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ORIGINAL PAPER

# Identification of the *Babesia*-responsive leucine-rich repeat domain-containing protein from the hard tick *Haemaphysalis longicornis*

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**Abstract** *Haemaphysalis longicornis* is a tick known for transmitting *Babesia* parasites, including *Babesia gibsoni*, in East Asian countries. The vector tick must have strategies to control *Babesia* parasites, while *Babesia* parasites are also considered to establish an evasive mechanism from the tick's innate immunity. Due to this mutual tolerance, *H. longicornis* is considered to be a vector of *Babesia* parasites. Recent studies have shown the important roles of leucine-rich repeat (LRR) domain-containing proteins in innate immunity in many living organisms. Some LRR domain-containing proteins were identified in ticks; however, their functions are still unknown. In this study, a novel LRR domain-containing protein was identified from *H. longicornis* (HlLRR). HlLRR contains two LRR domains, and the expression levels of mRNA and proteins were upregulated during blood feeding, particularly in the salivary glands and midgut. In addition, recombinant HlLRR (rHlLRR) demonstrated growth inhibition activity against *B. gibsoni* in vitro without a hemolytic effect at any concentration used. Moreover, the diameters of *Babesia* merozoites treated with rHlLRR were significantly larger than those of the control group. These results strongly indicate the key roles of HlLRR in the tick's innate immunity against *Babesia* parasites. Furthermore, HlLRR might be a potential alternative drug to treat babesiosis.

**Keywords** Leucine-rich repeat · Tick · *Babesia* · Recombinant · Parasite

Hiroki Maeda and Koshi Kurisu contributed equally to this work.

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## Introduction

Babesiosis is caused by intraerythrocytic apicomplexan parasites belonging to the genus *Babesia* and is mainly transmitted by tick vectors to a variety of vertebrate hosts, including wild and domestic animals as well as humans. With the worldwide distribution of ixodid ticks, babesiosis is the second most common blood-borne disease of mammals (Homer et al. 2000; Hunfeld et al. 2008; Schmidt et al. 2014; Schnittger et al. 2012; Yabsley and Shock 2013). Patients with babesiosis show variable symptoms, and several anti-babesial drugs have been used for treatment; however, they are ineffective because of some problems with toxicity and the appearance of drug-registrant parasites (Homer et al. 2000; Vial and Gorenflot 2006). Thus, the development of an effective therapeutic agent against

babesiosis with high specificity to the parasites and low adverse effects to the hosts is urgently needed.

A repeating amino acid motif has been considered an important component of proteins. Leucine-rich repeats (LRRs) are one of the repeating amino acid segments present in a number of proteins with diverse functions related to protein-protein interactions, such as hormone receptors, enzyme inhibitors, cell adhesion, and signaling (Kobe and Deisenhofer 1994, 1995; Kobe and Kajava 2001). Typical LRRs consist of 20–30 amino acids and are unusually rich in the hydrophobic-amino acid leucine. Three-dimensional structures of LRRs were determined and showed that the structural variability might be related to the functional versatility of this protein superfamily (Bella et al. 2008; Kajava 1998). The sequences of the variable part suggest that the superfamily of LRR-containing proteins can be subdivided into several different subfamilies (Kajava 1998). This structural information provides the functional prediction of LRR-containing proteins, and experimental data are needed to support the hypothesis.

In tick research, some LRR-containing proteins have been identified from *Ixodes scapularis* (Smith and Pal 2014); however, their functions are still unknown. Unlike in ticks, the roles of LRR-containing proteins from other arthropods are relatively well understood, especially in the mosquitoes as reviewed by Cirimotich et al. (2010). They reviewed the importance of LRR-containing proteins in thioester-containing protein 1 (TEP1)-mediated anti-*Plasmodium* immunity in mosquitoes. These reports strongly suggest the key roles of LRR-containing proteins in the immunity of arthropods. In this report, a novel LRR-containing protein has been identified and characterized from the hard tick *Haemaphysalis longicornis* (HlLRR). HlLRR has two LRR domains, and recombinant HlLRR shows a growth inhibition activity on *Babesia gibsoni* in vitro. The present data indicate the key role of LRR-containing proteins in the innate immunity of ticks.

## Materials and methods

### Ticks and animals

The parthenogenetic Okayama strain of *H. longicornis* has been maintained by blood feeding on the ears of Japanese white rabbits (Kyudo, Kumamoto, Japan) in the Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University (Fujisaki 1978).

Rabbits and mice were cared for in accordance with the guidelines approved by the Animal Care and Use Committee of Kagoshima University (Approval no. VM13007). They were maintained under regulated conditions throughout the experiments.

### Identification and characterization of cDNA encoding the LRR domain-containing protein

The putative LRR domain-containing protein was identified using an expressed sequence tags (EST) database constructed from the cDNA library of the fat body. A pGCAP1 plasmid containing an HlLRR gene-encoding insert was extracted using a Qiagen® Plasmid Mini Kit (Qiagen, Hilden, Germany). The insert was sequenced by the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) using the Applied Biosystems® 3500 XL Genetic Analyzer (Applied Biosystems).

The deduced amino acid translation of the HlLRR sequence was determined by GENETYX version 7.0 software (GENETYX, Tokyo, Japan). To search homologous genes from GenBank (http://www.ncbi.nlm.nih.gov/genbank), a BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. The domain structure was determined by the SMART program (http://smart.embl-heidelberg.de/). Besides this domain prediction, an LRR highly conserved segment (LRR-HCS) was scanned by the LRR search application (http://www.lrrsearch.com) (Bej et al. 2014). For the three-dimensional structure prediction of HlLRR, the Phyre2 Protein Fold Recognition Server (http://www.sbg.bio.ic.ac.uk/phyre2/) was used (Kelley and Sternberg 2009). The theoretical molecular mass and isoelectric point were computed using a ProtParam tool (http://web.expasy.org/protparam/). Putative signal peptide cleavage sites and N-linked glycosylation sites were determined by the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively.

### Expression and purification of recombinant proteins

Recombinant HlLRR (rHlLRR) was expressed as a histidine-tagged (His-tag) protein using the expression vector pRSET A (Invitrogen, Carlsbad, CA, USA). The HlLRR open reading frame (ORF) sequence without the putative signal peptide was amplified by polymerase chain reaction (PCR) using a forward primer (HlLRR F-*Bam*H I) containing a *Bam*H I recognition site and a reverse primer (HlLRR R-*Bgl* II) containing a *Bgl* II recognition site (Table 1). The amplified PCR product was then purified using a GENECLEAN® II KIT (MP Biomedical, Solon, OH, USA) and subcloned into the frame of pRSET A. For the control plasmid, LRR domains were removed by *Hin*d III digestion (rHlLRR-ND: recombinant LRR with no domains).

Recombinant plasmids were transformed into an *Escherichia coli* BL21(DE3) strain. rHlLRR and rHlLRR-ND were expressed by induction with 1 mM isopropyl-β-D(-)-thiogalactopyranoside (IPTG) at 37 °C for 4 h. Expressed recombinant proteins were purified by a His-trap FF column (GE

**Table 1** Gene-specific primers used in this paper

| Primers | Sequence (5′→3′) |
|---|---|
| HlLRR F-*BamH* I | CGGGATCCAGTCCATCTTGCCACGAT |
| HlLRR R-*Bgl* II | GAAGATCTTTCAAGCGCCATCGGCA |
| HlLRR RT-F | ATTCTCGGATATGGCAGCAC |
| HlLRR RT-R | TCAGAACGAATCGCAGTTTC |
| HlLRR T7-F | TAATACGACTCACTATAGGGAGCGGGCTCAATCTTCTCA |
| HlLRR T7-R | TAATACGACTCACTATAGGTTCTCCAAACGCGACAACC |
| HlLRR RNAi-F | GAGCGGGCTCAATCTTCTCA |
| HlLRR RNAi-R | TTCTCCAAACGCGACAACC |
| Actin F | CCAACAGGGAGAAGATGACG |
| Actin R | ACAGGTCCTTACGGATGTCC |

Underline shows the T7 RNA polymerase promoter sequence

Healthcare, Buckinghamshire, UK) using a Bio Logic Duo Flow Base System (BIO-RAD, Tokyo, Japan). The purified recombinant proteins were dialyzed against phosphate buffered saline (PBS). The concentrations of rHlLRR and rHlLRR-ND were determined by a Micro BCA™ protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), and the recombinant proteins were stored at −30 °C until use.

### Size-exclusion chromatography

The molecular size of purified rHlLRR was also measured by size-exclusion chromatography. One milligram per milliliter concentration of rHlLRR was loaded into a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) at a flow rate of 0.8 ml/min using the ÄKTAprime plus chromatography system (GE Healthcare) (Miyata et al. 2011).

### Production of an antiserum against rHlLRR

One hundred micrograms of rHlLRR completely mixed with Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) was intraperitoneally injected into ddY female mice (4 weeks old, Kyudo, Saga, Japan). After 2 weeks, these mice were injected with 100 μg of rHlLRR with Freund's incomplete adjuvant (Sigma-Aldrich) twice at 2-week intervals to boost the generation of antibodies against rHlLRR. Their blood was collected 2 weeks after the third immunization to obtain the specific antisera for rHlLRR.

### RNA extraction and cDNA synthesis

To extract total RNA, whole ticks were homogenized using an Automill (Tokken, Chiba, Japan), dissected organs were disrupted using a pellet pestle motor (Sigma-Aldrich), and then TRI® reagent (Sigma-Aldrich) was added. The extracted RNA was purified with a Turbo DNA-*free*™ Kit (Applied Biosystems). cDNA synthesis was performed with ReverTra Ace-α-® (TOYOBO, Osaka, Japan) following the manufacture's protocol, using 1 μg of total RNA. Synthesized cDNA was analyzed by RT-PCR using specific primers (HlLRR RT-F and HlLRR RT-R, Table 1). The bands were normalized by actin using a primer set, Actin F and Actin R (Table 1).

### Protein extraction and Western blot analysis

Homogenized ticks were suspended in PBS and ultrasonicated three times, 2 min each (Vibra Cell™; Sonics and Materials, CT, USA), on ice and finally centrifuged at 500×*g*. The supernatant was resolved in a 15 % SDS-PAGE gel under reducing conditions. After SDS-PAGE, the proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon®-P; Millipore, MA, USA). The membrane was blocked overnight with 5 % skim milk in PBS (blocking solution), then incubated with a 1:500 dilution of anti-rHlLRR mouse sera in blocking solution at 37 °C for 1 h. Tubulin was used as a control protein Umemiya-Shirafuji et al. (2012). After washing five times in PBS containing 0.05 % Tween20 (PBS-T), the membrane was incubated with a 1:30,000 dilution of horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (Dako, Glostrup, Denmark) in blocking solution at 37 °C for 1 h. After washing five times in PBS-T, bands were detected using Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) and viewed using FluorChem®FC2 software (Alpha Innotech, CA, USA).

### RNA interference

Two separate PCR reactions of approximately 543 bp with a single T7 promoter were generated by using the following primer sets: a T7-attached gene-specific forward primer (HlLRR T7-F) and gene-specific reverse primer (HlLRR RNAi-R) and a T7-attached gene-specific reverse primer (HlLRR T7-R) and gene-specific forward primer (HlLRR RNAi-F) (Table 1). After gel purification of PCR products using a GENECLEAN® II KIT (MP Biomedical), dsHlLRR RNA was synthesized using the T7 RiboMax™ Express

RNAi kit (Promega, Madison, WI, USA) with two separate single promoter templates according to the manufacturer's protocol. The firefly *luciferase* (*Luc*) gene was used for control. One microgram of the dsHlLRR or dsLuc was injected into each of 30 unfed ticks in the experimental or control group, respectively, through the fourth coxae into the hemocoel. Injected ticks were kept at 25 °C before being infested for 1 day on the same rabbit at separate ears. Three days after attachment, three ticks were collected for the confirmation of gene silencing by RT-PCR. The remaining ticks were allowed to feed until engorgement, and the total number of engorged ticks, the weight of engorgement, survival, and oviposition were assessed.

### Culture of *B. gibsoni*

The culture of *B. gibsoni* (Aomori strain) was maintained as reported previously (Matsuu et al. 2008) and kept in an incubator with a temperature of 37 °C and a humid atmosphere containing 5 % $CO_2$.

### Effect of rHlLRR on *Babesia* parasites in vitro

The culture medium of *B. gibsoni* was changed daily and rHlLRR or rHlLRR-ND was added each day at concentrations of 0.05, 0.5, and 5 μM. An equal volume of PBS was used for the control group. Blood smears stained with Giemsa were prepared daily to determine the parasitemia and observe the morphology of *Babesia* parasites. Parasitemia was calculated as the percentage of infected red blood cells (RBCs) to 1,000 total RBCs counted. The morphology of *Babesia* parasites was observed, and the diameter of the ring-form merozoites was measured using a confocal laser scanning microscopy (LSM700, Carl Zeiss, Jena, Germany).

### Hemolysis assay

The hemolytic activity of rHlLRR and rHlLRR-ND was determined as described previously (Stark et al. 2002). Briefly, normal canine RBCs were washed with PBS three times, then 0.01 to 5 μM concentrations of rHlLRR or rHlLRR-ND were mixed with canine RBCs in a 96-well plate (Nunc, Roskilde, Denmark). The plate was incubated at 37 °C for 1 h and centrifuged at 1,000×*g* for 5 min. The supernatant was collected and the degree of hemolysis was measured by reading the absorbance at 550 nm in a microplate reader Model 680 (BIO-RAD). PBS and Triton-X were used as agents for preparing 0 and 100 % hemolysis.

### Statistical analysis

All experiments were conducted in two or three separate trials. Data were statistically analyzed by using the Mann–Whitney *U* test and results are presented as mean±SE; $P<0.05$ was considered statistically significant.

## Results

### Identification of HlLRR

The cDNA-encoding LRR domain-containing protein (HlLRR; accession no. LC011457) was isolated from EST clones from the fat body cDNA libraries of *H. longicornis*. The HlLRR ORF consists of 945 bp encoding 314 amino acids (Fig. 1). A polyadenylation consensus signal sequence variant was identified upstream of the poly A tail. The predicted molecular mass of HlLRR is 35.2 kDa, and the theoretical isoelectric point (p*I*) is 5.4. A putative signal peptide cleavage site was identified between residues 22 (A) and 23 (S). HlLRR has two LRR domains from positions 157 to 242 (LRR_8) and 266 to 310 (LRR C-terminal domain; LRRCT). In the LRR_8 domain, three LRR highly conserved segments (HCSs) were found (183-193, 208-218, and 232-242). N-linked glycosylation sites (asparagine) were determined at positions 41, 82, 256, and 297.

### Expression of rHlLRR and rHlLRR-ND

Sequences encoding the HlLRR ORF were subcloned into a pRSET A vector. For the control, LRR domains were removed by a *Hin*d III restriction enzyme (Fig. 2a). His-tag recombinant fusion proteins were produced in *E. coli*. IPTG-induced bacterial cells were used for the confirmation of expression by SDS-PAGE. The expressed rHlLRR and rHlLRR-ND molecular masses were approximately 37.5 and 21.8 kDa, respectively, under a reducing condition (Fig. 2b). rHlLRR showed an extremely high molecular weight band under a non-reducing condition, whereas rHlLRR-ND appeared to have the same molecular mass as that under a reducing condition (Fig. 2c). To know the exact molecular size of rHlLRR, gel-filtration chromatography was performed, and a single peak of 612 kDa molecular mass was obtained (Fig. 2d).

### Transcription and protein expression profiles of HlLRR

The mRNA level of *HlLRR* in whole adult ticks and each organ during blood feeding and in the developmental stages (egg, larval, nymphal, and adult stages) were investigated by semiquantitative RT-PCR. *HlLRR* was gradually upregulated in the whole adult ticks, midgut, fat body, and hemocytes during blood feeding. On the other hand, *HlLRR* mRNA was expressed in salivary glands from day 1 of feeding and is constitutively expressed in the ovary (Fig. 3a). In all developmental stages, the expression level of *HlLRR* mRNA was clearly upregulated after blood feeding (Fig. 3b). In addition

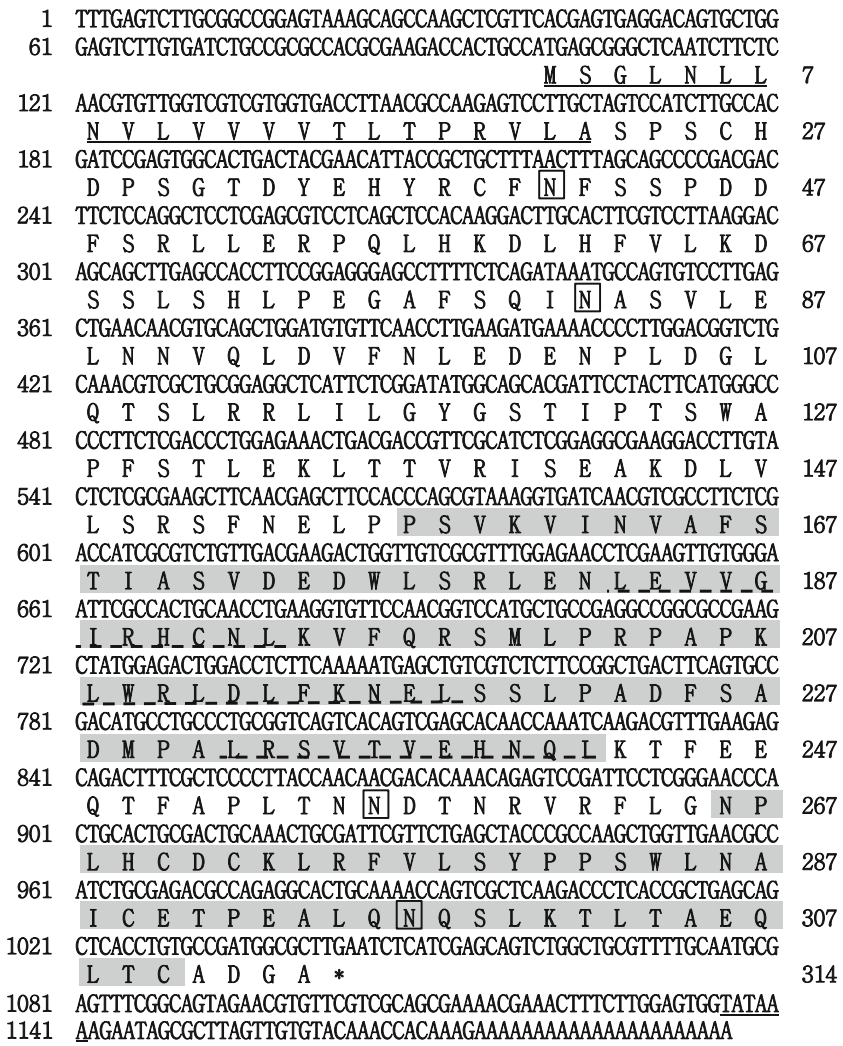


**Fig. 1** cDNA and deduced amino acid sequences of HlLRR from *H. longicornis*. The *underlined* amino acids in the N-terminal show the signal peptide. Putative glycosylated asparagines are *boxed*. Two LRR domains are represented as *gray-shaded letters* (LRR_8: 157 to 242, LRRCT: 266 to 310). Three LRR highly conserved segments (LRR-HCSs) are indicated with *dashed lines*. The putative polyadenylation signal variant after the stop codon (TGA) has been *underlined*

to analysis of *HlLRR* transcription, the protein expression of HlLRR was also confirmed by Western blotting using specific antisera. HlLRR expression increased and another band appeared approximately 2 kDa lower from the estimated band during blood feeding in all stages (Fig. 3c).

### Gene silencing effect of *HlLRR*

To clarify the functions of the *HlLRR* gene, gene silencing using an RNAi method was conducted. Clear gene silencing was confirmed by semiquantitative RT-PCR (Supplementary Fig. 1). However, no significant difference was observed on tick engorged body weight after infestation and laid-egg weight (Table 2).

### Impact of rHlLRR on the growth of *B. gibsoni* in vitro

The growth of *B. gibsoni* in vitro was inhibited dose-dependently and completely inhibited with 5 μM rHlLRR at 4–6 days (Fig. 4a). In addition, the LRR domain-removed rHlLRR (rHlLRR-ND) showed no effect on the growth of *B. gibsoni* at the concentration of 5 μM (Fig. 4a). Interestingly, in the presence of 5 μM rHlLRR, *Babesia* merozoites were sparsely observed, and their diameters were significantly larger than the in the PBS- and rHlLRR-ND-added control groups (Fig. 4b, c). Furthermore, no hemolytic effect was observed in canine RBCs incubated with any concentration of rHlLRR and rHlLRR-ND. The percentage of hemolysis was lower than 0.5 % and considered negligible at all concentrations of rHlLRR determined (Supplementary Fig. 2).

## Discussion

Tick-transmitted *Babesia* parasites are detrimental to animal health around the world. In addition to animals, human babesiosis is also a public health problem (Homer et al. 2000; Hunfeld et al. 2008; Schmidt et al. 2014; Schnittger et al. 2012; Yabsley and Shock 2013). Therefore, most research

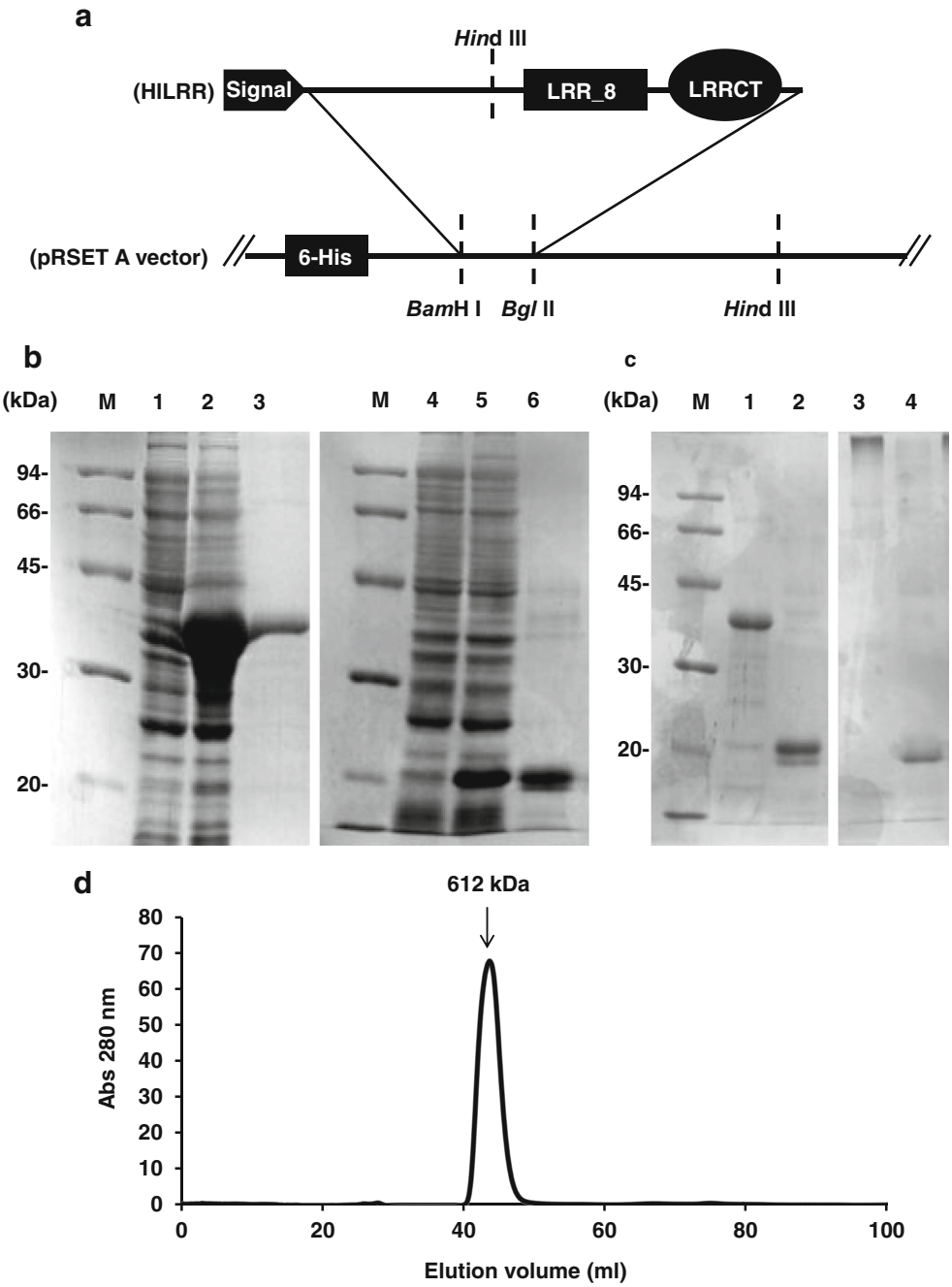


**Fig. 2** **a** A diagram of the recombinant plasmids. An HlLRR ORF without the signal peptide sequence is subcloned to a pRSET A vector using *Bam*H I and *Bgl* II recognition sites (rHlLRR). For the negative control, LRR domains were removed by cutting the *Hin*d III restriction enzyme (rHlLRR-ND). **b** SDS-PAGE analyses of expressed recombinant proteins, rHlLRR (*lanes 1–3*) and rHlLRR-ND (*lanes 4–6*). *M*, molecular weight marker; *lanes 1 and 4*, *E. coli* lysate before IPTG induction; *lanes 2 and 5*, *E. coli* lysate after IPTG induction; *lanes 3 and 6*, purified recombinant protein. **c** SDS-PAGE analyses of recombinant proteins under non-reduced conditions (*lanes 3 and 4*). M, molecular weight marker; *lane 1*, rHlLRR under a reduced condition; *lane 2*, rHlLRR-ND under a reduced condition; *lane 3*, rHlLRR under a non-reduced condition; *lane 4*, rHlLRR-ND under a non-reduced condition. **d** Determination of the HlLRR multimer's molecular weight using gel-filtration chromatography

on babesiosis is about medical treatments (Vial and Gorenflot 2006), while research focused on tick-*Babesia* interaction is scarce. *Babesia* parasites must cause serious damage to tick organs; however, the innate immune system of ticks might be able to control these effects. On the other hand, *Babesia* parasites have also developed strategies to avoid or limit the effects of tick immune responses to persist in the tick body while waiting for the opportunity to be transmitted to the vertebrate host (Chauvin et al. 2009; Florin-Christensen and Schnittger 2009). The existing and sustainable host-parasite relationship between ticks and *Babesia* parasites is assumed to be maintained on the basis of superb molecular mechanisms (Hajdušek et al. 2013).

LRR is a widespread structural motif with a characteristic structural repetitive sequence pattern rich in leucines and has been found in thousands of proteins with diverse functions in all life forms (Kobe and Deisenhofer 1994, 1995; Kobe and Kajava 2001), including toll-like receptors (TLRs), which are considered to have essential roles in the host defense mechanism (Bell et al. 2003). Thus, it is expected that the LRR-containing proteins have key roles as one of the central host defense systems. In mosquitoes, some LRR domain-

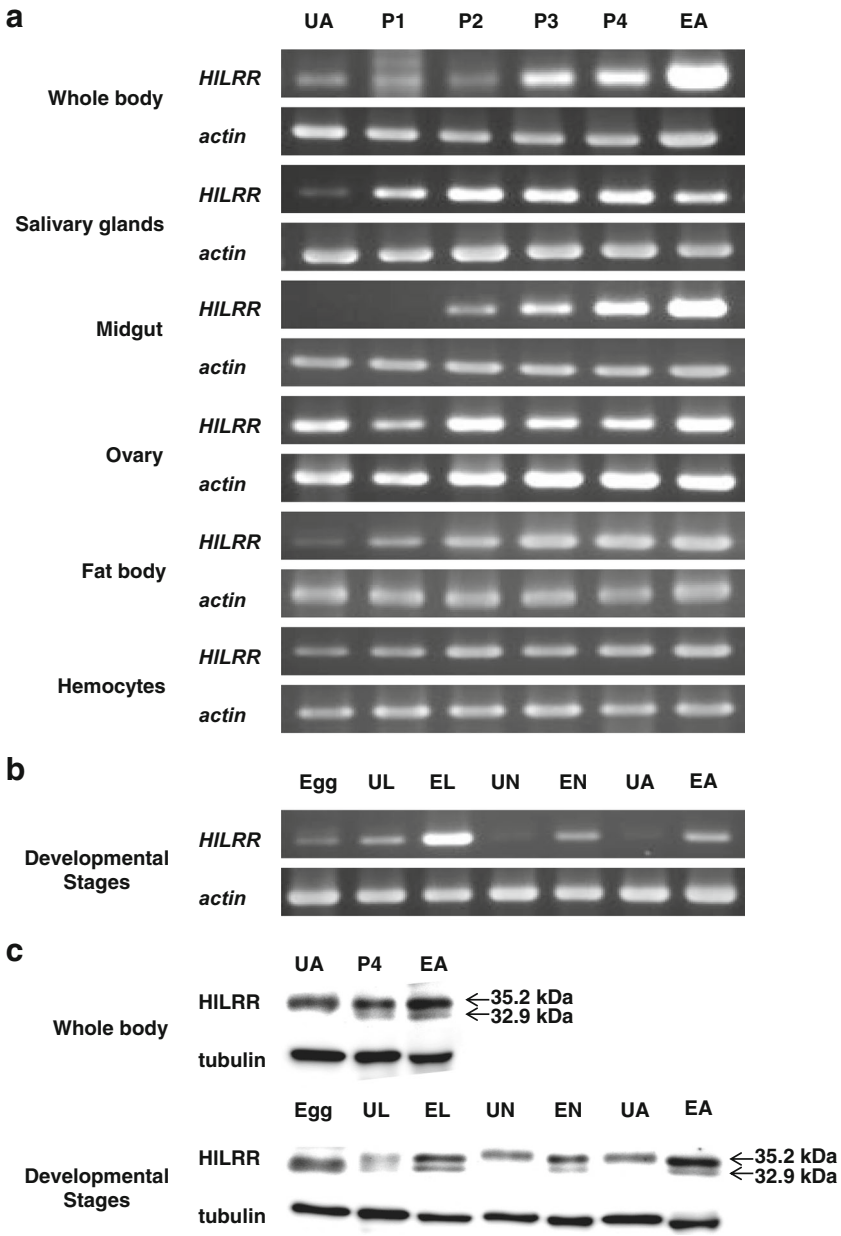


**Fig. 3** **a** Transcription profiles of *HlLRR* in whole ticks and different organs during blood feeding analyzed by semiquantitative RT-PCR. UA, unfed adults; P1-P4, adults partially fed for 1–4 days; *EA*, engorged adults. **b** Transcription profiles of *HlLRR* in developmental stages. *UL*, unfed larvae; *EL*, engorged larvae; *UN*, unfed nymphs; *EN*, engorged nymphs; *UA*, unfed adults; *EA*, engorged adults. **c** Protein expression profiles of HlLRR in whole adults and developmental stages

containing proteins have been identified and are well studied, especially in leucine-rich immune (LRIM) proteins and *Anopheles Plasmodium*-responsive leucine-rich repeat protein 1 (APL1) (Cirimotich et al. 2010; Waterhouse et al. 2010). It is well understood that the LRIM1 and APL1 complexes regulate TEP1-mediated complement-like immunity in *Anopheles gambiae* for defense against *Plasmodium* parasites (Baxter et al. 2010; Fraiture et al. 2009; Povelones et al. 2009, 2011). Although some genes encoding LRR domain-containing proteins were found i *Ixodes scapularis* ticks (Smith and Pal 2014) by a genome project (Hill and Wikel 2005; Pagel Van Zee et al. 2007), their biological roles in ticks remain unknown.

**Table 2** Gene silencing effects of *HlLRR*

| | Number of ticks | Engorged body weight (mg) | Egg weight (mg) |
|---|---|---|---|
| *dsLuc* | 30 | 131.9±48.9 | 60.6±28.4 |
| *dsHlLRR* | 30 | 119.4±44.1 | 58.0±27.7 |

In this study, we identified a novel LRR domain-containing protein from the *H. longicornis* cDNA library. The identified *HlLRR* sequence has an LRR_8 domain with three LRR-HCSs and an LRR-CT domain (Fig. 1), and the putative homologous gene was not found by BLAST analysis (data not shown). A signal peptide cleavage site, four N-linked glycosylation sites, and a polyadenylation consensus signal

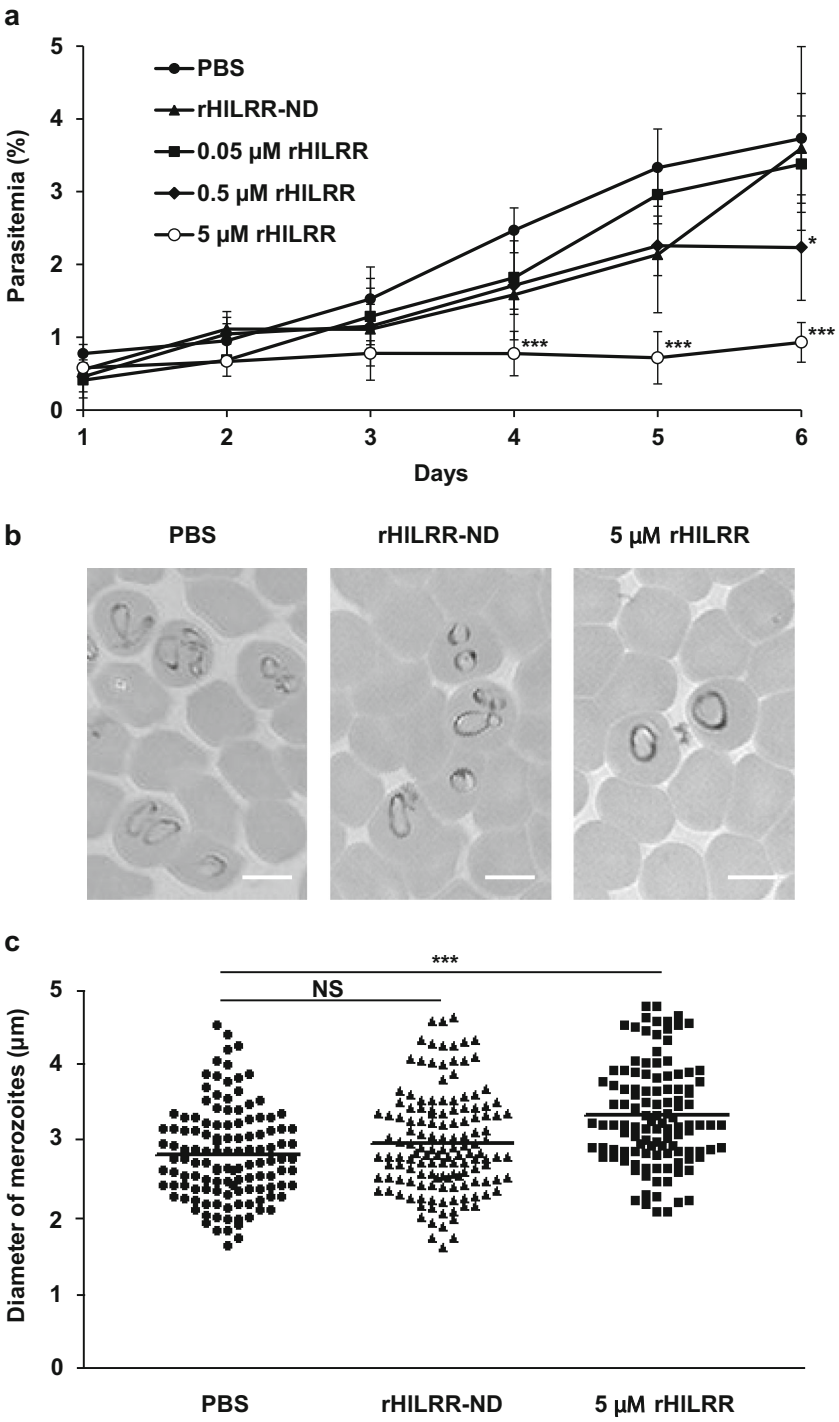


**Fig. 4** **a** Parasitemia for 6 days showing the effect of rHlLRR and rHlLRR-ND on the growth of *B. gibsoni*. **P*<0.05; ****P*<0.01, significantly different, control vs. recombinant protein-treated groups. **b** Differential interference contrast images of Giemsa-stained blood smear from day 6 showing parasite morphology from PBS control, 5 μM HlLRR-ND, and 5 μM HlLRR treatment. *Bar*=5 μm. **c** Diameter of merozoites at day 6. Each dot indicates the diameter of an individual parasite. *Black bar* indicates the average of diameter of parasites measured. *NS*, no significant difference, control vs. rHlLRR-ND-treated group; ****P*<0.01, significantly different, control vs. rHlLRR-treated group

sequence variant (Lutz 2008) were also determined in the sequence (Fig. 1). Unlike the other reported LRR domain-containing proteins possessing other types of domains such as TLRs, LRIM, APL1, and other immune-related LRR domain-containing proteins (Bell et al. 2003; Povelones et al. 2011; Waterhouse et al. 2010), HlLRR contains only LRR domains. Hence, it is considered that the HlLRR function might reflect the function of LRR domains. Recombinant proteins, including or excluding LRR domains (rHlLRR and rHlLRR-ND), were successfully expressed using *E. coli* according to the estimated molecular weight (Fig. 2b). rHlLRR showed a high molecular weight under a non-reducing conditions, while rHlLRR-ND failed to form a multimer (Fig. 2c). Core cysteine residues play a central role in stability and folding cooperativity were found in LRR domains (Rämisch et al. 2014). Thus, LRR domains are essential for the formation of a multimer linked by disulfide bonds. *HlLRR* mRNA expression was upregulated during blood feeding, particularly in

the salivary glands and midgut, and is constitutively expressed in the ovary (Fig. 3a). Similarly, in developmental stages, *HlLRR* mRNA expression was elevated after blood feeding (Fig. 3b). These results suggest the important roles of HlLRR during blood feeding. Furthermore, HlLRR protein expression was also upregulated after blood feeding, and a new band was detected below the estimated position (Fig. 3c). This band was ~2 kDa lower and is considered to be the mature HlLRR secreted by cutting the signal peptide. To clarify the HlLRR function, gene silencing experiments were conducted; however, there were no significant effects on the ticks' engorged body weight or egg laying (Table 2). Taken together, these results suggest that HlLRR might play crucial roles as a secreted form during blood feeding but is not involved in the blood ingestion or oviposition of ticks.

To elucidate the possible function of HlLRR in tick immunity, different concentrations of rHlLRR were added to an in vitro culture of *B. gibsoni*. *H. longicornis* has been considered the natural vector of *B. gibsoni* (Uilenberg 2006). Consequently, *H. longicornis* must have control mechanisms for *B. gibsoni* to decrease the adverse effects, while *B. gibsoni* can still evade the tick's innate immunity. This mutual tolerance between the anti-babesial function in *H. longicornis* and the evasion mechanism of *B. gibsoni* from the tick's innate immunity has been already established (Chauvin et al. 2009; Florin-Christensen and Schnittger 2009). In the present study, rHlLRR showed a growth inhibitory effect on *B. gibsoni* in vitro in a dose-dependent manner, and the growth was completely inhibited at a 5 μM concentration (Fig. 4a). rHlLRR showed a growth inhibition of *B. gibsoni* in vitro at a lower or similar concentration to those of reported agents, including doxycycline hydrochloride, azithromycin, ketoconazole, and so on (Matsuu et al. 2008). Moreover, 5 μM rHlLRR-ND did not exhibit any inhibitory action on the growth of *B. gibsoni*. Thus, LRR domains of HlLRR are thought to have potentially powerful anti-babesial activity. Furthermore, an additional hemolysis test of recombinant proteins revealed that it does not cause any cytotoxic effect on RBCs (Supplementary Fig. 2). Interestingly, the diameter of the merozoites in the 5 μM rHlLRR-treated group displayed an abnormally drastic increase compared to the control groups (Fig. 4b, c). This phenomenon might be because HlLRR can affect the osmoregulatory or metabolic system of *Babesia* parasites. Due to its LRR_8 domain, the three-dimensional structure of HlLRR showed some similarity to the Wnt-activated inhibitory factor (Waif, data not shown). Waif was known to relate Wnt/β-catenin signaling, which regulates numerous cellular processes, including cell proliferation and tissue homeostasis (Kagermeier-Schenk et al. 2011; MacDonald et al. 2009). *Babesia* merozoites were influenced by HlLRR, presumably due to the involved Wnt/β-catenin signaling. As shown in Fig. 2, the mRNA expression level of HlLRR increased in the salivary glands and midgut during blood feeding and was constitutively observed in the ovary. These organs play critical roles in the multiplication and transmission of *Babesia* parasites in vector ticks (Chauvin et al. 2009; Florin-Christensen and Schnittger 2009). Therefore, HlLRR might be related to the tick's immune response to the *Babesia* parasite.

In conclusion, this study suggests the key role of HlLRR in the tick's innate immunity against *Babesia* infection. In vitro experimentation suggests that HlLRR might be a potential alternative chemotherapeutic agent against babesiosis. A deeper understanding of LRR-containing protein families would lead to the design of new control strategies against ticks and tick-borne pathogens.

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