

# フタトゲチマダニの唾液腺で発現しているセメント物質様抗原 の遺伝子

誌名	The journal of veterinary medical science
ISSN	09167250
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巻/号	68巻11号
掲載ページ	p. 1155-1160
発行年月	2006年11月

## Identification of Genes Encoding Cement-Like Antigens Expressed in the Salivary Glands of *Haemaphysalis longicornis*

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(Received 31 March 2006/Accepted 6 July 2006)

**ABSTRACT.** A cDNA expression library from the salivary glands of hard tick, *Haemaphysalis longicornis*, was constructed. Immunoscreeing was performed using sera of the rabbit repeatedly infested with ticks and seventeen positive clones were obtained. A BLASTP search suggested that 8 sequences matched with that of hypothetical *H. longicornis* sequence and one clone encoded HL35 antigen U from the same tick species. Eight of 17 gave no match to any sequence reported in the database. The proteins expected from these novel sequences possess common characteristics with cement proteins which assist ticks in their attachment to the host during blood feeding. The expression of these genes in salivary glands was confirmed by RT-PCR. Four of the 8 sequences showed to be upregulated upon blood feeding. These immunodominant antigens are of particular interest as candidates for future cement protein based-tick vaccine.

**KEY WORDS:** cDNA library, *Haemaphysalis longicornis*, immunoscreeing, salivary gland, tick.

*J. Vet. Med. Sci.* 68(11): 1155–1160, 2006

Ticks are medically important vectors of human and animal diseases. In addition to transmitting pathogens to mankind, tick infestations in animals produce worldwide economic losses [3]. Although tick control strategies have relied extensively on the use of chemical acaricides, this type of control is becoming less sustainable due to the development of resistance against acaricides among ticks and the undesirable contamination of the environment and animal food products [26]. Therefore, the development of alternative control strategies is necessary, in which vaccination against ticks is a desirable method. A concealed antigen based-vaccine against *Rhiphicephalus (Boophilus) microplus* [7] infestation was successfully commercialized [28]. A concealed antigen in the tick gut, however, is unlikely for the boost of the immune response by natural tick exposure compared with salivary glands antigens.

As a vaccine target, salivary glands are a rich source of exposed antigens that can stimulate host immune response following natural infestation. Tick saliva contains a mixture of peptides and proteins serving various functions that are essential for the creation and maintenance of the blood pool or the lesion in the host skin for feeding. Several bioactive molecules in tick saliva that affect the host's hemostatic, inflammatory, and immune systems have been extensively studied [16–18, 23]. The discovery of novel proteins and genes expressed in salivary glands has been accomplished by massive sequencing of full-length cDNA libraries together with approaches involving proteomics and functional genomics. This information assists in the exploration of protective antigens for vaccine development.

A hard tick *Haemaphysalis longicornis*, which com-

monly infests cattle and dogs in Japan and other parts of East Asia, is a major vector that transmits pathogens, such as *Babesia*, *Theileria*, and *Borrelia*, to domestic and wild animals in these areas [5]. Several vaccine candidates have been studied and tested for vaccine potency against tick infestation [8, 11, 22]. However, the animals vaccinated with these molecules conferred partial protection against certain stages of tick. The identification of other the candidates for use in a cocktail vaccine is still required.

In this research, of particular interest are the immunogens produced from the salivary glands of ticks. A cDNA library from the salivary glands of the hard tick, *H. longicornis* would provide valuable material, as it would contain a wide variety of genes encoding putative vaccine candidates. The immunoscreeing method was used to recruit immunodominant key molecules that might be useful for the tick control strategy.

### MATERIALS AND METHODS

**Ticks:** The adults and nymphs of parthenogenetic Okayama strain of the tick *H. longicornis* maintained by feeding on rabbits and mice for several generations in our laboratory since 1997 were used in this experiment.

**Animals:** All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

**Extraction of total RNA and poly (A)+ RNA:** Salivary glands from 160 adult ticks fed on rabbits for 3 days were homogenized using a homogenizer (Ultra Turrax T8; IKA Labor Technik, Hohenstaufen, Germany) in a Tri reagent (Sigma, St Louis, MO, U.S.A.). The total RNA was subsequently isolated according to the manufacturer's instruc-

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tions. Approximately 600  $\mu\text{g}$  of total RNA was obtained from 320 pairs of salivary glands. The poly(A)<sup>+</sup> RNA was purified using an Oligotex-dT30 (Super) mRNA isolation kit (Takara, Tokyo, Japan). The yield of purified mRNA was 1% of the total RNA.

**Construction of a cDNA library:** The construction of the cDNA library was performed with 5  $\mu\text{g}$  of mRNA using a ZAP-cDNA<sup>®</sup> synthesis kit (Stratagene, CA, U.S.A.) according to the manufacturer's instructions. After size fractionating, the cDNA was ligated into an *EcoRI/XhoI*-ended UniZap XR<sup>®</sup> vector. The ligation product was packaged using a ZAP-cDNA<sup>®</sup> Gigapack<sup>®</sup> III packaging extract (Stratagene, CA, U.S.A.). Plating and titrating of the primary library was performed to determine the titer and ratio of the recombinant/non-recombinant clones. The construction of the cDNA library yielded  $1.65 \times 10^5$  pfu of the primary library. The determination of the ratio of recombinant to non-recombinant clones with the use of 5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) and Isopropyl-1-thio- $\beta$ -D-thiogalactopyranoside (IPTG) showed that the library contained  $1.61 \times 10^5$  recombinant clones and 3,500 non-recombinant clones. Half the amount of the primary library was used for further amplification. The amplified library with  $4.0 \times 10^9$  pfu/ml was aliquoted and kept at  $-85^\circ\text{C}$  in 7% dimethylsulfoxide (DMSO).

**Immunoscreening of the cDNA library:** Approximately 20,000 pfu per plate of the amplified library was used for plaque lifting. The library was grown on NZY agar for 3.5–4.0 hr at  $42^\circ\text{C}$ . Then, IPTG induction was performed at  $37^\circ\text{C}$  for 3.5 hr by placing nitrocellulose membranes soaked with 10 mM IPTG onto the surface of the agar. For secondary and tertiary screening, 600 and 200 pfu were used for plating, respectively. The total of  $5 \times 10^5$  pfu of the cDNA library was screened using a polyclonal rabbit anti-*H. longicornis* tick immune serum prepared as previously described [30]. Immunoscreening was performed using a Picoblue<sup>™</sup> immunoscreening kit (Stratagene, CA, U.S.A.), according to the manufacturer's instructions. After blocking a non-specific binding site with 1% bovine serum albumin (BSA) for 1 hr, the membranes were allowed to react with polyclonal rabbit anti-tick saliva serum diluted 1:200 in 1% BSA in Tris-Buffered Saline (TBS) for 3 hr. After washing 3 times with Tris-Buffered Saline and 0.05% Tween 20 (TBS-T), the membranes were further incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG at a dilution of 1:10,000. The Nitroblue Tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl Phosphate (BCIP) substrate was used for the visualization of the positive signal. The positive plaques were subjected to further secondary and tertiary screening until the isolated clones were obtained. The cDNA inserts of the positive clone were then excised from the lambda phages to obtain the plasmid DNA for sequencing.

**DNA sequence analysis:** The nucleotide sequences of cDNA fragments were determined using a BigDye terminator cycle sequencing kit (PerkinElmer Life Sciences, Tokyo, Japan) and an automated sequencer (ABI PRISM 310 Genetic Analyzer, Tokyo, Japan). The sequences were

obtained by using vector-specific T3 and RPV primers. The nucleotide sequences were translated into amino acid sequences by means of an ExPASy server [6] (<http://www.expasy.org>). The deduced amino acid sequences were submitted to a similarity search against a non-redundant protein database using the BLASTP program at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) [1]. The FASTA files of the translated amino acid sequences were submitted to the SIGNALP server [13] (at <http://www.cbs.dtu.dk/services/SignalP/>) to determine the presence of signal peptides. Multiple sequence alignment of the nucleic and amino acids was performed using ClustalW [14] at the EMBL-EBI server (<http://www.ebi.ac.uk/clustalw/#>) and edited using the Genedoc program (<http://www.psc.edu/biomed/genedoc>).

**RT-PCR analysis of mRNA expression in salivary glands:** The expression of immunodominant mRNA transcripts in the salivary glands of *H. longicornis* was studied. RT-PCR was performed using a TAKARA one-step RNA PCR kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. For the PCR amplification of the total RNA from the salivary glands of unfed and partially fed ticks, the specific primers of each cDNA clone were used. The primers specific for the *H. longicornis* actin gene were also used as an internal control for the RT-PCR reaction. A negative-control reaction was performed under the condition without adding reverse-transcriptase to exclude some contamination of genomic DNA.

## RESULTS

The amplified salivary gland cDNA library was screened with polyclonal rabbit anti-*H. longicornis* tick immune serum. Screening of  $5 \times 10^5$  pfu yielded 17 positive clones. PCR amplification showed that positive clones contained inserts with sizes ranging from 0.5 to 1.7 kbp. As shown in Table 1, the BLASTP search indicated that 8 amino acid sequences were novel proteins; 8 matched with hypothetical proteins in *H. longicornis*; and one with the *H. longicornis* HL35 antigen U [22]. Ten of 17 sequences were found to possibly contain a secretory signal peptide. The remaining 7 sequences appeared to be truncated since starting methionine or 5'UTR were not found (marked as "T"). Multiple amino acid sequence alignments of 8 novel proteins are shown in Fig. 1. These sequences shared 23–97% identity to each other. Three sequences, designated hlim10, hlim11, and hlim20, shared greater than 90% identity were, therefore, considered to be the same. The amino acid sequence alignments suggested that these proteins would have unique features of cement proteins contained in tick saliva [2], as indicated by the presence of several GL[G/Y/S/L/F] tripeptide repeats.

One sequence, designated hlim3 (nucleotide accession number AB252633), matched with the previously reported HL35 antigen U [22]. This sequence also possessed the characteristics of a cement component [2]. The multiple sequence alignments of hlim3, HL35 (nucleotide accession

Table 1. Categorization of the positive clones from immunoscreening

Sequence Id	Accession number	Genbank match*	Species of NR match	E-value	Signal peptide
hlim1	AB259293	No matches found	–	–	T
hlim2	AB259292	No matches found	–	–	T
hlim3	AB252633	>gi 12060354 dbj BAB20558.1  HL35 antigen U	<i>H. longicornis</i>	1e-119	S
hlim4	AB259294	No matches found	–	–	S
hlim6	–	>gi 68051227 dbj BAE02556.1  hypothetical protein	<i>H. longicornis</i>	0.055	S
hlim7	–	>gi 68051227 dbj BAE02556.1  hypothetical protein	<i>H. longicornis</i>	0.055	S
hlim8	–	>gi 68051227 dbj BAE02556.1  hypothetical protein	<i>H. longicornis</i>	0.055	S
hlim9	–	>gi 68051227 dbj BAE02556.1  hypothetical protein	<i>H. longicornis</i>	0.055	S
hlim10	AB259299	No matches found	–	–	T
hlim11	–	No matches found	–	–	T
hlim12	–	>gi 68051227 dbj BAE02556.1  hypothetical protein	<i>H. longicornis</i>	0.055	T
hlim13	AB259296	No matches found	–	–	S
hlim14	AB259298	>gi 68051227 dbj BAE02556.1  hypothetical protein	<i>H. longicornis</i>	0.055	S
hlim17	–	>gi 68051227 dbj BAE02556.1  hypothetical protein	<i>H. longicornis</i>	0.055	S
hlim18	–	>gi 68051227 dbj BAE02556.1  hypothetical protein	<i>H. longicornis</i>	0.055	S
hlim20	AB259297	No matches found	–	–	T
hlim21	AB259295	No matches found	–	–	T

\* BLASTP performed with the Blosom62 matrix against the non-redundant (NR) protein database of NCBI; S, a secretory signal sequence was found; T, truncated sequence. Note; hlim10 and hlim11 are identical sequences (nucleotide accession number AB259299), 8 hypothetical proteins are the same sequences (nucleotide accession number AB259298).

gi|12060354), and its homologue, HL34 (nucleotide accession gi|10336521), are shown in Fig. 2. Even though the amino acid sequence of hlim3 shared greater than 80% identity with HL35, several amino acid substitutions and insertions were observed, suggesting the presence of sequence polymorphism.

The remaining identical 8 sequences shared weak similarity with the hypothetical protein of *H. longicornis* [12]. The calculated molecular weight was approximately 12 kDa and acidic in nature. The prediction from the SIGNALP server suggested that the sequence contained a secretory signal peptide.

The expression of the mRNA transcripts of immunoscreening-positive clones in the salivary glands of unfed and partially fed ticks was studied using RT-PCR (Fig. 3). Based on the amino acid sequence identity, the clones can be arbitrarily grouped into 8 categories, namely, 6 novel sequences (5 singletrons and one tripletron), the *H. longicornis* hypothetical protein, and the HL35 antigen U-like protein. The results suggested that all 8 sequences studied were expressed in the salivary glands of partially fed ticks and 7 sequences, namely hlim1, hlim2, hlim4 (faint band), hlim13, hlim14 (faint band), hlim20, and hlim21, were also expressed in unfed ticks. No obvious in the expression level of 4 transcripts (hlim2, hlim13, hlim20, and hlim21)

between RNA of unfed and fed ticks could be observed, whereas the remaining 4 (hlim1, hlim3, hlim4, and hlim14) appeared to be upregulated during blood feeding.

## DISCUSSION

The cloning and expression of several key molecules for use as tick vaccine candidates are ongoing worldwide. The study on antigens that elicit an antibody response is a useful strategy to identify vaccine antigens. For this purpose, the cDNA expression library of salivary glands from the adult female tick of *H. longicornis* was constructed in the present study. The genes encoding for immunodominant proteins were identified by immunoscreening using rabbit sera after repeated infestation with *H. longicornis* [30]. The BLASTP search of 17 positive clones revealed that 8 clones did not yield any similarities with known proteins, 8 clones gave matches with hypothetical proteins of *H. longicornis* and one clone, hlim3, shared similarity with the HL35 antigen U. The vaccination effect of HL34, the homologue of HL 35, has been evaluated, suggesting its suitability as a tick vaccine candidate [22]. The clone hlim3 shared 83% identity with the HL35 protein; however, the substitution and insertion of several amino acids were observed from the sequence alignments, indicating the presence of gene poly-

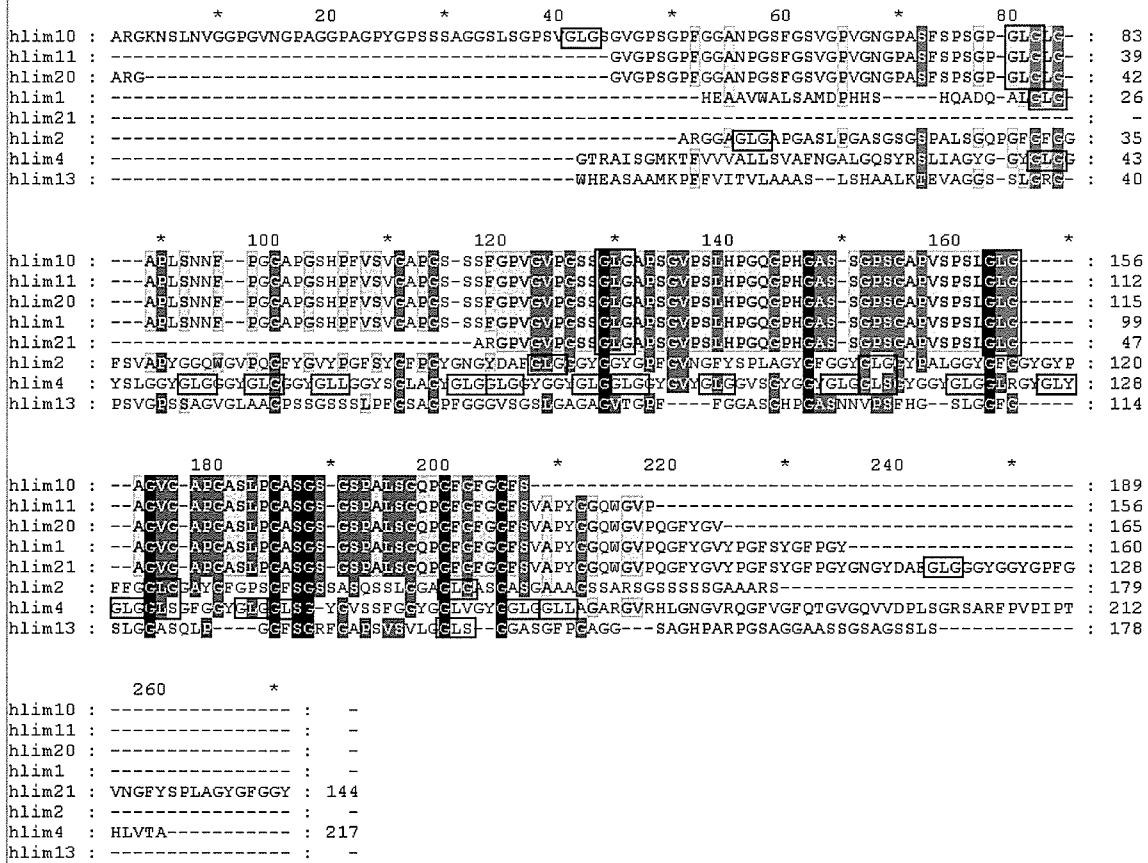


Fig. 1. Multiple amino acid sequence alignment of novel proteins. The CLUSTAL alignments of 8 novel proteins obtained by immunoscreening are shown (hlim1, hlim2, hlim4, hlim10, hlim11, hlim13, hlim20 and hlim21; nucleotide accession number AB2529293, AB2529292, AB2529294, AB2529299, AB2529296, AB2529297 and AB2529295, respectively). The unique features of cement protein, GLX repeats, are highlighted by boxes. Residues conserved in all sequences are marked with black shadow. Similarity is marked with a dark grey and light grey shadow. Note; hlim10 and hlim11 are identical sequences (nucleotide accession number AB2529299).

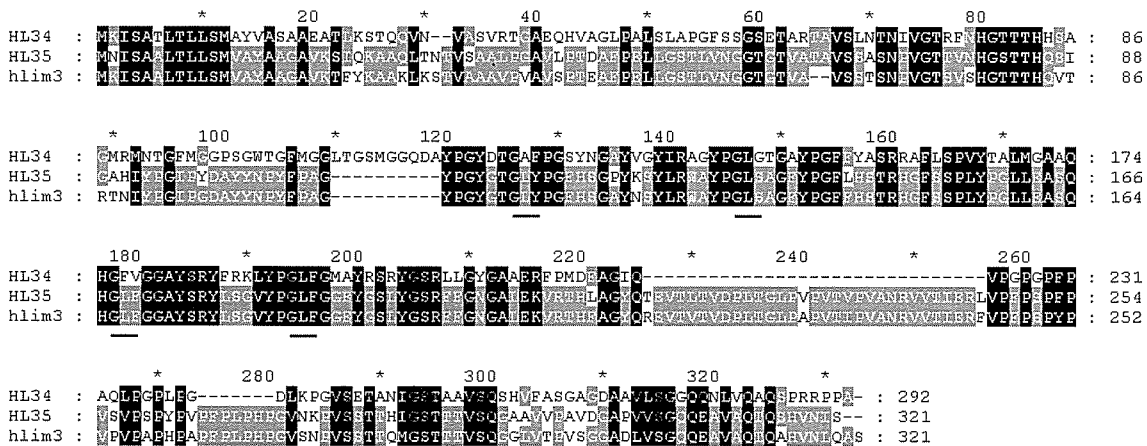


Fig. 2. Multiple sequence alignments of the HL35 antigen U-like protein. Amino acid sequence alignments of hlim3 (nucleotide accession number AB252633), previously reported HL35 (nucleotide accession number gi|12060354), and its homologue, HL34 (nucleotide accession number gi|10336521), are shown. The GLX repeats are underlined. Residues conserved in all sequences are marked with black shadow. Similarity is marked with a dark grey shadow.

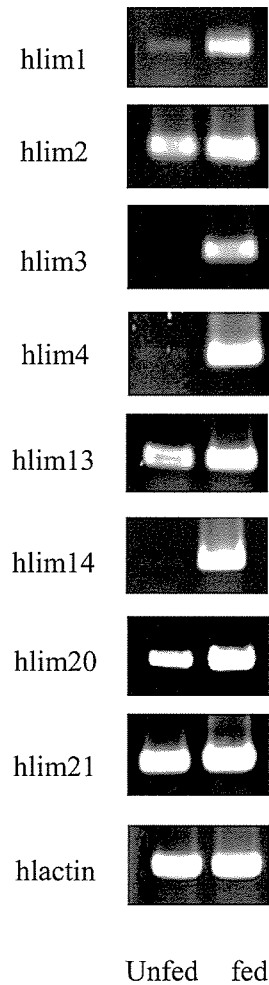


Fig. 3. Analysis of mRNA encoding for immunodominant antigens in unfed and partially fed salivary glands. Total RNA was isolated from the salivary glands of unfed and partially fed *H. longicornis* adults. The RT-PCR reaction with specific primers of each gene was performed. Primers specific for the *H. longicornis* actin gene were used as an internal control for the RT-PCR reaction.

morphism.

Eight cDNA clones gave best matches with the hypothetical protein from *H. longicornis*, indicating that this gene was abundantly expressed. The RT-PCR results showed that the mRNA transcript was upregulated in salivary glands during blood feeding. The peptides are possibly related to a secretory product, as they display a secretion signal. These findings implied that this protein plays an important role during blood feeding, although its function is still unknown.

The deduced amino acid sequences of the 8 novel proteins possessed striking feature of cement substances even though no hits to any sequence reported in the database were obtained, [2]. The presence of glycine triplet repeats and the high content of glycine are characteristic features of the vertebrate extracellular matrix protein [2]. One clone, hlim4,

contained a signal peptide and an 18 GLX repeat, a representative feature of cement substances. The cement components are initially secreted to assist in the attachment to the host tissue. A cement protein 36 (RIM36) of the tick *Rhipicephalus appendiculatus* is regarded as an immunodominant molecule and a promising target of eliciting antibodies in vaccinated cattle [2].

With regard to vaccine targeting, no consensus agreement is obtained as to whether an exposed or a concealed antigen is the best choice [9, 10, 27]. The use of a concealed antigen, as the commercial vaccines in cattle, can successfully induce protective immunity against tick feeding. However, immunity to concealed antigens may not prevent the attachment and feeding of the ticks or the transmission of tick-borne infections. In addition, immunity from this vaccine is short-lived because natural infestation does not stimulate a response to the concealed antigen [4, 29].

Salivary gland proteins are exposed antigens that are injected into the host during blood feeding. Resistance to salivary gland proteins is naturally acquired by multiple tick infestation or immunization with crude tick extracts [23, 24]. The positive aspects of salivary gland antigens as potential candidates for the regulation of tick attachment and feeding and transmission blocking vaccines [15] are superior beyond the drawbacks of the reduction of their antigenicity under the pressure of host immunity [19] or their inferior immunity compared to that induced by concealed antigens [25]. In particular, the immune response induced by the exposed antigen is expected to be continually boosted by the natural exposure of ticks to livestock in the field.

The salivary glands of ticks produce a cocktail of molecules having different functions [16–18, 23]. One of the categories is the cement components that are initially secreted from the salivary glands and enable the tick to attach to the host. Several studies have shown the attractiveness of the cement protein as vaccine candidates. Vaccination with the recombinant p29, the glycine-rich extracellular matrix protein from *H. longicornis*, led to a significant reduction of the engorged body weight in adult ticks and the mortality of both larvae and nymphs [11]. Another candidate, HL34, could induce morbidity and mortality in adult and nymphal ticks of the same tick species [22]. The recombinant cement cone protein RIM36 has been characterized from *R. appendiculatus*, in which it was indicated that this cement component could potentially elicit strong antibody responses in cattle exposed to feeding ticks [2]. When a putative tick cement protein (64P) from *R. appendiculatus* was examined as a vaccine target, a cross-reactivity of anti-sera raised against several truncated forms of recombinant 64P with several tissue extracts, suggesting that this cement protein was a promising candidate targeting both exposed and concealed antigens [21]. Moreover, it was shown that vaccination with the recombinant 64P cement protein induced protective humoral and cell-mediated immune responses in addition to antigenic cross-reactivity with the other tick species, suggesting its potential for use as a broad-spectrum anti-tick vaccine [20].

In the present study, we identified immunodominant antigens from the salivary glands of *H. longicornis* and several novel putative cement proteins were obtained. Further study on the recombinant expressed individual protein will clarify the role of these proteins for tick attachment and their possible use in a recombinant cocktail vaccine.

**ACKNOWLEDGEMENTS.** This work was supported by grants from the Bio-orientated Technology Research Advancement Institution (BRAIN), the 21st Century COE program (A-1) from the Ministry of Education, Science, Sports, and Culture of Japan, and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Sciences.

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