8. Plasmid subclones in BM25.8 were isolated and then transformed into the host strain JM109. The templates for sequencing were generated by purification of recombinant plasmid DNA using TaKaRa MiniBEST Plasmid Purification Kit (TaKaRa, Japan). Positive clones, confirmed by PCR amplification using siTag primer (5′-CGAACGCCAGCACATGGACA-3′) and T7 terminator primer (5′-GCTAGTTATTGCTCAGCGG-3′), were sequenced by TaKaRa company.

Sequences analysis

Gene sequences were analyzed by BLASTN. And HqL07, HqL08, HqL09, and HqL11 sequences were submitted to GenBank.

Recombinant protein expression in *E. coli*

Recombinant plasmids rHqL07, rHqL08 and rHqL09, conceiving larval *H. qinghaiensis* cDNA fragments, were extracted from JM109. Recombinant proteins fused with the T7 gene 10 protein were produced by expressing recombinant plasmids in *E. coli* BL21(DE3) pLysE. For induction of recombinant protein expression, IPTG to a final concentration of 1 mmol L^{-1} was added, and expression was induced at 31°C. The control protein, T7 gene 10 protein with a predicted molecular weight of 36 kDa, was produced from *E. coli*.

Immunogenicity analysis of recombinant proteins

Recombinant proteins rHqL07 and rHqL09, from 500 mL cultured medium for each positive clone, were purified and used to immunize rabbits as antigens following the same procedure of the generation of rabbit anti-larval *H. qinghaiensis* protein serum. 500 μg antigen was used for each inoculation. Positive serum against rHqL07 and positive serum against rHqL09 collected from the rabbits were used in Western blot assay as positive sera to probe corresponding recombinant proteins.

Recombinant proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were either viewed directly by staining the gel with Coomassie brilliant blue or transferred to NC membranes and subjected to Western blot analysis. The prepared blots were sequentially incubated with positive sera respectively. Positive signals were detected with alkaline phosphatase conjugated goat anti-rabbit IgG (Novagen, USA), NBT and BCIP as a substrate.

Animal care and manipulations

Conduct of animal experiments in this research was in accordance with the instructions of Animal House of Lanzhou Veterinary Research Institute and ordinances on Animal Welfare and adhered to the Guide for the Care and Use of Laboratory Animals.

RESULTS

Construction and immunoscreening of cDNA library of larval *H. qinghaiensis* ticks

The concentrations of extracted total RNA and subsequently mRNA of larval *H. qinghaiensis* were 2.82 mg

mL^{-1} and 215 μg mL^{-1}, respectively. The values of OD_{260}/OD_{280} were 1.823 for total RNA and 2.012 for mRNA. Sizes of synthesized cDNAs from the purified mRNA ranged from 100 to above 2 000 bp (Fig.1). A primary cDNA library with a title of 5.0×10^5 PFU mL^{-1} was successfully constructed. The titer of the amplified library was 8.0×10^9 PFU mL^{-1}. PCR results appeared that 5 out of 8 plaques randomly picked from the cDNA library contained inserts greater than 500 bp (Fig.2). The library was immunoscreened using rabbit anti-larval *H. qinghaiensis* protein immune-serum. Totally 20 positive signals were screened after a primary screening. Phage plugs were harvested respectively from the LB plate according to the sites of those 20 positive signals on the membrane. Rescreening was performed for those 20 signals until all the signals on membranes were positive. 11 out of the 20 signals were proved real positive after several rounds of rescreening and their corresponding inserts were sequenced. The 11 positive clones were named as from HqL01 to HqL11. Sequence analysis showed that there was no repeats among the 11 positive clones and 5 (HqL02 to HqL06) out of the 11 positive clones shared 95% or even higher homology with 5 genes of adult *H. qinghaiensis*. Nucleotide sequence and their predicted amino acid sequences of HqL07, HqL08, HqL09, and HqL11 were deposited in the GenBank/NCBI database. Detailed information of the positive clones and accession numbers of HqL07, HqL08, HqL09, and HqL11 were listed in Table.

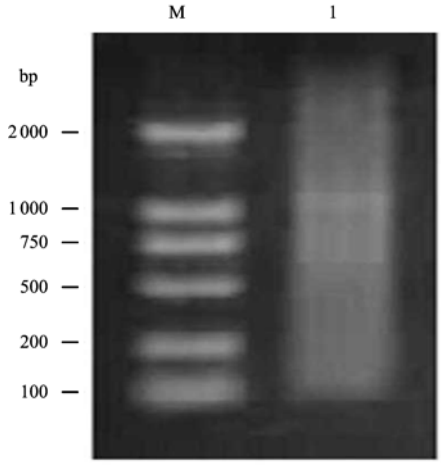

Fig. 1 Agarose electrophoresis analysis of the double strand cDNA. M, DNA marker 2000 bp; 1, cDNA.

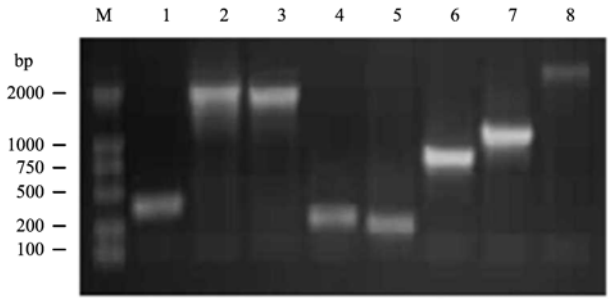

Fig. 2 PCR amplification of the cDNA inserts in the cDNA library. Results showed 5 out of 8 randomly selected plaques containing inserts with sizes greater than 500 bp. M, DNA marker 2000 bp; 1-8, cDNA clones from the library.

In vitro expression of recombinant proteins in *E. coli*

Recombinant plasmids, conceiving cDNA fragments of HqL07, HqL08 and HqL09, respectively, were expressed *in vitro* in BL21(DE3)pLysE. All the expressed proteins were fused with T7 gene 10 protein. Results indi-

Table BLASTN analysis of the selected genes from our larval tick cDNA library

Clone no.	Size (bp)	Homologous with adult gene	Homologous with other gene	Identity (%)	GenBank accession no.
HqL01	126	-	-	-	-
HqL02	763	Hq05	-	98.2	-
HqL03	1 102	Hq02	-	99.0	-
HqL04	364	Hq20	-	96.3	-
HqL05	394	Hq15	-	97.1	-
HqL06	817	Hq12	-	98.6	-
HqL07	610	-	-	-	EF605263
HqL08	1 068	-	*Haemaphysalis flava* mitochondrial DNA	90.0	EF605264
HqL09[1)]	1 605	-	*Haemaphysalis longicornis* tropomyosin mRNA	96.0	EF605265
HqL10	118	-	-	-	-
HqL11	606	-	-	-	EF605266

[1)] It containes an open reading frame (ORF) from position 2 to 874 bp.

-, blank.

cated that 6 h after the beginning of IPTG induction, high amount of recombinant proteins rHqL09 and T7 gene 10 protein were obtained, while the optimal expression time for rHqL07 and rHqL08 were both 8 h after induction. As shown in Fig.3, the molecular weight of the T7 gene 10 protein was estimated to be approximately 36 kDa, while those of rHqL07 and rHqL09 were around 60 and 70 kDa respectively, which consistented with the expected molecular weights. The molecular weight of expressed rHq08 was 35 kDa considering that the expression vector itself also produced a recombinant protein with a size of 36 kDa, thus rHqL08 was expressed unsuccessfully.

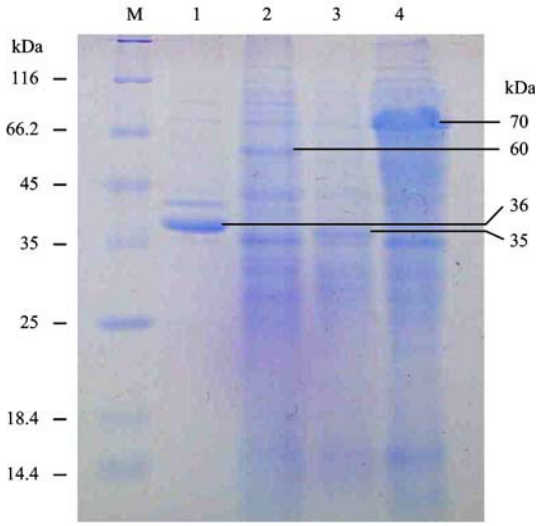

Fig. 3 SDS-PAGE electrophoresis of expressed proteins. M, protein marker; 1, blank vector; 2, rHqL07; 3, rHqL08; 4, rHqL09.

Immunogenicity analysis of recombinant proteins

Positive signals were detected by Western blot. Results showed that both rHqL07 and rHqL09 appeared immunogenicity though lysate of *E. coli* BL21(DE3)pLysE and normal rabbit serum as negative control (Fig.4).

DISCUSSION

This study constructed the first cDNA expression library of the larval *H. qinghaiensis*. Two positive clones, HqL07 and HqL09, were successfully identified from the library by immunoscreening and expressed as T7 gene 10 fusion-protein. Both of them showed

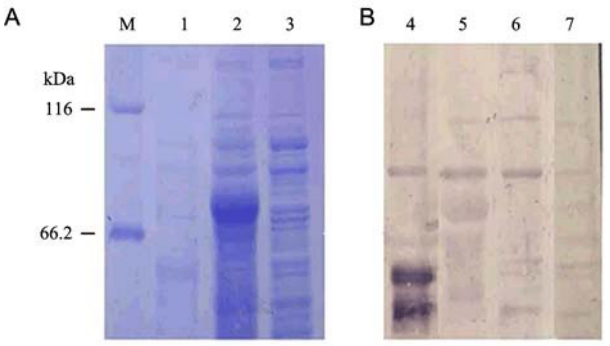

Fig. 4 SDS-PAGE (A) and Western blot analysis (B) of the expressed proteins: rHqL07 and rHqL09. M, protein marker; 1, rHqL07; 2, rHqL09; 3, lysate of *E. coli* BL21(DE3)pLysE as negative control; 4, rHqL07 incubated with positive serum against rHqL07; 5, rHqL09 incubated with positive serum against rHqL09; 6, lysate of *E. coli* BL21(DE3)pLysE incubated with rabbit anti-larval *H. qinghaiensis* protein serum; 7, lysate of *E. coli* BL21(DE3)pLysE with normal rabbit serum.

immunogenicity.

It is not clear about the nature of HqL07 gene at the moment. Though BLASTN searching showed that it shared 73% identities with *Mus musculus* chromosome 3 at its sites between 82-212 nucleosides. BLASTP searching result, using predicted amino acid sequence of HqL07, showed that it shared 55% identities with conserved hypothetical protein of *Ixodes scapularis*, and it shared 62% identities with vitellogenin of *Dermacentor variabilis* at its sites between 1 702-1 768 amino acids. So rHqL07 maybe similar to vitellogenin of *D. variabilis*. However, the strong reaction of rHqL07 with rabbit positive serum in Western blot indicate that rHqL07 might be a vaccine candidate for *H. qinghaiensis*. The predicted amino acid sequence of HqL09 shared 94, 96 and 80% identities with the tropomyosin mRNA of *H. longicornis*, the tropomyosin mRNA of *B. microplus* and the tropomyosin mRNA of tick *Aleuroglyphus ovatus* respectively (NCBI/BLASTN). Therefore, HqL09 gene most likely encodes tropomyosin protein of *H. qinghaiensis*. It is known that tick myoideum plays an important part in hemophagia. You *et al*. (2001) screened the gene P27/30 from the cDNA library of *H. longicornis*, and found that its amino acid sequence shared homology (58% amino acid identities and 11% amino acid identities) with the tropomyosin subunit E2 of *Drosophila melanogaster*. All these evidences indicate that the encoding product of HqL09 might be a vaccine candidate for *H. qinghaiensis*.

Besides the genes we expressed (HqL07 and HqL09), HqL05 is also a positive clone identified from immunoscreening of our larval tick cDNA library. The reason why we did not express the protein of HqL05 was that HqL05 shared 97.5% homology with Hq15, a gene recently identified from the adult tick cDNA expression library in our lab (Gao *et al*. 2006, 2007). Sequence analysis indicated that HqL05 was an adhesion molecule. It was reported that adhesion molecules had strong immunogenicity (Zhou 2004). McKenna *et al*. (1998) reported that adhesion molecules had significant effects on *B. microplus* immunity. Bishop *et al*. (2002) also found that on the surface of excreting cells, from *Rhipicephalus appendiculatus* salivary gland, there exists an adhesive protein that can induce cattle's strong immune response. Therefore, HqL05 is a promising gene to be further studied for the development of vaccines against tick infestation.

Antigens screened from cDNA library were expressed in *E. coli* BL21(DE3)pLysE fused with T7 gene 10 protein. As *E. coli* is one of the most widely distributed bacteria in nature, some positive signals detected by rabbit anti-larval *H. qinghaiensis* protein might not be the protein products originated from *H. qinghaiensis*, but rather from *E. coli*, as there is a slim probability of *E. coli* protein contamination. That is, some positive clones screened from the library might be false positive ones. In order to eliminate this type of false positive clones, we treated rabbit anti-larval *H. qinghaiensis* protein serum with *E. coli* lysates before being used. The results from analysis the 11 genes using NCBI/BLASTN showed that our selected genes shared no homology with the genes from *E. coli* or other types of bacteria. Though BLASTP searching result, using predicted amino acid sequence of HqL11, showed that it shared 40% identities with conserved hypothetical protein of *Burkholderia* sp. H160 at its sites between 351-409 amino acids. *Burkholderia* sp. H160 isn't a common bacteria existing in laboratory, so there is slim opportunity to touch with it. So there were two possibilities where HqL11 came from, the first was that it came from *H. qinghaiensis* but rHqL11 maybe similar to conserved hypothetical protein from a bacteria *Burkholderia* sp. H160, another was that the ticks were infected by *Burkholderia* sp. H160.

Development of genetic vaccines for controlling ticks has been painfully slow since the successful production of TickGARD™ and Gavac™ for controlling *B. mircoplus* in 1995. To curb the economic loss in animal husbandry caused by *H. qinghaiensis*, in this study we successfully constructed a cDNA expression library of the larval stage of this tick species. Some relevant functional genes were identified based on immunogenicity through screening the library. Among these genes, two candidate genes were identified for the future production of genetically engineered vaccines. Therefore, this study has laid the potential foundation for the immunological control of *H. qinghaiensis*.

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