

Gene Cloning, Expression and Immunogenicity of the Protective Antigen Subolesin in *Dermacentor silvarum*

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Abstract: Subolesin (4D8), the ortholog of insect akirins, is a highly conserved protective antigen and thus has the potential for development of a broad-spectrum vaccine against ticks and mosquitoes. To date, no protective antigens have been characterized nor tested as candidate vaccines against *Dermacentor silvarum* bites and transmission of associated pathogens. In this study, we cloned the open reading frame (ORF) of *D. silvarum* 4D8 cDNA (*Ds4D8*), which consisted of 498 bp encoding 165 amino acid residues. The results of sequence alignments and phylogenetic analysis demonstrated that *D. silvarum* 4D8 (*Ds4D8*) is highly conserved showing more than 81% identity of amino acid sequences with those of other hard ticks. Additionally, *Ds4D8* containing restriction sites was ligated into the pET-32(a+) expression vector and the recombinant plasmid was transformed into *Escherichia coli* rosetta. The recombinant *Ds4D8* (rDs4D8) was induced by isopropyl β-D-thiogalactopyranoside (IPTG) and purified using Ni affinity chromatography. The SDS-PAGE results showed that the molecular weight of rDs4D8 was 40 kDa, which was consistent with the expected molecular mass considering 22 kDa histidine-tagged thioredoxin (TRX) protein from the expression vector. Western blot results showed that rabbit anti-*D. silvarum* serum recognized the expressed rDs4D8, suggesting an immune response against rDs4D8. These results provided the basis for developing a candidate vaccine against *D. silvarum* ticks and transmission of associated pathogens.

Key words: *Dermacentor silvarum*, protective antigen, subolesin, 4D8, rDs4D8, vaccine

The hard tick *Dermacentor silvarum* Olenov, one of the principal vectors of Russian spring-summer encephalitis virus, *Babesia equi*, *Babesia caballi*, *Rickettsia slovaca*, and *Rickettsia raoultii*, is widely distributed in northern China, Russia, and Mongolia, and has an impact on human health as well as economic losses [1,2]. Previous studies on *D. silvarum* mainly focused on its life cycle and biological characteristics [3], and no candidate vaccines against biting *D. silvarum* have been reported. Thus, searching for a protective antigen as an effective vaccine against *D. silvarum* infection is necessary.

Tick subolesin (4D8) is the ortholog of insect akirins, which are evolutionarily conserved proteins that affect the innate immune response, reproduction, and development of ticks [4-6]. Vaccination trials with the recombinant 4D8/akirins antigens reduced fertility and survival, not only in ticks, but also in

mosquitoes and sand flies [7]. Here, the *D. silvarum* 4D8 (*Ds4D8*) gene was cloned, and immunogenicity of the recombinant *Ds4D8* (rDs4D8) was analyzed by western blot.

Adult *D. silvarum* were collected from infested sheep at the Xiaowutai National Natural Conservation Area, Hebei province, China and reared as described previously [3]. To clone the *Ds4D8* gene, 2 μg of total RNA was extracted from adult females using RNA purification kit (Axygen, Union City, California, USA) and reverse-transcribed to synthesize cDNA templates using a cDNA synthesis kit (ThermoScript RT-PCR system, Invitrogen, Carlsbad, California, USA). The forward primer FP-RA4D85 and reverse primer RP-RA4D83 (Table 1) were used to clone the open reading frame (ORF) of *Ds4D8* cDNA (*Ds4D8*). PCR samples were amplified at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 59°C for 30 sec, 68°C for 1 min, and a final extension at 72°C for 10 min. Subsequently, *Ds4D8* protein sequences were aligned using ClustalX, and the output of a graphic file was used with DNAMAN software. Phylogenetic analysis was constructed using MEGA version 3.1. A bootstrap resampling analysis with 1,000 replicates was performed to evaluate the inferred tree topology.

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The gene-specific primers with *EcoRI/XhoI* restriction enzyme sites, the forward primer FP-*EcoRI*-RA4D85 and reverse primer RP-*XhoI*-RA4D83 (Table 1), were designed to clone the *Ds4D8*. PCR amplification was performed at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 59°C for 30 sec, 72°C for 50 sec, and a final extension for 10 min at 72°C. The PCR products were ligated into the pMD19-T cloning vector (Takara, Ohtsu, Japan), digested with restriction enzymes, and ligated into the pET-32 (a+) expression vector (Takara) to form the recombinant plasmid pET-32(a+)-4D8 which was transformed into *Escherichia coli* rosetta. The recombinant protein rDs4D8 was fused with 22 kDa histidine-tagged thioredoxin protein (TRX), following the induction by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 5 hr at 37°C. *E. coli* liquids of different treatment were centrifuged by 10,000 rpm for 10 min at 4°C and the precipitate was broken up by ultrasonic disruption (power, 400 W), and 20 µg protein samples per lane were separated by 10% SDS-PAGE.

The immunogenicity analysis of rDs4D8 was performed by western blot. A total of 20 µg protein sample per lane, including prestained protein marker (Fermentas, Ontario, Canada), purified rDs4D8, and IPTG-induced *E. coli* containing pET-32(a+)-4D8, were separated by 10% SDS-PAGE and transferred

to polyvinylidene difluoride (PVDF) membrane. The membrane was cut into 2 strips and blocked for 2 hr in PBS-Tween-20 (PBST) containing 5% fat-free milk, and then incubated with rabbit anti-*D. silvarum* serum (1:2,000) and rabbit negative serum overnight at room temperature, respectively. Following overnight incubation, they were washed with PBST and incubated with diluted peroxidase-conjugated affinipure goat anti-rabbit IgG (1:2,000) for 1.5 hr (Proteintech, Chicago, Illinois, USA). Positive signals were detected using supersignal® west dura extended duration substrate (Thermo Scientific, Rockford, Illinois, USA).

The *Ds4D8* sequence, consisted of 498 bp and encoding 165 amino acid residues, was submitted to GenBank (no. JX856138) (Fig. 1). The predicted amino acid sequence was compared with ORF from 12 tick species belonging to 6 different genera (Fig. 2). The results showed that *Ds4D8* shared 100% identity with *Dermacentor marginatus*, 98.2% identity with *Dermacentor variabilis*, 95.4% identity with *Hyalomma marginatum*, 93.9% identity with *Rhipicephalus sanguineus*, 93.0% identity with *Rhipicephalus appendiculatus* and *Rhipicephalus microplus*, and 81.0-90.2% identity for ticks in the other 3 genera. In addition, the phylogenetic tree showed 2 major monophyletic clades (excluding outgroup sequences) and sequences clustered in each clade were classified as a subfamily (Fig. 3). One monophyletic clade for 4D8 included *Ixodes ricinus* and *Ixodes scapularis* as a subfamily with bootstrap scores 100. The other monophyletic clade included *Rhipicephalus*, *Dermacentor*, *Hyalomma*, *Haemaphysalis*, and *Amblyomma* spp.

As shown in 10% SDS-PAGE gel (Fig. 4), the rDs4D8 expression was induced by IPTG and its molecular weight was about

Table 1. Primer pairs used for *Ds4D8* amplification by PCR

Primer name	Sequence
FP-RA4D85	ATGGCTTGTGCGACATTAAGC
RP-RA4D83	TTACGACAAATAGCTGGGCGTAGC
FP- <i>EcoRI</i> -RA4D85	GAATTCATGGCTTGTGCGACATTAAGC
RP- <i>XhoI</i> -RA4D83	CTCGAGTTACGACAAATAGCTGGGCGTAGC

The orientation of the primer is from 5' end to 3' end.

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1  ATGGCTTGTGCGACATTAAGCGGAACACACGACTGGGATCCCCTGCACAGTCCGAACGGCAGATCGCCGAAAAGACGGAGATGCATGCCT
1  M A C A T L K R T H D W D P L H S P N G R S P K R R R C M P

91  CTGTCAGTCTCCCCACCAGCGCCTCCAACAAGGGCGCACCAAAATCAATCCCCTCGCCGTTCCGAGACGTTGCCACCGAAGTTGACTTCAGAG
31  L S V S P P A P P T R A H Q I N P S P F G D V P P K L T S E

181 GAGATAGCAGCCAACATCCGCGAGGAGATGCGACGGCTGCAACGGCGCAAGCAGCTCTGCTTCCAGGGAAACCGACCTGAATCCCAGCCC
61  E I A A N I R E E M R R L Q R R K Q L C F Q G T D P E S Q P

271 ACAAGCGGCTCTTTATCTCCCGTCCGTCGAGACCAGCCTCTGTTTACCTTCCGCCAGGTGGGGCTCATTGCGAGCGGATGATGAAGGAG
91  T S G L L S P V R R D Q P L F T F R Q V G L I C E R M M K E

361 CGAGAGAGCCAGATACGGGAGGAGTATGACCACGTGCTTCCACCAAGCTCGCAGAACAATACGACACATTTGTTAAATTTACCTATGAC
121 R E S Q I R E E Y D H V L S T K L A E Q Y D T F V K F T Y D

451 CAAATTCAGAAGCGGTTTGAGGGTGCTACGCCAGCTATTTGTCGTAA
151 Q I Q K R F E G A T P S Y L S *
    
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Fig. 1. Nucleic acid and deduced amino acid sequence of *Ds4D8*. The start codon is underlined and the stop codon is marked with an asterisk.

40 kDa, which was consistent with the expected molecular mass considering that the expression vector produced a recombinant protein fused with a 22 kDa TRX protein (Fig. 4). The

reactivities of the purified rDs4D8 and IPTG-induced *E. coli* containing pET-32(a+)-4D8 with rabbit anti-*D. silvarum* serum were tested by western blot. The results showed that the rDs4D8

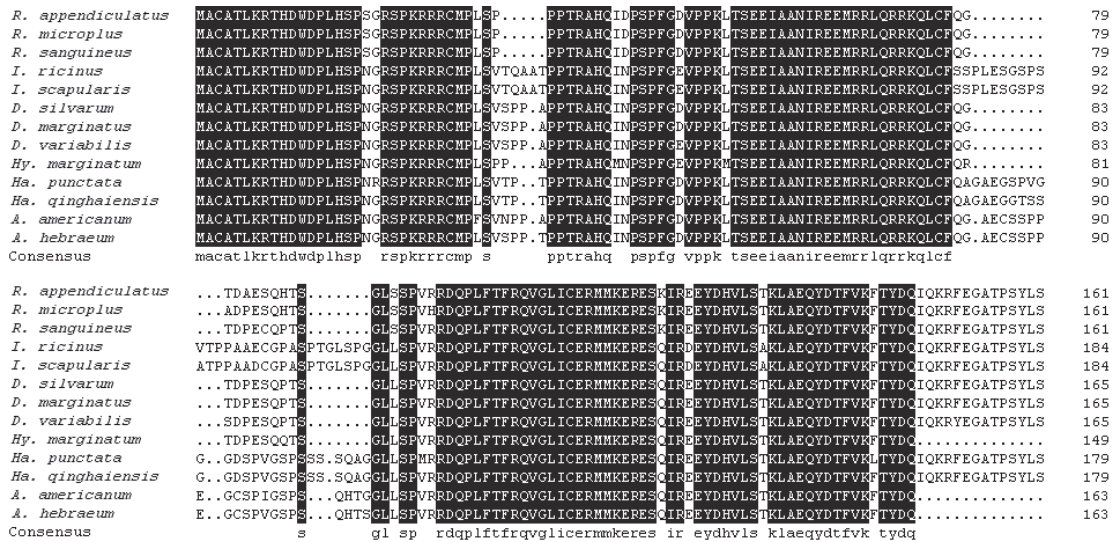


Fig. 2. Multiple alignment of 4D8 protein sequences for various tick species. Alignments were accomplished with Clustal X software. The graphic file was constructed by DNAMAN software program. GenBank accession numbers were *Rhipicephalus appendiculatus*, ABA62331; *Rhipicephalus microplus*, ABZ89745; *Rhipicephalus sanguineus*, ABA62332; *Ixodes ricinus*, ABA62325; *Ixodes scapularis*, AAV67031; *Dermacentor silvarum*, AFY52599; *Dermacentor marginatus*, ABA62333; *Dermacentor variabilis*, AAV67034; *Hyalomma marginatum*, ABA62335; *Haemaphysalis punctata*, ABA62336; *Haemaphysalis qinghaiensis*, ACA09713; *Amblyomma americanum*, ABA62326; *Amblyomma hebraeum*, ABY84524. The dark black shade shows identity.

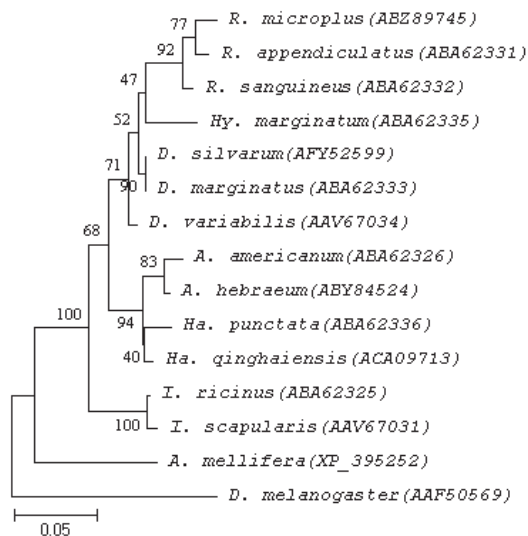


Fig. 3. Phylogenetic relationship of 4D8 sequences for various tick species. The outgroup sequence is 4D8 sequences of *Apis mellifera* (XP_395252) and *Drosophila melanogaster* (AAF50569). Numbers at the nodes represent bootstrap values on 1,000 replicates. The accession numbers of the other 4D8 sequences from ticks are shown in parentheses.

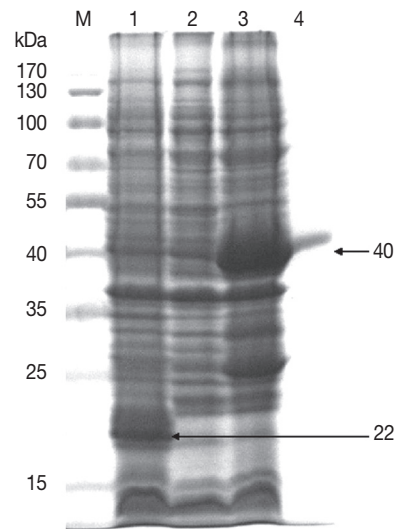


Fig. 4. SDS-PAGE analysis of rDs4D8 expressed in *E. coli*. The *E. coli* lysates were electrophoresed on 10% SDS-PAGE and stained with Coomassie brilliant blue R-250. M, molecular weight marker; lane 1, IPTG-induced *E. coli* with pET-32(a+); lane 2, uninduced *E. coli* with pET-32(a+)-4D8; Lane 3, IPTG-induced *E. coli* with pET-32(a+)-4D8; Lane 4, purified rDs4D8. TRX protein (22 kDa) and rDs4D8 (40 kDa) are shown by arrow.

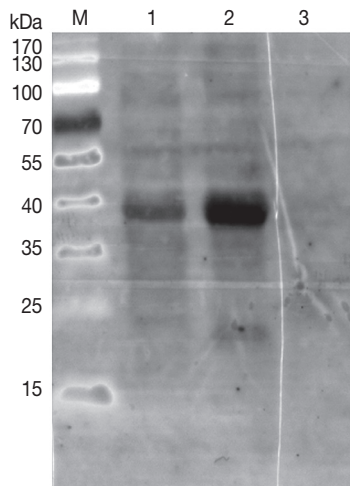


Fig. 5. Immunogenicity analysis of the expressed rDs4D8. M, molecular weight marker; Lane 1, purified rDs4D8 incubated by rabbit anti-*D. silvarum* serum; Lane 2, IPTG-induced *E. coli* with pET-32(a+)-4D8 incubated by rabbit anti-*D. silvarum* serum; Lane 3, IPTG-induced *E. coli* with pET-32(a+)-4D8 incubated by rabbit negative serum.

(40 kDa) in lane 1 and lane 2 could be recognized by rabbit anti-*D. silvarum* serum, while rabbit negative serum did not react with the rDs4D8 expressed in *E. coli* of lane 3 (Fig. 5).

The 4D8 plays a potential role in tick control. For example, de la Fuente et al. [4] verified that the silencing of 4D8 expression after RNAi resulted in the reduction of tick weight, oviposition, and survival. Oviposition was reduced in over 90% of all tick species evaluated. Vaccination with recombinant tick 4D8 may control *R. microplus* tick infestations in white-tailed deer to reduce tick populations, where the overall vaccine efficacy was 83% [8]. In addition, Merino et al. [9] confirmed that 4D8 vaccines also reduced the infection levels by 2 of the most important pathogens transmitted by cattle ticks, *Anaplasma marginale* and *Babesia bigemina*. To date, no 4D8 orthologs have been fully characterized nor tested as recombinant antigens in *D. silvarum*.

In this study, we cloned *Ds4D8* containing 498 bp coding for 165 amino acids with a predicted molecular weight of 18 kDa, which was similar to the values observed for most 4D8 proteins [4]. The results of multiple alignment and phylogenetic relationship demonstrated that 4D8 protein sequences are highly conserved among *Dermacentor*, *Rhipicephalus*, and *Hyalomma*.

The 4D8 cDNA from *Ornithodoros erraticus* was subcloned into the expression vector pQE-30 and expressed in *E. coli* M15 cells [10]. The western blot results showed that the molecular

weight of *O. erraticus* recombinant 4D8 was about 23 kDa containing 6×His-tagged recombinant polypeptides, while rDs4D8 had a larger molecular weight of 40 kDa when fused with pET-32(a+) tag protein. This may be due to the difference of expression vectors. In addition, Bensaci et al. [11] cloned *I. scapularis* 4D8 into vaccinia virus vector, and it was expressed from mammalian cells infected with the recombinant virus. The results suggested that immunized mice with *I. scapularis* 4D8 by oral gavage inhibited tick feeding and transmission of *Borrelia burgdorferi*. This provided a new way to analyze the function of rDs4D8 in *D. silvarum*.

By combining the results of peptide and phage-display libraries scan analysis, tick 4D8 linear B-cell epitopes (T1, T2, and T3) were identified [12]. Subsequently, the Q38 and Q41 chimeras were designed by combining 4D8 and akirin protective epitopes [12]. Studies of immunized mice showed that tick molting from larvae to nymphs was significantly reduced by 14-48% when compared to controls [13]. Our results of western blot showed that rDs4D8 demonstrated immunities against rabbit anti-*D. silvarum* serum. These data provided a basis for future studies on immunization with rDs4D8 or 4D8 epitopes to prevent attachment of *D. silvarum* ticks on target vertebrate hosts.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest related with this work.

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