TABLE 1
Effect of storage of samples on sensitivity and specificity and on mean antibody unit with acetone treated urine ELISA

		Measurement time		
		2001 February	2003 May	2007 November
	Sensitivity	91.1% (51/56)	91.1% (51/56)	82.1% (46/56)
	Specificity	95.3% (201/211)	97.2% (205/211)	98.1% (207/211)
Mean antibody unit (%)	VL	103.10 (100.0)	83.86 (81.3)	61.11 (59.3)
	Non-VL	0.93 (100.0)	0.94 (101.2)	0.89 (95.5)

The rKRP42 Urine ELISA. A total of 115 VL and 240 non-VL samples were tested for IgG using rKRP42 antigen (Figure 1). With the NEHC group, the cut-off was determined as 57.9 U, based on which the test showed a sensitivity of 94.0% (108 positives among 115 VL samples) and a specificity of 99.6% (239 negatives among 240 non-VL samples). Of the 108 positive VL samples, only 10 had titers < 1,000 U. There was no difference between the sensitivities when calculated with the parasite-confirmed VL group (92.5% with 40 samples) and clinical VL group (94.7% with 75 samples; χ^2 test, $P > 0.64$). The specificities for EHC, NEHC, M, TB, and CL were 100%. Only one sample of other diseases category (aplastic anemia with nephrotic syndrome) became positive by the test.

KAtex. The urinary antigen detection assay KAtex showed a low sensitivity of 55.6% (60 positives among 108 VL samples) and a high specificity of 100% with 240 non-VL samples. Among the 108 VL samples, 36 were parasite positive, the rest (72) were clinical VL, and the KAtex positive rate for the former group was 63.9% (23 positives) and for the latter group was 51.4% (37 positives). No significance difference was obtained between the groups (χ^2 test, $P > 0.21$). In this study, 13 samples (of which 5 were parasitologically confirmed) were collected before initiation of treatment, and the rest (95 samples) were collected at various times in the course of treatment. Further analysis showed that the pre-treatment samples had a high sensitivity of 76.9%, whereas the treated samples had much lower sensitivity (44–54%; Table 2). However, the difference between the pre- and undertreatment (all combined) samples was not significant (χ^2 test, $P > 0.09$).

DISCUSSION

Because most of the urine samples used in this study were stored at 4°C for years, it would be essential to study the effect of storage on sensitivity, specificity, and the level of antibody unit, and for the study, acetone-treated urine

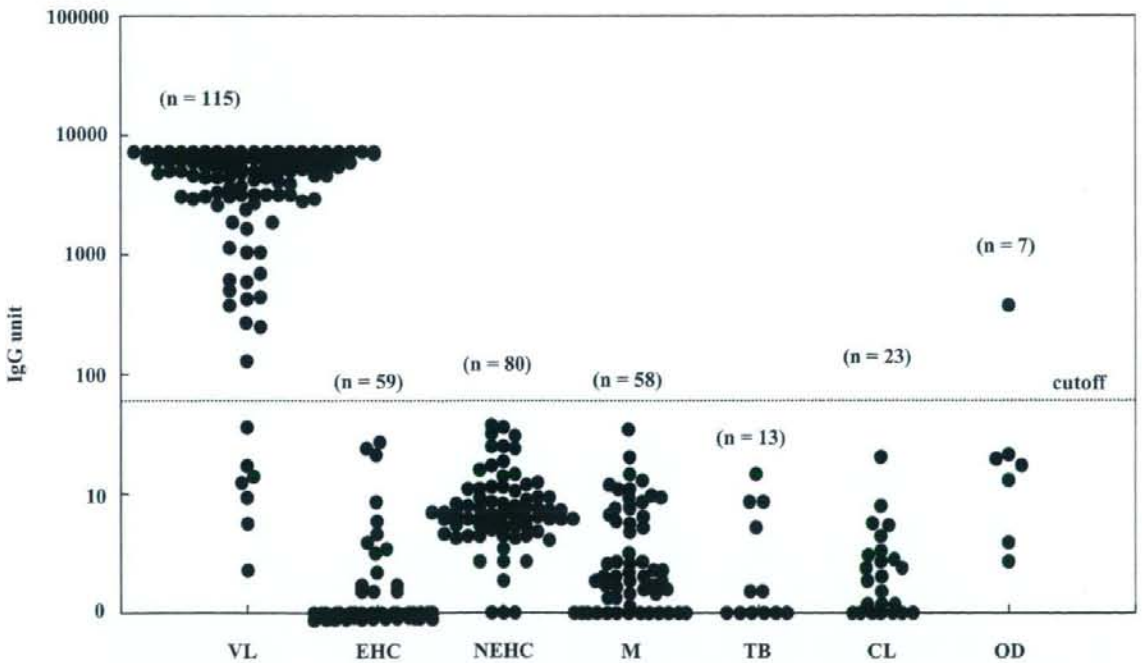

FIGURE 1. Detection of anti-rKRP42 IgG in urine of visceral leishmaniasis patients, healthy individuals, and controls with various diseases by ELISA. Each symbol (•) stands for a single urine sample. The horizontal dotted line represents the cut-off value: 57.9 U. VL = visceral leishmaniasis; EHC = endemic healthy controls; NEHC = non-endemic healthy controls; M = malaria; TB = tuberculosis; CL = cutaneous leishmaniasis; OD = other diseases; n = number of samples.

TABLE 2

KAtex-positive rate in relation to sample collection day with treatment

Sample collected on the day after initiation of treatment	Tested samples	KAtex positives
0 (before initiation of treatment)	13	76.9% (10/13)
1–10	9	44.4% (4/9)
11–20	15	53.3% (8/15)
21–30 or more	6	50.0% (3/6)
No clear history*	65	53.8% (35/65)

* In the patient's data sheets, the exact date of collection was not mentioned, but all the samples were collected within the course of treatment

ELISA was applied. The storage clearly decreased mean antibody units at a rate of 6% annually in the VL group. To the best of our knowledge, this is the first quantitative report on the reduction of *L. donovani*-specific antibody after long-term storage of NaN_3-added urine samples at 4°C. Thus, theoretically, urine storage could decrease sensitivity and at the same time might improve specificity. However, such effects will be only on borderline positive titers. In this study, up to 6 years and 8 months of storage did not affect the sensitivity and specificity at a statistically significant level.

The rKRP42 urine ELISA with samples kept for years showed a high sensitivity of 94%. The ELISA also resulted in a very high specificity of 99.6%. These results are comparable to our previously reported figures obtained by the acetone-treated urine ELISA, urine DAT, and rKRP42 serum ELISA. The commercially available rK39 dipstick test (InBios International, Seattle, WA) also gave similar results in our study in Bangladesh (Table 3).

When three different methods for urinary antibody detection (rKRP42 urine ELISA, acetone-treated urine ELISA, and urine DAT) were taken into consideration, a total 90.1% (64 of 71) VL samples were positive and 95.9% (211 of 220) of non-VL samples were negative by all three tests. Variable results, i.e., positive by one test but negative by other test(s), were obtained in 3 of 71 VL samples (4.2%), and 8 of 220 non-VL samples (3.6%). All these methods failed to detect four VL cases, of which three were parasite positives, or cross-reacted with one non-VL sample that was collected from a patient of aplastic anemia with nephrotic syndrome.

When we compared the rKRP42 urine ELISA with the rKRP42 serum ELISA and the rK39 dipstick test using 71 VL cases, 65 (91.5%) samples became positive and 4 (4.6%) samples were negative in all assays. Two samples from one parasite-confirmed and one clinical VL gave false-negative results with rKRP42 urine ELISA but were positives by rKRP42 serum ELISA. Of the four false negatives, three were collected from parasitologically confirmed patients. These three samples gave false-negative results with conventional serum-based DAT[23] and with urinary antibody detection assays (acetone-treated urine ELISA and urine DAT).

There is always a fraction of infected persons without clinical manifestations. Khalil and others[26] reported that, in eastern Sudan, the ratios of clinical and subclinical cases in Um-Salala village, respectively, in 1994/95 and 1995/96, were 1.2:1 and 2.6:1, and in Mashrau Koka village were 1:11 and 1:2.5. In another study conducted in Bihar state, India, 69% of asymptomatic seropositves by rK39 ELISA and dipstick developed kala-azar within 1 year,[27] suggesting that many of the asymptomatic cases were in a pre-clinical state. In predicting possible clinical cases, an ELISA, which is quantitative, would be

TABLE 3

Sensitivity and specificity of rKRP42 urine ELISA for the diagnosis of visceral leishmaniasis

Samples	Methods	Sensitivity*	Specificity†						References
		VL	EHC	NEHC	M	TB	CL	OD	Total	
Urine	rKRP42 urine ELISA	94.0% (108/115)	100% (59/59)	100% (80/80)	100% (58/58)	100% (13/13)	100% (23/23)	85.7% (1/7)	99.6% (239/240)	
	Acetone- treated urine ELISA	95.0% (57/60)	100% (59/59)	100% (53/53)	91.5% (54/59)	100% (13/13)	87.0% (20/23)	71.4% (5/7)	95.3% (204/214)	Islam and others (23)
	Urine DAT	90.1% (68/75)	96.6% (57/59)	100% (65/65)	100% (58/58)	92.3% (12/13)	87.0% (20/23)	71.4% (5/7)	96.4% (217/225)	Islam and others (24)
	KAtex	55.6% (60/108)	100% (59/59)	100% (80/80)	100% (58/58)	100% (13/13)	100% (23/23)	100% (7/7)	100% (240/240)	
Serum	rKRP42 serum ELISA	94.6% (70/74)		99.3% (148/149)					99.3% (148/149)	Takagi and others (19)
	rK39 dipstick test	93.2% (69/74)								Takagi and others (19)

* Number positive/total number of VL samples.
† Number negative/total number of non-VL samples in each category.
VL = visceral leishmaniasis; EHC = healthy controls from endemic areas; NEHC = healthy controls from non-endemic areas; M = malaria; TB = tuberculosis; CL = cutaneous leishmaniasis; OD = other diseases.

more advantageous than a dipstick format, that is, high antibody titers, or increase in antibody titers with time could be indicative of possible clinical cases. Such early diagnosis will have a practical importance now that oral treatment with miltefosine has become available.[28] However, frequent collection of blood often hinders the field surveys. Urine samples that can be easily and safely collected will solve many of the difficulties experienced with blood collection in a field study, especially with children.

Antigen detection is more useful than antibody detection for diagnosing active VL cases. KAtex has been widely evaluated in many places such as in Nepal[29] and India[30] and found to have a sensitivity of 47.7% and 73.5%, respectively. In this study, the sensitivity and specificity of KAtex was 55.6% and 100%, respectively. Because the study included urine samples from clinically suspected cases and undertreatment cases, the sensitivity could be an underestimate. In our separate study, it was observed that six of seven KAtex-positive cases turned negative after completion of 28-day course of sodium stibogluconate treatment, suggesting its diagnostic and prognostic value.

The rKRP42 urine ELISA has been shown to be highly sensitive and specific. The stability of urinary antibody at 4°C even after prolonged storage has also been confirmed. Our rKRP42 urine ELISA would be a better alternative for the diagnosis of VL in a field survey, especially when repeated sampling is needed. KAtex that uses urine would be good adjunct for determining the active VL cases among rKRP42 urine ELISA positives.

Received June 3, 2008. Accepted for publication July 17, 2008.

Acknowledgments: We thank Drs. Rusella Mirza, Farzana Matin, Mohsin Ali, A. K. M. Shamsuzzaman Choudhury, Akram Hossain, Nilufar Begam, and Sultana Monira Hussain for helping in collection of VL samples and Dr. Judson. L. Leafasia for providing malaria samples.

Financial support: This study was supported by Grant-in-Aid for Scientific Research for JSPS Postdoctoral Fellowship for Foreign Researchers 16. 04227, Grant-in-Aid for Scientific Research (B) 15406018, and Grant-in-Aid for Scientific Research (B) 18406013 from the Japan Society for the Promotion of Science, and also by the Special Coordination Funds for Promoting Science and Technology of MEXT, Japan, 1200015.

Authors' addresses: Mohammad Zahidul Islam, Makoto Itoh, Hidekazu Takagi, Atsuhide Takesue, and Eisaku Kimura: Department of Parasitology, Aichi Medical University School of Medicine, Nagakute, Aichi-ken 480-1195, Japan, Tel: 81-52-264-4811, Fax: 81-561-63-3645, E-mails: zahid@aichi-med-u.ac.jp, macitoh@aichi-med-u.ac.jp, htakagi@aichi-med-u.ac.jp, atakesue@aichi-med-u.ac.jp, and kimura@aichi-med-u.ac.jp. Anwar Ul Islam and Ajijur Rahman, Department of Pharmacy, University of Rajshahi, Rajshahi, Bangladesh, Tel: 88-721-750041-9, Fax: 88-721-750064, E-mails: profanwarulislam@yahoo.com and shamim_ru2003@yahoo.com. A. R. M. Saifuddin Ekram, Department of Medicine, Rajshahi Medical College, Rajshahi, Bangladesh, Tel: 88-721-776001-9, Fax: 88-721-772174, E-mail: armsekram@yahoo.com. Yoshihisa Hashiguchi, Department of Parasitology, Kochi Medical school, Kochi University, Nankoku City, Kochi 783-8505, Japan, Tel: 81-88-880-2415, Fax: 81-88-880-2415, E-mail: hasiguti@kochi-u.ac.jp.

Reprint requests: Mohammad Zahidul Islam, Department of Parasitology, Aichi Medical University School of Medicine, Nagakute, Aichi-ken 480-1195, Japan, E-mails: zahid@aichi-med-u.ac.jp and islammz@yahoo.com.

REFERENCES

1. Collin S, Davidson R, Ritmeijer K, Keus K, Melaku Y, Kipngetich S, Davies C, 2004. Conflict and kala-azar: determinants of adverse outcome of kala-azar among patients in Southern Sudan. *Clin Infect Dis 38:* 612–619.
2. Ahluwalia IB, Bern C, Costa C, Akter T, Chowdhury R, Ali M, Alam D, Kenah E, Amann J, Islam M, Wagatsuma Y, Haque R, Breiman RF, Maguire JH, 2003. Visceral leishmaniasis: consequences of a neglected disease in a Bangladeshi community. *Am J Trop Med Hyg 69:* 624–628.
3. World Health Organization, 2007. *Global Plan to combat Neglected Tropical Diseases, 2008–2015.* Geneva, Switzerland: World Health Organization.
4. Zijlstra EE, Ali MS, el-Hassan AM, el-Toum IA, Satti M, Ghalib HW, Kager PA, 1992. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. *Trans R Soc Trop Med Hyg 86:* 505–507.
5. Kaul P, Malla N, Kaur S, Mahajan RC, Ganguly NK, 2000. Evaluation of a 200-kDa amastigote-specific antigen of *L. donovani* by enzyme-linked immunosorbent assay (ELISA) for the diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg 94:* 173–175.
6. Raj VS, Ghosh A, Dole VS, Madhubala R, Myler PJ, Stuart KD, 1999. Serodiagnosis of leishmaniasis with recombinant ORFF antigen. *Am J Trop Med Hyg 61:* 482–487.
7. Zijlstra EE, Daifalla NS, Kager PA, Khalil EAG, El-Hassan AM, Reed SG, Ghalib HW, 1998. rK39 enzyme-linked immunosorbent assay for diagnosis of *Leishmania donovani* infection. *Clin Diagn Lab Immunol 5:* 717–720.
8. Harith AE, Kolk AHJ, Kager PA, Leeuwenburg J, Muigai R, Kiugu S, Laarman JJ, 1986. A simple and economical direct agglutination test for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis. *Trans R Soc Trop Med Hyg 80:* 583–587.
9. Harith AE, Kolk AHJ, Leeuwenburg J, Muigai R, Huigen E, Jelsma T, Kager PA, 1988. Improvement of a direct agglutination test for field studies of visceral leishmaniasis. *J Clin Microbiol 26:* 1321–1325.
10. Burns JM, Shreffler WG, Benson DR, Ghalib HW, Badaro R, Reed SG, 1993. Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African American visceral leishmaniasis. *Proc Natl Acad Sci USA 90:* 775–779.
11. Maalej IA, Chenik M, Louzir H, Salah AB, Bahloul C, Amri F, Dellagi K, 2003. Comparative evaluation of ELISAs based on ten recombinant or purified leishmania antigens for the serodiagnosis of Mediterranean visceral leishmaniasis. *Am J Trop Med Hyg 68:* 312–320.
12. Sundar S, Reed SG, Singh VP, Kumar PCK, Murray HW, 1998. Rapid accurate field diagnosis of Indian visceral leishmaniasis. *Lancet 351:* 563–565.
13. Sundar S, Pai K, Sahu M, Kumar V, Marray HW, 2002. Immunochromatographic strip-test detection of anti-K39 antibody in Indian visceral leishmaniasis. *Ann Trop Med Parasitol 96:* 19–23.
14. Bern C, Jha SN, Joshi AB, Thakur GD, Bista MB, 2000. Use of the recombinant K39 dipstick test and the direct agglutination test in a setting endemic for visceral leishmaniasis in Nepal. *Am J Trop Med Hyg 63:* 153–157.
15. Delgado O, Feliciangeli MD, Coraspe V, Silva S, Perez A, Arias J, 2001. Value of a dipstick based on recombinant rK39 antigen for differential diagnosis of American visceral leishmaniasis from other sympatric endemic diseases in Venezuela. *Parasite 8:* 355–357.
16. Jelinek T, Eichenlaub S, Löscher T, 1999. Sensitivity and specificity of a rapid immunochromatographic test for diagnosis of visceral leishmaniasis. *Eur J Clin Mirobiol Infect Dis 18:* 669–670.
17. Zijlstra EE, Nur Y, Desjeux P, Khalil E, El-Hassan AM, Groen J, 2001. Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from the Sudan. *Trop Med Int Health 6:* 108–113.
18. Carvalho SF, Lemos EM, Corey R, Dietze R, 2003. Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. *Am J Trop Med Hyg 68:* 321–324.
19. Takagi H, Islam MZ, Itoh M, Islam MAU, Ekram ARMS, Hussain SM, Hashiguchi Y, Kimura E, 2007. Production of recombinant kinesin-related protein of *Leishmania donovani* and its

application in the serodiagnosis of visceral leishmaniasis. *Am J Trop Med Hyg 76:* 902–905.

20. Attar ZJ, Chance ML, el-Safi S, Carney J, Azazy A, El-Hadi M, Dourado C, Hommel M, 2001. Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. *Acta Trop 78:* 11–16.
21. Sarkari B, Chance M, Hommel M, 2002. Antigenuria in visceral leishmaniasis: detection and partial charecterisation of a carbohydrate antigen. *Acta Trop 82:* 339–348.
22. Islam MZ, Itoh M, Shamsuzzaman SM, Mirza R, Matin F, Ahmed I, Choudhury AKMS, Hossain MA, Qiu XG, Begam N, Furuya M, Leafasia JL, Hashiguchi Y, Kimura E, 2002. Diagnosis of visceral leishmaniasis by ELISA using urine samples. *Clin Diagn Lab Immunol 9:* 789–794.
23. Islam MZ, Itoh M, Mirza R, Ahmed I, Ekram ARMS, Sarder AH, Shamsuzzaman SM, Hashiguchi Y, Kimura E, 2004. Direct agglutination test with urine samples for the diagnosis of visceral leishmaniasis. *Am J Trop Med Hyg 70:* 78–82.
24. World Health Organization, 1996. *Manual on Visceral Leishmaniasis Control.* Geneva, Switzerland: World Health Organization.
25. World Health Organization, 1984. *Leishmaniasis.* Geneva, Switzerland: World Health Organization.
26. Khalil EAG, Zijlstra EE, Kager PA, El Hassan MA, 2002. Epidemiology and clinical manifestations of *Leishmania donovani* infection in two villages in an endemic area in eastern Sudan. *Trop Med Int Health 7:* 35–44.
27. Singh S, Kumari V, Singh N, 2002. Predicting kala-azar disease manifestations in asymptomatic patients with latent *Leishmania donovani* infection by detection of antibody against recombinant K39 antigen. *Clin Diagn Lab Immunol 9:* 568–572.
28. Jha TK, Sundar S, Thakur CP, Backmann P, Karbwang J, Fischer C, Vos A, Berman J, 1999. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. *N Engl J Med 341:* 1795–1800.
29. Rijal S, Boelaert M, Regmi S, Karki BMS, Jacquet D, Singh R, Chance ML, Chappuis F, Hommel M, Desjeux P, Van der Stuyft P, Le Ray D, Koirala S, 2004. Evaluation of urinary antigen–based latex agglutination test in the diagnosis of kala-azar in eastern Nepal. *Trop Med Int Health 9:* 724–729.
30. Sundar S, Sing RK, Bimal SK, Gidwani K, Mishra A, Mauriya R, Singh SK, Manandar KD, Boelaert M, Rai M, 2007. Comparative evaluation of parasitology and serological tests in the diagnosis of visceral leishmaniasis in India: a phase III diagnostic accuracy study. *Trop Med Int Health 12:* 284–289.

International Journal for Parasitology 39 (2009) 97–107

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Legumains from the hard tick *Haemaphysalis longicornis* play modulatory roles in blood feeding and gut cellular remodelling and impact on embryogenesis

M. Abdul Alim [a], Naotoshi Tsuji [a,*], Takeharu Miyoshi [a], M. Khyrul Islam [a], Takeshi Hatta [a], Kozo Fujisaki [b,c]

[a] Laboratory of Parasitic Diseases, National Institute of Animal Health, National Agricultural and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan
[b] National Research Centre for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
[c] Laboratory of Emerging Infectious Diseases, School of Frontier Veterinary Medicine, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan

ARTICLE INFO

Article history:
Received 15 April 2008
Received in revised form 21 May 2008
Accepted 7 June 2008

Keywords:
Tick
Legumains
RNA Interference
Blood digestion
Midgut remodelling
Embryogenesis
Haemaphysalis longicornis

ABSTRACT

The biology and vectorial capacity of haematophagous ticks are directly related to effective blood feeding and digestion. The midgut-associated proteases in ticks are involved in the blood (Hb) digestion cascade, the molecular mechanisms of which are yet poorly understood. Our previous studies indicated that *Haemaphysalis longicornis* midgut-specific asparaginyl endopeptidases/legumains, HlLgm and HlLgm2, act in the Hb digestion cascade. Here, we investigated the potential of these enzymes in blood feeding and digestion, midgut remodelling and reproduction of ticks by employing RNA interference (RNAi) techniques. Injection of *HlLgm*- and *HlLgm2* gene-specific double-stranded RNAs into unfed adult female *H. longicornis* caused gene-specific transcriptional and translational disruptions. RNAi impacted on tick blood feeding leading to death of the feeding ticks, failure of ticks to reach repletion and significant reductions in engorged tick body weight. Histological examination revealed that deletion of legumains resulted in damage to the midgut tissues and disruption of normal cellular remodelling during feeding. Gene knock-down also caused significantly delayed onset of oviposition, reduced number of eggs and, most strikingly, structurally deformed eggs that failed to hatch suggesting imperfect embryogenesis. Synergistic impacts of RNAi were reflected on all parameters evaluated when *HlLgm* and *HlLgm2* were silenced together. These findings suggest that legumains may play modulatory roles in blood feeding and digestion, midgut cellular remodelling and embryogenesis in *H. longicornis*. Deletion of legumains in *H. longicornis* would help in controlling the tick population and thereby transmission of diseases to their hosts.

1. Introduction

The life cycles of the three-host non-nidicolous tick species are characterised by feeding for a few days to a few weeks on a host and a long period of diapause in habitats where either they moult into the next stage or the adult females lay eggs and die. The newly moulted stage or the newly hatched larva repeats the process of blood feeding on a different host (Ostfeld et al., 2006). This unique feature of the life cycle history of the non-nidicolous ticks provides an opportunity for many bacterial, viral, protozoal and rickettsial species to use ticks as effective vehicles for dispersal from one vertebrate host to another (Soulsby, 1986; Grubhoffer et al., 2005; Klompen, 2005).

Host blood feeding and digestion of blood (Hb) provides nutrition and energy for the moulting, development and vitellogenesis of ticks (Grandjean, 1984). Also, in ticks, the microbial pathogens generally do not reproduce and typically do not cause obvious disease until the ticks begin feeding on a host (Klompen, 2005; Ostfeld et al., 2006). The ingested blood meal supplies the energy reserves, especially proteins, required for yolk formation on a massive scale, and more than 50–60% of the engorged female body weight is converted into eggs (Sonenshine et al., 2002; Hatta et al., 2007). The high fecundity and the unique capacity of ticks for transmitting various viruses, bacteria, rickettsiae and protozoa to their progeny by way of transovarial transmission (Klompen, 2005; Oliveira et al., 2005) make ticks highly successful as disease vectors. *Haemaphysalis longicornis* is a common, prevalent three-host tick in East Asia and Australia (Hoogstraal et al., 1968; Fujisaki et al., 1994). *H. longicornis* sucks a considerable amount of blood and causes damage to the host skin. It transmits many bacterial, viral, protozoal and rickettsial diseases of humans and animals (Hoogstraal et al., 1968). In particular, *H. longicornis* is the primary vector for *Babesia* sp. parasites in Japan (Tsuji et al., 2007) and also serves as a major vector of *Coxiella burnetti* in cattle, dogs and humans (Ho et al., 1995). To combat ticks and tick-borne diseases, a variety of control measures, including chemical and biological strategies have been attempted worldwide. However, to avoid

* Corresponding author. Tel.: +81 29 838 7749; fax: +81 29 838 7750.
E-mail address: tsujin@affrc.go.jp (N. Tsuji).

0020-7519/$34.00 – see front matter © 2008 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijpara.2008.06.012

toxicity to humans and animals and to avoid wide-spread multi-acaricide resistance development (Zaim and Guillet, 2002; Bianchi et al., 2003), vaccination or the knock-down of the vital tick genes seems to be the most promising alternative to chemical pesticide application for controlling tick and tick-borne diseases. Recently, some bioactive molecules of vaccine and drug-target importance have been identified and characterised in the ixodid ticks *H. longicornis* (Miyoshi et al., 2004; Boldbaatar et al., 2006; Hatta et al., 2006) and *Boophilus microplus* (Mendiola et al., 1996; Renard et al., 2000). Previously, we cloned and partially characterised two cDNAs from the midgut of adult *H. longicornis* that encode the asparaginyl endopeptidases/legumains, HlLgm (Alim et al., 2007) and HlLgm2 (Alim et al., 2008). Endogenous legumains were localised in the midgut and the recombinant proteins efficiently digested the blood proteins, Hb and bovine serum albumin (BSA) Alim et al., 2007, 2008). Legumain from *Ixodes ricinus* (IrAE) has been reported to digest Hb and to *trans*-process and activate the cathepsin B1 zymogen, an enzyme in the Hb digestion cascade in the gut of the blood fluke, *Schistosoma mansoni* (Sojka et al., 2007).

Here, we investigated the biological roles of *H. longicornis* legumain genes (*HlLgm* and *HlLgm2*) on tick blood feeding, gut remodelling and reproduction by using the nucleic acid-based reverse genetic approach, RNA interference (RNAi). In recent years, RNAi has successfully been employed to investigate and identify the role of proteins hypothesised to be involved in blood feeding by different ticks (Aljamali et al., 2003; Narasimhan et al., 2004; Pal et al., 2004; Karim et al., 2005; Ramamoorthi et al., 2005; Hatta et al., 2007; Huang et al., 2007) and also for screening tick protective antigens (de la Fuente et al., 2005, 2006; Nijhof et al., 2007)and *HlLgm2* genes by RNAi treatment resulted in reduced tick survival, blood feeding, disruption of midgut cellular development and differentiation, reduced oviposition and strikingly, impaired embryogenesis suggesting their critical roles in development and reproduction of ixodid ticks.

2. Materials and methods

2.1. Ticks and experimental animals

The parthenogenetic Okayama strain of *H. longicornis* has been maintained in the Laboratory of Parasitic Diseases, National Institute of Animal Health (NIAH), Tsukuba, Ibaraki, Japan by feeding on rabbits as described previously (Alim et al., 2007). The infested rabbits were checked daily and the ticks were collected when engorged or after the indicated period of attachment. The animals were adapted to the experimental conditions for 2 weeks prior to the experiment and were treated in accordance with the protocols approved by the Animal Care and Use Committee, NIAH (Approval nos. 441, 508, 578).

2.2. In vitro transcription of HlLgm and HlLgm2 gene double-stranded RNA

The open reading frames (ORF) of *HlLgm* and *HlLgm2* genes inserted into *Escherichia coli* expressed pTrcHisB/*HlLgm* and pTrcHisB/*HlLgm2* plasmids (Alim et al., 2007, 2008) were used as templates and cloned into pBluescript II SK+ plasmid (Toyobo, Osaka, Japan). In parallel, PBS and double-stranded RNA (dsRNA) complementary to the non-functional portion of the *E. coli* malE gene (*malE*) for ticks that encodes the maltose-binding protein was used as a negative control (Cheon et al., 2006). mRNA from *E. coli* (BL21 strain, Invitrogen, Carlsbard, CA, USA) was isolated using an mRNA isolation kit (QIAGEN Sciences, Germantown, MA, USA) and following the manufacturer's protocol. The mRNA template was employed to prepare single-stranded cDNA by reverse transcriptase (RT)-PCR using a Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) and following the protocol provided by the manufacturer. The cDNA of *malE* was cloned into pBlueskript II SK+ plasmid using the oligonucleotides 5′-CCGCTCGAGCGGTTAT GAAAATAAAAACAGGTGCA-3′ and 5′-GAATTCGCTTGTCCTGGAACG CTTTGTC-3′ as forward and reverse primers, respectively. The inserted sequences for HlLgm, HlLgm2 and malE were amplified by PCR using the oligonucleotide T7 (5′-GTAATACGACTCACTA TAGGGC-3′) and CMo422 (5′-GCGTAATACGACTCACTATAGGGAA CAAAAGCTGGAGCT-3′) as primers to attach T7 promoter recognition sites on both the 5′ and 3′ ends (T7-HlLgm-T7, T7-HlLgm2-T7 and T7-malE-T7). The PCR products were purified by agarose gel electrophoresis using the QIAquick® Gel Extraction kit (QIAGEN Sciences, Germantown, MA, USA) and following the manufacturer's protocol. Using approximately 2 μg T7-HlLgm-T7, T7-HlLgm2-T7 and T7-malE-T7 as templates, 50–100 μg dsRNA complementary to the sequences encoding ORFs of *HlLgm*, *HlLgm2* and *malE*, respectively, was synthesised by an in vitro transcription method using T7 RNA polymerase (Ribomax™ Express Large Scale RNA Production System, Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The purity of the synthesised dsRNAs was checked by 1% agarose gel electrophoresis and their concentration was determined by spectrophotometer (GE Healthcare Biosciences KK, NJ, USA).

2.3. Injection of ticks with dsRNA and tick feeding

The ticks were microinjected with dsRNA as described previously (Tsuji et al., 2007). Briefly, 1 μg of *HlLgm* dsRNA and 1 μg of *HlLgm2* dsRNA either separately or in combination in 0.5 μl of PBS was injected into the haemocoel through the fourth coxa in 10–14 day post-moult adult unfed female ticks fixed on a glass slide with adhesive tape. The control ticks were injected with 0.5 μl of PBS alone or 0.5 μl of PBS containing 1 μg dsRNA of *malE*. We used 75, 110, 73, 65 and 115 ticks which were injected with PBS alone, *malE* dsRNA, *HlLgm* dsRNA, *HlLgm2* dsRNA and with a combination of *HlLgm* and *HlLgm2* dsRNA, respectively. Following treatment, the ticks were allowed to rest for 18–24 h at 25 °C and then placed on the ears of rabbits for attachment. The ticks that dropped on repletion were picked up and those that did not engorge on day 7 post-infestation were removed forcibly using forceps.

2.4. Analysis to confirm gene silencing by reverse transcriptase (RT)-PCR and quantitative RT-PCR

The midguts from three randomly collected ticks from control and RNAi groups during feeding (24 h, 48 h, 72 h and 96 h), repletion, pre-oviposition (4 days post-engorgement (PE)) and oviposition period (10 days PE) were dissected and used for mRNA detection by RT-PCR and quantitative RT-PCR as described previously (Alim et al., 2008). Immediately after collection, the midgut tissues were stored in RNA*later* RNA Stabilisation Reagent (QIAGEN). The total RNA from these samples was extracted by using an RNeasy Mini Kit (QIAGEN) in accordance with the manufacturer's protocol and either used immediately or stored at −80 °C until used. RT-PCR was carried out with a template of 500 ng of total RNA for each 10-μl reaction using a Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) and following the manufacturer's instructions. PCRs were performed using 500 ng of each cDNA synthesis reaction and oligonucleotides specific for either *HlLgm* (forward primer 5′-CGACGAGCAAATCGTAGTCA-3′ and reverse primer 5′-ACTTTTCCGCTTCCTCCATT-3′) or *HlLgm2* (forward primer 5′-CCTTCGCAACAAGCTAAAGG-3′ and reverse primer 5′-TCAGAA GTCCTTCGGTGCTT-3′) or primers specific for positive control cDNA encoding β-actin in a final volume of 20 μl. PCR was performed for

5 min at 95 °C and for 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 1.5 min at 72 °C followed by elongation at 72 °C for 5 min. The PCR products were electrophoresed in 1% agarose gel and the size of amplified fragments was checked by comparison with a DNA molecular weight marker (100 bp DNA Ladder, Promega). The quantitative RT-PCR was performed in a LightCycler 1.5 instrument (Roche Instrument Center AG, Roikreuz, Switzerland) using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Nonnenwald, Germany). The reaction mixture of 20 µl contained 4 mM $MgCl_2$, 0.5 µM of each primer (forward and reverse as described above), 2 µl of LightCycler FastStart DNA Master SYBR Green I and 2 µl of the single-stranded DNA template. The data obtained were analysed using LightCycler Software Version 3.5.

2.5. Protein expression analysis by Western blotting

The impact of RNAi on protein translation was determined by Western blotting. Midguts from six partially fed (72 h) ticks of all RNAi and control groups were dissected separately in PBS and antigens were prepared as described previously (Alim et al., 2007). Equal amounts of protein (40 µg) from control and RNAi groups were separated by SDS–PAGE under reducing condition and processed for immunoblotting. For detection of endogenous legumains, the immunoblots were incubated with mouse anti-rHlLgm serum (Alim et al., 2007) and mouse anti-rHlLgm2 serum (Alim et al., 2008) separately at a dilution of 1:250. The membranes were washed with Tris buffered saline–Tween (TBS–T) and the bound antibodies were detected by using alkaline phosphatase-conjugated goat anti-mouse IgG (H + L) (ZYMED, San Francisco, CA, USA) as a secondary antibody. The immunoblots were developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT, Promega, Madison, WI, USA).

2.6. Immunofluorescent staining of the midgut tissues

The protein translational disruption by RNAi was also investigated by immunofluorescent staining of *H. longicornis* midgut tissues. The midgut tissues from 72 h fed ticks of the *HlLgm* and *HlLgm2* dsRNA dually injected RNAi group and the *malE* dsRNA treated control group were dissected separately in PBS and thin sections were prepared as described previously (Tsuji et al., 2007). The midgut tissue sections were incubated with mouse anti-rHlLgm serum (1:250) overnight at 4 °C. The slides were rinsed with ice cold PBS and reacted with fluorescence labelled secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG (H + L) (Invitrogen)) for 1 h at room temperature. After washing thoroughly with ice cold PBS, the slides were mounted with VECTASHIELD® (Vector) with DAPI (Vector Laboratories, Burlingame, CA, USA), cover slipped and photographed with a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using appropriate filter sets. Images were collected by using Leica FW4000 software.

2.7. Analysis of impact of RNAi on midgut tissues

The tick midgut cells undergo changes during different phases of blood feeding and post-feeding periods for effective digestion of blood meal (Agyei and Runham, 1995). The impacts of RNAi on midgut tissues were investigated on the basis of gross and histological changes of the midgut tissues compared with the control. The midgut tissues were obtained from ticks from the combined *HlLgm* dsRNA and *HlLgm2* dsRNA injected group and the *malE* dsRNA injected control group during feeding (24 h, 48 h, 72 h and 96 h) and immediately after repletion. We randomly collected three ticks from each group during each phase. Dissection of midgut tissues and preparation of fixed tissue sections were performed as above. The midgut sections of each phase of feeding from each group were stained with H&E and examined under a microscope (DM 4000B, Leica) to analyse the impact of legumain gene silencing on midgut epithelium.

2.8. Determination of effect of HlLgm and HlLgm2 gene silencing on tick blood feeding and reproduction

The effects of RNAi on tick blood feeding and reproductive parameters were investigated by measuring the attachment rate, death of the ticks after attachment, blood feeding periods, number of ticks engorged, engorged tick body weight, pre-oviposition periods, weight of egg mass, number of eggs per tick and egg conversion ratio (total egg mass/engorged body weight). The Reproductive Efficiency Index (REI) (number of eggs/weight of engorged female at the time of host detachment) (Drummond and Whetstone, 1970) and Reproductive Fitness Index (RFI) (number of eggs that hatch into larvae/weight of the engorged female at the time of host detachment) (Chilton, 1992) were measured for each tick. To count the number of eggs laid by each tick, arbitrarily, we weighed 200 eggs taken from each of three randomly selected ticks from each group. The total number of eggs/tick was calculated as (weight of total egg mass (mg)/mean weight of 200 eggs (mg)) × 200. The weight of individual tick and egg mass/tick was taken by using a digital balance (Model: 321-3357, Shimadzu, Kyoto, Japan). Photographs of ticks were taken with a digital camera (Cannon, Tokyo, Japan). For convenience of investigating the effects of RNAi on reproduction, we measured only the engorged ticks from all groups.

2.9. Statistical analysis

Statistical analyses of data obtained on feeding and reproductive parameters for RNAi treated and control groups of ticks were performed by using Student's *t*-test with unequal variance. Tick mortality was compared between the RNAi treated and control ticks by χ^2-test. *P* values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. Demonstration of gene silencing by RT-PCR and quantitative RT-PCR

The total RNA isolated from the midgut of ticks during different phases of feeding, engorgement, pre-oviposition (4 days PE), oviposition periods (10 days PE) and eggs of control groups (PBS and *malE* dsRNA injected), and RNAi treated groups (*HlLgm* and *HlLgm2* dsRNA injected separately or in combination) were analysed by RT-PCR and quantitative RT-PCR to evaluate the effect of RNAi on *HlLgm* and *HlLgm2* mRNA expression as described above. The results demonstrated that there was significant reduction or absence of detectable mRNA expression corresponding to the *HlLgm* and *HlLgm2* genes in ticks injected with *HlLgm* dsRNA and *HlLgm2* dsRNA either individually or in combination while equal levels of *HlLgm* and *HlLgm2* mRNA expression were detected in PBS and *malE* dsRNA injected ticks (Fig. 1A and B) indicating that disruption of *HlLgm* and *HlLgm2* mRNA transcription was achieved by RNAi. There were no detectable *HlLgm2* amplicons in engorged ticks of either control groups which is consistent with our previous observations on the expression profile of the *HlLgm2* gene during different phases of blood feeding until engorgement in adult *H. longicornis* (Alim et al., 2008). Interestingly, the present study revealed that the *HlLgm2* mRNA expression profile was bimodal where the transcript was further expressed during pre-oviposition

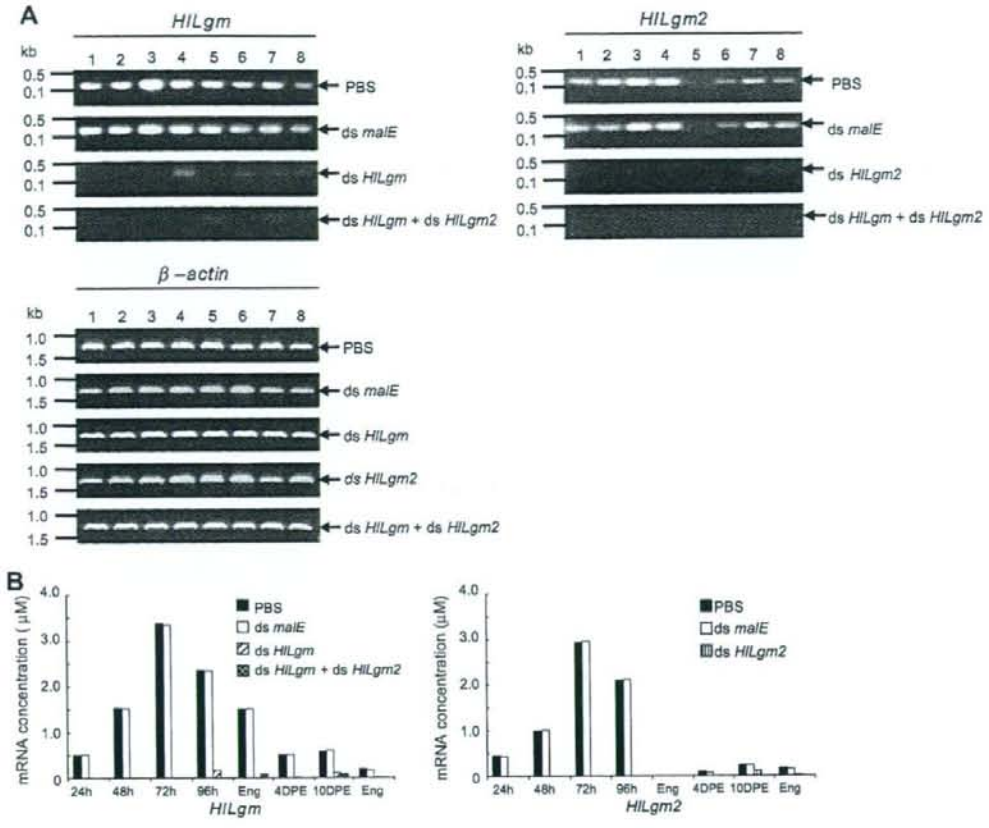

Fig. 1. Silencing of *HlLgm* and *HlLgm2* genes from adult *Haemaphysalis longicornis* by treating with double-stranded RNA (dsRNA). (A) Reverse transcriptase (RT)-PCR analysis. The control groups of ticks were injected with either PBS alone or PBS containing 1 μg of *Escherichia coli malE* dsRNA. The RNA interference groups of ticks were treated with 1 μg *HlLgm* dsRNA and 1 μg *HlLgm2* dsRNA either individually or in combination. The level of expression of *HlLgm*- and *HlLgm2*-specific mRNAs are indicated by arrows. Actin is shown as an internal control. Lane 1, 24 h post-attachment; lane 2, 48 h post-attachment; lane 3, 72 h post-attachment; lane 4, 96 h post-attachment; lane 5, engorged; lane 6, 4 days post-engorgement; lane 7, 10 days post-engorgement; lane 8, eggs. (B) Quantitative RT-PCR was performed using the same total RNA and the same primers specific for *HlLgm* and *HlLgm2* as in (A).

and oviposition periods and also in eggs. However, the reasons for the missing *HlLgm2* signal in engorged ticks are unclear and await elucidation. There is no obvious sequence homology between *E. coli malE* and the *H. longicornis* legumain family (*HlLgm* and *HlLgm2*), and therefore we did not expect any direct interaction between the *malE* dsRNA and legumain genes. Targeting *HlLgm* and *HlLgm2* mRNA did not affect the transcription of the *H. longicornis* β-actin control gene (Fig. 1 A) indicating that dsRNA treatment was gene-specific. Although not consistent in different RNAi groups of ticks, there was detectable expression of *HlLgm* and *HlLgm2* mRNAs during 96 h of blood feeding and beyond (Fig. 1A and B). This may be due to variation in the tick population. We used pools of midgut extracts from three randomly collected ticks for mRNA detection during each phase and it is very likely that RNAi targeting was not uniform in each microinjected tick. Also, dilution of injected dsRNA in vivo during the prolonged period of tick blood feeding (Karim et al., 2005) may be a cause of detectable expression of legumains at a later phase of blood feeding and onward. However, the results suggest that RNAi targeting effectively silenced the expression of legumains mRNAs in *H. longicornis*. RNAi is a robust and target-specific phenomenon that abrogates only the mRNAs complementary to the introduced dsRNA leading to the silencing of the specific genes (Fire et al., 1998; Parrish et al., 2000). However, off-target silencing can be achieved as RNAi can trigger the destruction of mRNAs containing significant stretches of nucleotide sequence identity (Jackson et al., 2003; Narasimhan et al., 2004). *HlLgm* and *HlLgm2* mRNA nucleotide sequences (GenBank Accession Nos. AB279705 and AB353127, respectively) share 78% identity to each other and individual injection of dsRNA corresponding to the ORFs of *HlLgm* and *HlLgm2* genes caused a 45.56% and 39% reduction of *HlLgm2* and *HlLgm* mRNA expression levels, respectively, compared with PBS and *malE* dsRNA injected control ticks as revealed by quantitative RT-PCR (Fig. 2A and B), suggesting some off-target silencing of the homologues.

3.2. Demonstration of gene silencing by immunoblot and immunofluorescence analyses

Protein extracts from the midguts of 72 h fed ticks injected with *HlLgm* and *HlLgm2* dsRNA individually or in combination and *malE* dsRNA and PBS alone were analysed by immunoblotting. On immunoblots of the antigens from RNAi and control groups probed with mouse anti-rHlLgm serum and mouse anti-rHlLgm2 serum, we observed reactive bands of ~38 kDa protein corresponding to

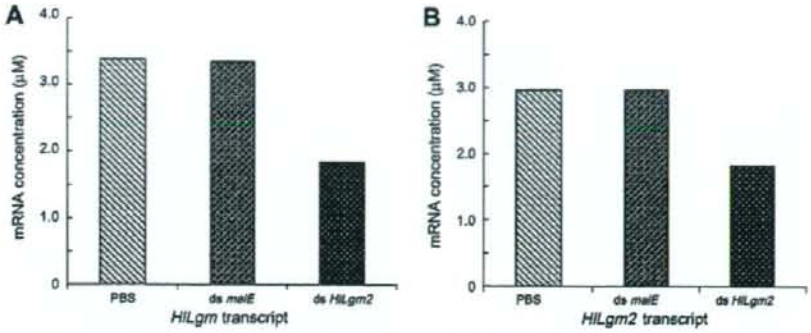

Fig. 2. Off-target silencing of *HlLgm* and *HlLgm2* transcripts in RNA interference groups compared with controls ticks. (A) Off-target silencing of *HlLgm* demonstrated by quantitative reverse transcriptase-PCR analysis using the total RNA isolated from 72 h fed *HlLgm2* double-stranded RNA (dsRNA) treated ticks and the primers specific for *HlLgm*. (B) Off-target silencing of *HlLgm2* transcript demonstrated by the same way using the total RNA from *HlLgm* dsRNA treated ticks and primers specific for *HlLgm2*.

both the endogenous HlLgm (Alim et al., 2007) and HlLgm2 (Alim et al., 2008) in control groups whereas no visible reactive band in the combined *HlLgm* and *HlLgm2* dsRNA introduced group was seen. However, positive reactive bands with a lower intensity were detected in either individual *HlLgm* and *HlLgm2* knock-down tick extracts (Fig. 3A) that might be due to the cross-reactivity of the polyclonal antibodies against the legumain isomers. HlLgm and HlLgm2 share 75% identity at the protein level (Alim et al., 2008) and we observed that polyclonal antibodies raised against recombinant HlLgm (rHlLgm) and recombinant HlLgm2 (rHlLgm2) cross-reacted with rHlLgm2 and rHlLgm, respectively (unpublished data). Immunofluorescence analysis using fixed midgut sections of 72 h fed ticks from control (*malE* dsRNA injected) and RNAi groups (combined *HlLgm* and *HlLgm2* dsRNA injected) incubated with mouse anti-rHlLgm serum (1:250) and fluorescence labelled goat anti-mouse IgG (H + L) revealed strong fluorescence reaction in the sections from the control group while there was very little or no reaction in the sections prepared from the RNAi group (Fig. 3B). These results indicate the blocking of translation of endogenous proteins by RNAi.

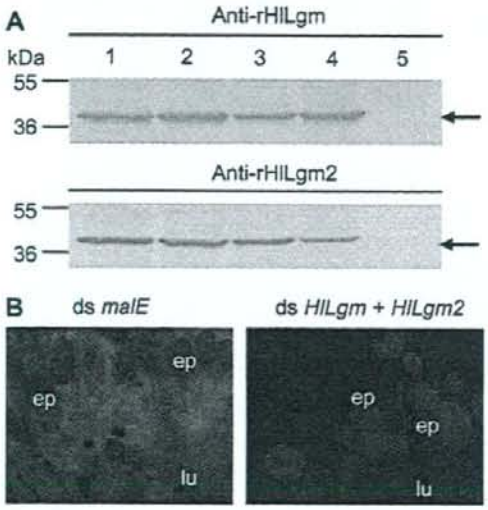

Fig. 3. Translational effects of *HlLgm* and *HlLgm2* gene knock-down by introduction of double-stranded RNA (dsRNA). Ticks were injected with PBS alone, *malE* dsRNA or *HlLgm* and *HlLgm2* dsRNA either individually or in combination. (A) Comparison of endogenous HlLgm and HlLgm2 expression in between the control and RNA interference treated groups by Western blot analysis. Lane 1, ticks injected with PBS alone; lane 2, ticks injected with *malE* dsRNA; lane 3, ticks injected with *HlLgm* dsRNA; lane 4, ticks injected with *HlLgm2* dsRNA; lane 5, ticks injected with a combination of *HlLgm* and *HlLgm2* dsRNA. Arrows indicate endogenous legumain. (B) Immunofluorescence analysis of the midgut tissues. Midgut tissues from partially fed (72 h) ticks of the *malE* dsRNA treated group and combined *HlLgm* and *HlLgm2* dsRNA treated group. Bm, basal membrane; ep, midgut epithelial cells; lu, midgut lumen. Data reported here is from one of the three replicates.

3.3. Impact of gene silencing on blood feeding of H. longicornis

The impact of legumain gene silencing on tick feeding was evaluated by comparing attachment rate, feeding period, engorged tick body weight and mortality of ticks among the control and RNAi groups. None of the ticks from any group died during the post-injection incubation period prior to infesting rabbits for feeding. All ticks from each group were found to attach successfully on rabbit ears 24 h post-placement. None of the ticks from the control groups, either PBS injected or *malE* dsRNA injected, died after attachment. By contrast, death of the ticks in RNAi groups was observed 48 h after successful attachment and blood feeding (Fig. 4A). Among the RNAi groups, four (5.48%) *HlLgm* dsRNA injected and two (3.1%) *HlLgm2* dsRNA injected feeding ticks died and the mortality rate was relatively higher (11 ticks, 9.6%) when the ticks were injected with *HlLgm* and *HlLgm2* dsRNA together (data not shown). All ticks from control groups fed to repletion, however, two (2.73%) *HlLgm* dsRNA injected, one (1.54%) *HlLgm2* dsRNA injected and 11 (9.6%) combined *HlLgm* and *HlLgm2* dsRNA injected ticks failed to engorge (data not shown). Phenotypically, the RNAi treated engorged ticks were smaller than those in control groups. Additionally, the cuticle of these ticks appeared to be tense and there was no cuticular wrinkling on the dorsum of ticks (Fig. 4B). There was no notable variation in the blood feeding period toward repletion among the PBS injected (5.11 ± 0.35 days), *malE* dsRNA injected (5.20 ± 0.40 days), *HlLgm* dsRNA injected (5.35 ± 0.48 days) and *HlLgm2* dsRNA injected (5.34 ± 0.37 days) groups of ticks. However, introduction of combined *HlLgm* dsRNA and *HlLgm2* dsRNA resulted in slower tick blood feeding (Fig. 4A) and the blood feeding period of this group (6.28 ± 0.73 days) of ticks was significantly longer ($P < 0.01$) than all other groups of ticks (Fig. 4C). The mean body weight of engorged ticks in PBS and *malE* dsRNA injected control groups was identical, 285.3 ± 30.05 mg and 281.9 ± 27.90 mg, respectively. A significant ($P < 0.01$) decrease in mean engorged body weight compared with control groups was observed in *HlLgm* dsRNA (196.82 ± 34.50 mg)

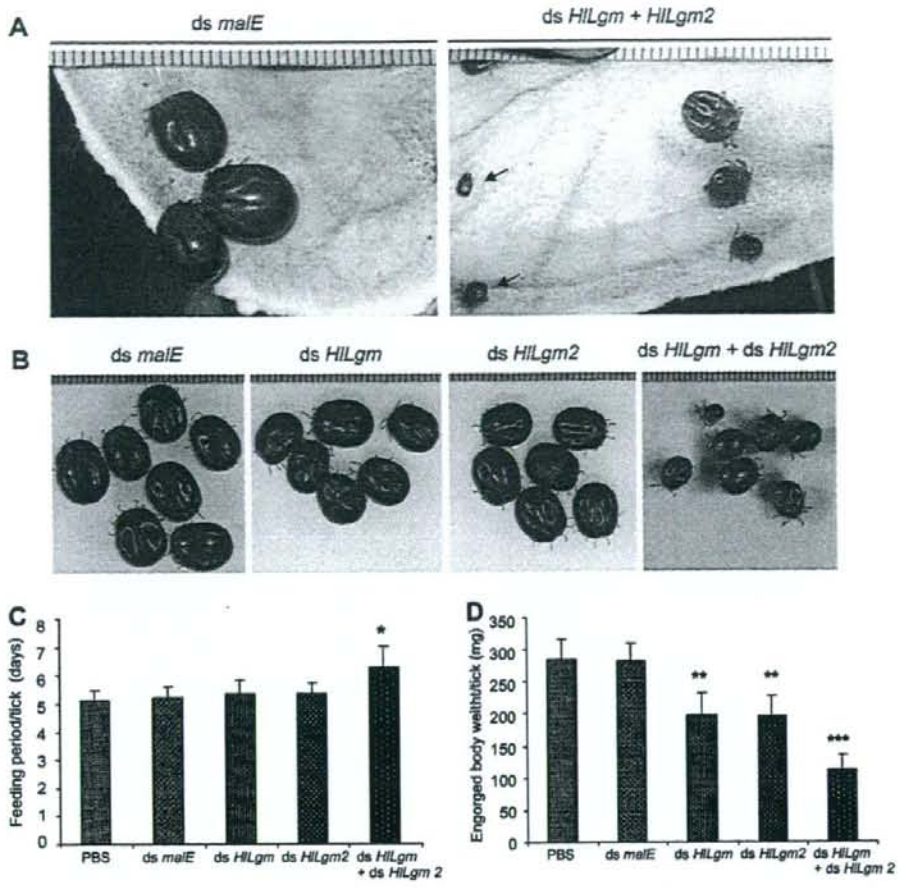

Fig. 4. Effects of *HlLgm* and *HlLgm2* gene silencing on blood feeding and survival of adult *Haemaphysalis longicornis*. Ticks were injected with 0.5 μl PBS alone (control) or 0.5 μl PBS containing 1 μg of *malE* double-stranded RNA (dsRNA) (control) or 1 μg each of *HlLgm* and *HlLgm* dsRNA either individually or in combination and allowed to feed on rabbit for 7 days. (A) Disruption of blood feeding and death of feeding ticks in the legumains (*HlLgm* and *HlLgm2*) knock-down group compared with control ticks (*malE* dsRNA). Photographs were taken on day 5 post-infestation. Arrows indicate the dead ticks. (B) Comparison of the phenotype of the engorged ticks from control and RNA interference (RNAi) treated groups. The replete and spontaneously dropped ticks (from all groups) and those that were attached at 7 days post-infestation (combined *HlLgm* and *HlLgm2* dsRNA injected groups) were forcibly collected. Phenotypically, the engorged ticks from all RNAi treated groups were smaller compared with the control ticks. Some individuals from *HlLgm* and *HlLgm2* dsRNA dually treated group failed to reach repletion and the engorged ticks from this group were much smaller, rounder and most of them had no wrinkling on the dorsum. Scale: 1 unit = 1 mm. (C) Blood feeding period (days) toward repletion. Blood feeding period was calculated only for the engorged ticks from the time of attachment to spontaneous dropping-off the host on repletion. (D) Engorged body weight. The bars represent mean values and the error bars indicate SD. Asterisks (*) denotes the difference compared with the control group is significant as determined by Student's *t*-test with unequal variance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

and *HlLgm2* dsRNA (195.99 ± 30.57 mg) groups separately injected. Interestingly, when the ticks were injected with *HlLgm* and *HlLgm2* dsRNA together, the impact on body weight gain was more pronounced with a highly significant ($P < 0.001$) reduction of the mean engorged body weight (111.8 ± 23.30 mg) compared with the control groups (Fig. 4D). Taken together, the results indicate that HlLgm and HlLgm2 play a vital role in blood feeding and survival of *H. longicornis*.

3.4. Impact of gene silencing on remodelling of midgut epithelium during different phases of blood feeding

The effects of *HlLgm* and *HlLgm2* gene silencing on *H. longicornis* midgut tissues were analysed macroscopically and microscopically using sections prepared from partially fed (24 h, 48 h, 72 h and 96 h) and engorged ticks of control (*malE* dsRNA) and RNAi (*HlLgm* dsRNA and *HlLgm2* dsRNA) treated groups. Macroscopically, the midguts of RNAi treated ticks were atrophied with narrower lumina compared with the controls (data not shown). The midgut epithelia in control ticks showed cellular changes during different phases of blood feeding. Following attachment and feeding, the midgut epithelial cells underwent dramatic changes including massive proliferation and differentiation of the stem cells (Walker and Fletcher, 1987; Agyei and Runham, 1995). The stem cells are dome shaped cells with rounded nuclei and are located on the basement membrane. These cells were found to maintain their original morphology throughout the feeding period. The prodigest cells which are morphologically slightly elongated cells with large rounded nuclei appeared soon after attachment and blood feeding. With the progression of feeding and enlargement of the lumen, the

prodigest cells rapidly transformed into sessile digest cells and residual sessile digest cells during 48 h and 72 h of feeding, respectively. The sessile digest cells are comparatively large cells, columnar in shape, having a broad base in contact with the basement membrane. The residual sessile digest cells are larger club-shaped cells which are thinly attached to the basement membrane and appear to move into the lumen. Sometimes there is apical movement of nucleus. Beyond 96 h of feeding, the residual sessile digest cells started sloughing off into the lumen resulting in reduction of sessile and residual sessile digest cells on the basement membrane of the midgut. In the engorged ticks, the midgut was distended with blood and its epithelial lining consisted mainly of stem cells. Strikingly, no such cellular events were observed in RNAi group of ticks; rather there was damage to midgut tissues and loss of the epithelium with retention only of the stem cells and sometimes predigest cells which were sparsely distributed on the basement membrane of the midgut tissues throughout the feeding periods (Fig. 5A). These findings suggest that legumains play an important role in midgut epithelial cell development and differentiation and in maintaining the integrity of the midgut tissues in ticks.

3.5. Role of blood-induced proliferating and differentiated mature midgut epithelial cells in legumain expression

Striking developmental variations in the midgut epithelium during feeding indicative of major functional differences were seen between the control and RNAi groups of ticks. The role of the proliferating and differentiated mature midgut cells in legumain expression was therefore investigated. Immunofluorescent staining using fixed midgut sections of 24 h and 96 h fed control (*malE* dsRNA injected) and RNAi treated (*HlLgm* and *HlLgm2* dsRNA injected) ticks revealed strong expression of endogenous legumains in the sessile digest cells and residual sessile digest cells of 96 h fed control ticks. Interestingly the stem cells and the prodigest cells in both 24 h fed and 96 h fed control tick sections showed little fluorescence. Furthermore, there was very little or no reaction in the counterparts of these sections of RNAi treated ticks (Fig. 5B). These results show that endogenous legumains are mostly expressed in the proliferating and differentiated midgut cells.

3.6. Effects of gene silencing on reproduction

The impact of RNAi on tick reproduction was investigated by determining the pre-oviposition period, egg mass weight and number of eggs laid per tick, egg conversion ratio, REI, number (%) of eggs hatched and RFI. All these phenotypic parameters of reproduction were significantly affected by RNAi treatment, especially in *HlLgm* and *HlLgm2* gene knock-down ticks. Some ticks from control groups started ovipositing 4 days after repletion and ticks injected with *HlLgm* dsRNA and *HlLgm2* dsRNA separately, started laying eggs on day 5 post-repletion showing no significant variations in mean pre-oviposition periods among these groups. Interestingly, in ticks injected with a combination of *HlLgm* dsRNA and *HlLgm2* dsRNA, the onset of oviposition was on day 6 post-repletion and their mean pre-oviposition period was significantly ($P < 0.01$) longer than that of ticks of all other groups (Fig. 6A). Gene knock-down resulted in death of the engorged ticks where three (7.70%) *HlLgm* dsRNA injected and two (4.65%) *HlLgm2* dsRNA injected engorged ticks died without laying any eggs. Notably, the mortality of engorged ticks was significantly higher in *HlLgm* and *HlLgm2* dsRNA dually injected ticks (13, 16.88%) compared with the controls ($P < 0.05$). However, due to unknown reasons, one

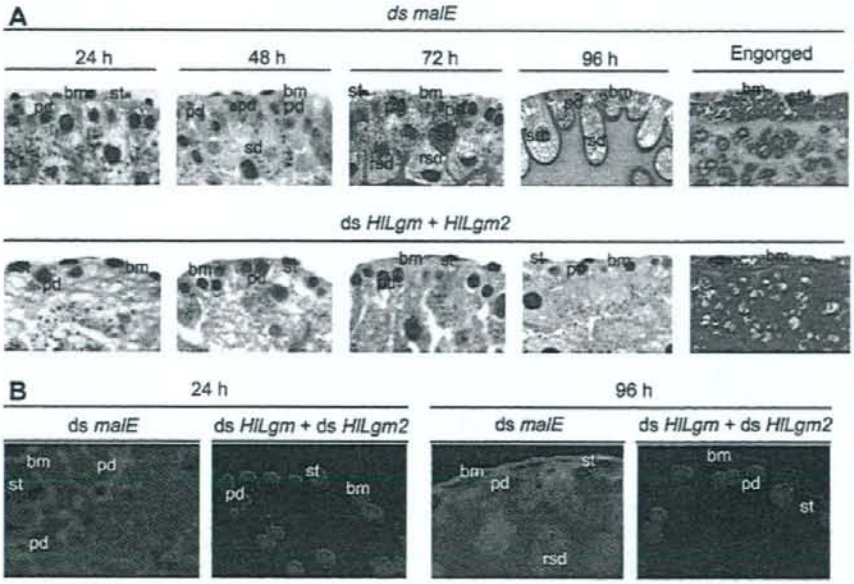

Fig. 5. Endogenous legumain expression by midgut cells during blood feeding and impact of legumain gene silencing on midgut cellular remodelling in adult *Haemaphysalis longicornis*. The midgut tissues were collected from the *malE* double-stranded RNA (dsRNA) injected control group and combined *HlLgm* and *HlLgm2* dsRNA group during different phases of feeding (24 h, 48 h, 72 h and 96 h) and engorgement. (A) Impacts of *HlLgm* and *HlLgm2* gene knock-down on midgut cellular remodelling. On attachment and progression of feeding, the midgut epithelial cells in the control group followed the normal cellular cycle characterised by massive proliferation and differentiation of cells. Compared with controls, no cellular remodelling in the midgut epithelium, but rather damage of midgut tissues, was observed in RNA interference treated ticks. (B) Immunofluorescent staining showing endogenous legumain expression by midgut epithelial cells during blood feeding. Bm, basal membrane; st, stem cells; pd, predigest cells; sd, sessile digest cells; rsd, residual sessile digest cells.

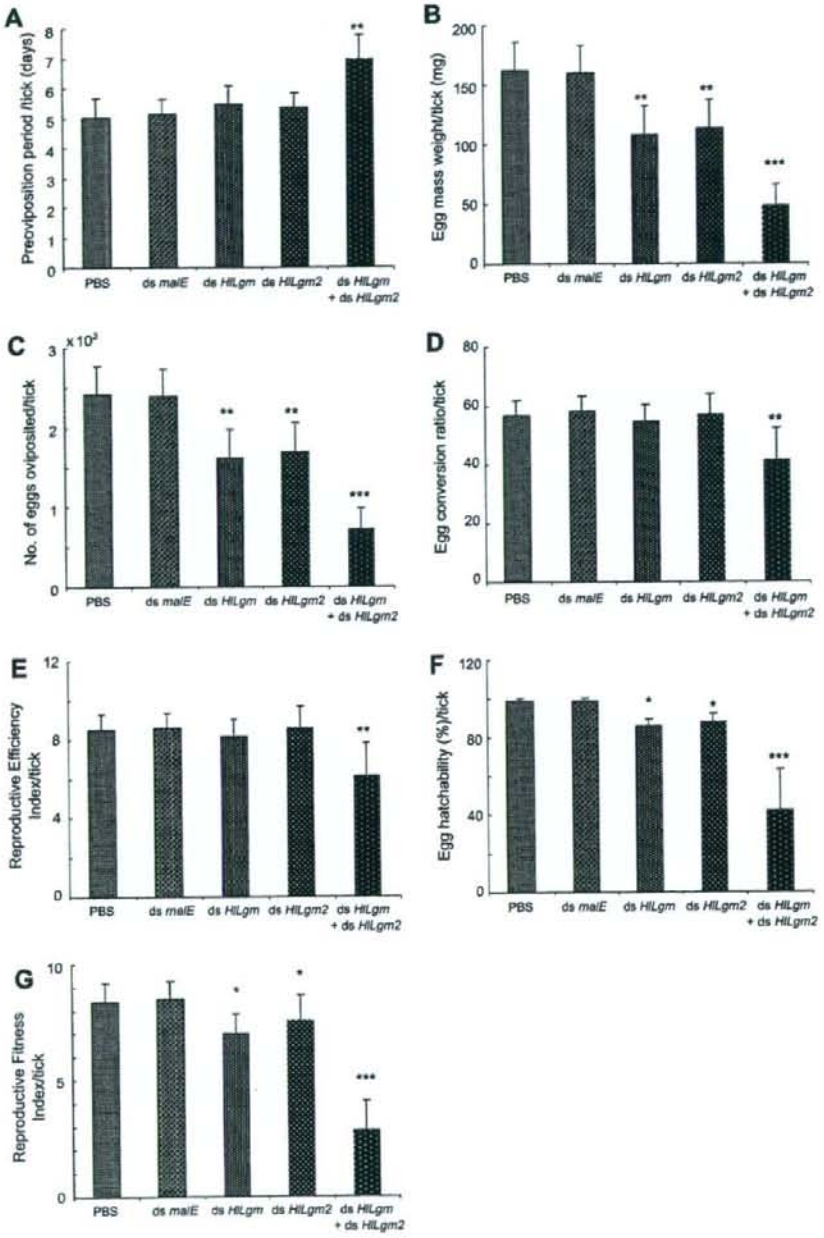

Fig. 6. Impacts of legumain gene knock-down on reproduction of adult female *Haemaphysalis longicornis*. The ticks that spontaneously dropped-off the host after engorgement were collected and incubated at 25 °C and 95% relative humidity. (A) Pre-oviposition period (days). (B) Egg mass weight (mg)/tick. (C) Number of eggs oviposited/tick. (D) Egg conversion ratio/tick. (E) Reproductive Efficiency Index (REI)/tick. (F) Egg hatchability (%)/tick. (G) Reproductive Fitness Index (RFI)/tick. The bars represent mean values and the error bars indicate SD. Asterisks (*) denote the difference compared with the control groups is significant as determined by Student's *t*-test with unequal variance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

(2.70%) PBS injected, and one (1.30%) *malE* dsRNA injected engorged ticks died during incubation for oviposition. It was observed that eggs laid by all groups of ticks were normal in shape with a glistening appearance indicating that they were successfully coated with the secretion from Gene's organ. The mean egg mass weight and total number of eggs laid by the ticks injected with *HlLgm* dsRNA (108.15 ± 24.12 mg and 1615.82 ± 360.61, respectively) and *HlLgm2* dsRNA (113.80 ± 23.20 mg and 1700.95 ±

351.96, respectively) were significantly ($P < 0.01$) lower than those of the PBS injected (162.41 ± 23.31 mg and 2450.84 ± 347.94, respectively) and *malE* dsRNA injected (159.91 ± 22.83 mg and 2391.81 ± 340.77, respectively) groups. In the case of the combined *HlLgm* dsRNA and *HlLgm2* dsRNA injected group, the mean egg mass weight (48.93 ± 17.14 mg) and the total number of eggs (730.11 ± 255.75) were significantly ($P < 0.001$) lower than those of control groups (Fig. 6B and C). Interestingly, the egg conversion ratio and REI of ticks injected with *HlLgm* dsRNA (54.78 ± 5.86% and 8.15 ± 0.88, respectively) and *HlLgm2* dsRNA (57.04 ± 6.97% and 8.60 ± 1.07%, respectively) were almost similar to PBS (56.73 ± 5.17% and 8.50 ± 0.81%, respectively) and *malE* dsRNA (58.13 ± 5.04% and 8.60 ± 0.76%, respectively) injected control groups. These two reproductive parameters were significantly ($P < 0.01$) lower in the *HlLgm* and *HlLgm2* knock-down group of ticks (41.27 ± 10.90 and 6.16 ± 1.64, respectively) than those of all other groups (Fig. 6D and E). The most significant impact of *HlLgm* and *Hllgm2* gene silencing was on the embryogenesis and hatching of eggs. Successful embryogenesis and hatching of eggs, showing a uniformly constant rate of hatchability and RFI, was detected in eggs laid by the PBS treated (98.80 ± 1.25% and 8.4 ± 0.78%, respectively) and *malE* dsRNA treated (98.83 ± 1.47% and 8.5 ± 0.77%, respectively) control ticks. The egg hatchability and RFI were significantly ($P < 0.05$) affected in individually *HlLgm* dsRNA (85.60 ± 3.56% and 7.6 ± 1.08%, respectively) and *HlLgm2* dsRNA (88.15 ± 4.1% and 7.6 ± 1.08%, respectively) introduced ticks compared with controls. Strikingly, the majority of the eggs oviposited by engorged females injected with combined *HlLgm* dsRNA and *HlLgm2* dsRNA failed to hatch and the mean hatchability (42.35 ± 30.92%) and RFI (2.88 ± 1.25%) were significantly lower compared with controls ($P < 0.001$) and other RNAi groups (Fig. 6F and G). These eggs showed an aberrant phenotype with an undifferentiated mass inside and the embryos did not develop within these eggs (Fig. 7). The eggs that failed to hatch eventually dried up and shriveled after incubation at 25 °C/95% relative humidity for 4 weeks. These results clearly show that *HlLgm* and *HlLgm2* play important roles in oviposition and RFI in *H. longicornis*.

4. Discussion

Successful blood feeding and blood meal digestion is essential for the survival and reproduction of haematophagous ticks. Blood meal digestion in ticks occurs as a slow intracellular process in the midgut epithelium (Coons et al., 1986), although the molecular basis of in vivo blood digestion in ticks is yet to be elucidated. It is reasonable to speculate that specific gene products are involved in blood meal digestion in ticks. In this article, we describe the physiological roles of our previously identified *H. longicornis* midgut-specific asparaginyl endopeptidase/legumain genes, *HlLgm* (Alim et al., 2007) and *HlLgm2* (Alim et al., 2008) in tick feeding and digestion, gut remodelling and reproduction in vivo by RNAi knock-down techniques.

The knock-down of legumain genes in adult *H. longicornis* (*HlLgm* and *HlLgm2*) impacted markedly on its midgut tissues and was characterised by atrophy, disruption of normal cellular development and differentiation and damage of the gut tissues. In ixodid ticks, the midgut epithelium follows a specific cellular cycle while feeding. During the preparatory phase or slow feeding period (1–3 days post-attachment) when continuous digestion of the blood meal takes place, there are rapid cellular changes in the midgut epithelium. The prodigest cells transform into sessile digest cells and later into residual sessile digest cells. In the rapid feeding period, a further 2 days, when digestion is limited, most of the active sessile and residual sessile digest cells slough off into the lumen (Tarnowski and Coons, 1989; Agyei and Runham, 1995). A recent report shows that legumain controls the extracellular matrix remodelling in mouse renal proximal tubular cells through degradation of fibronectin, a component of the extracellular matrix (Morita et al., 2007). Overexpression of legumain under stress conditions such as tumour hypoxia leads to increased tumour progression, angiogenesis and metastasis (Luo et al., 2006). Angiogenesis is the formation of new blood vessels which is essentially dependent on proliferation of endothelial cells and is a normal process in growth and development, as well as in wound healing (Francischetti et al., 2005). Modulation of angiogenesis and tumour growth by mammalian legumains indicates its involvement in cellular growth and differentiation. It is plausible that ticks have molecules which specifically modulate orderly proliferation and differentiation of midgut cells during feeding. The present study revealed that there was disruption of midgut cellular proliferation during feeding in legumains knock-down ticks, whereas the midgut cells underwent normal proliferation and differentiation (Agyei and Runham, 1995) in the control ticks. Furthermore, legumains were mostly expressed by the proliferating, differentiated mature midgut cells in the control ticks (Fig. 5A and B). From our observations, it is reasonable to speculate that legumains may play a vital role in maintaining midgut epithelial cell integrity and remodelling in ticks. However, the precise molecular pathway is yet to be defined. Disruption of midgut cellular developmental events and damage of midgut tissues by RNAi suggests that legumains may play roles in combating host defense systems and in gut-associated disease transmission since the tick midgut and its epithelial lining is a major physical barrier between the tick and the host defense mecha-

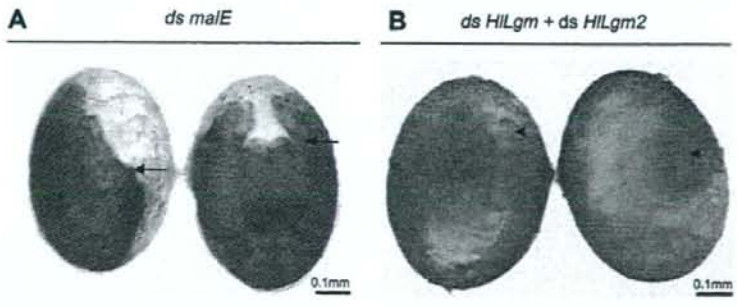

Fig. 7. Effects of RNA interference treatment on embryogenesis. (A) Representative eggs from female *Haemaphysalis longicornis* injected with *malE* double-stranded RNA (dsRNA) (A) or a combination of *HlLgm* and *HlLgm2* dsRNA (B) 18 days after oviposition. Arrows indicate normal development of embryo, arrowheads indicate an undifferentiated mass.

nisms (Agyei and Runham, 1995) and tick midguts and salivary glands are major sites of pathogen infection, development and transmission (Kocan et al., 2004).

Silencing of legumain genes resulted in perturbation of feeding leading to significant changes in feeding period, repletion, body weight gain and survival of the feeding and engorged ticks. Failure of ticks to feed to repletion and significant reduction in engorged body weight of legumain-silenced ticks support our assertion that HlLgm and HlLgm2 are involved in blood feeding and the Hb digestion cascade in *H. longicornis* (Alim et al., 2007, 2008). These findings are also in accord with the host Hb-degrading function of the orthologus legumain IrAE in the midgut of the haematophagous tick *I. ricinus* (Sojka et al., 2007). The causes of death of the feeding and engorged ticks are not clear but they might be due to extensive damage of the midgut tissues or blocking/inactivation of legumain-induced biological functions of other protease(s) essential for tick survival.

The most pronounced impacts of legumain RNAi was on tick reproduction. RNAi treatment impacted on *H. longicornis* oviposition, fecundity, embryogenesis and hatchability of eggs. The development of egg masses was directly related to the amount of blood imbibed by individual ticks and it was noticed that the egg conversion ratio and REI of *HlLgm* dsRNA and *HlLgm2* dsRNA separately injected groups were not affected by RNAi compared with those of control ticks. Assuming one larva from one egg, a significant impact of RNAi was on the number of eggs laid by individual ticks. However, the ultimate reproductive success of each tick depends on the number of progeny it can produce and it serves as the driving force behind tick infestations and transmission of disease. Decreased hatchability and RFI in the RNAi treated groups compared with those of the control ticks clearly indicate the critical roles of legumains in tick embryonic development. Simultaneous knock-down of two legumains impacted more on tick feeding, reproduction and survival than when the genes were silenced individually. Death and failure of more individuals to reach repletion and the dramatic falls in engorged body weight, egg conversion ratio, number of eggs laid, hatchability and RFI in the group with two legumains silenced suggest the synergistic effects of RNAi. These results also further reinforce the role of legumains in the modulation of tick feeding and reproduction. The disruption of oviposition and hatching in RNAi groups of ticks may be due to disturbances in oocyte development (vitellogenesis) and embryogenesis. Recently, it has been reported that vitellogenesis, the central event in reproduction, is regulated by the ingested blood meal in anautogenous mosquitoes (Attardo et al., 2005). As with mosquitoes, vitellogenesis is also induced in response to a blood meal in adult female ticks (Taylor and Chinzei, 2002). Pertinently, the absorbable amino acids of Hb digestion in ticks serve as raw materials and a trigger for vitellogenesis as it has been described for mosquitoes (Hansen et al., 2004; Attardo et al., 2005). Reduced egg mass and impaired embryogenesis in RNAi treated groups of ticks are obviously due to less blood intake and interference in Hb digestion to absorbable amino acids resulting from legumain deletion. However, ticks have several proteases that act in a cascade to effect complete digestion of blood meal (Sojka et al., 2008). It is likely that expression of multiple midgut-specific proteases would have to be ablated simultaneously to abolish tick feeding and reproduction. Recombinant HlLgm and HlLgm2 proteins share almost similar biochemical properties and digest BSA and Hb in vitro in the same manner (Alim et al., 2008). It was expected that there would be functional compensation by the homologue in case of individual silencing of either *HlLgm* or *HlLgm2*. It is clear that this expected functional compensation was hampered due to off-target silencing of the homologue.

The data above suggested highly specialised and conserved biological functions for legumains. RNAi experiments provided evidence that legumains play key roles in mediation of blood feeding, digestion, midgut cellular remodelling and, most critically, in vitellogenesis and embryo development in *H. longicornis*. Deleterious effects of legumain knock-down on gut architecture also suggest that legumains may be used as therapeutic targets. Deletion of *HlLgm* and *HlLgm2* genes in *H. longicornis* would likely impact on both feeding and reproductive ability of ticks, thereby reducing the field population size and hence disease transmission. Understanding the mechanisms underlying legumain-mediated blood feeding, midgut cellular remodelling and amino acid signalling relating to vitellogenesis are of paramount importance to develop legumain-based novel strategies for controlling ticks and tick-mediated disease transmission.

Acknowledgements

This work was supported by the 21st Century COE Program (A-1, to K.F.) and Grant-in-Aids (to N.T. and K.F.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported by a grant (to N.T. and K.F.) for Promotion of Basic Research Activities for Innovative Biosciences from the Bio-oriented Technology Research Advancement Institution. We are very thankful to H. Shimada and M. Kobayashi of the Histopathology Core Group for their help in preparing tick paraffin sections.

References

Agyei, A.D., Runham, N.W., 1995. Studies on the morphological changes in the midguts of two ixodid tick species *Boophilus microplus* and *Rhipicephalus appendiculatus* during digestion of the blood meal. Int. J. Parasitol. 25, 55–62.

Alim, M.A., Tsuji, N., Miyoshi, T., Islam, M.K., Huang, X., Hatta, T., Fujisaki, K., 2008. HlLgm2, a member of asparaginyl endopeptidases/legumains in the midgut of the ixodid tick *Haemaphysalis longicornis*, is involved in blood-meal digestion. J. Insect Physiol. 54, 573–585.

Alim, M.A., Tsuji, N., Miyoshi, T., Islam, M.K., Huang, X., Motobu, M., Fujisaki, K., 2007. Characterization of asparaginyl endopeptidase, legumain induced by blood feeding in the ixodid tick *Haemaphysalis longicornis*. Insect Biochem. Mol. Biol. 37, 911–922.

Aljamali, M.N., Bior, A.D., Sauer, J.R., Essenberg, R.C., 2003. RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum*. Insect Mol. Biol. 12, 299–305.

Attardo, G.M., Hansen, I.A., Raikhel, A.S., 2005. Nutritional regulation of vitellogenesis in mosquitoes: implications for anautogeny. Insect Biochem. Mol. Biol. 35, 661–675.

Bianchi, M.W., Barre, N., Messad, S., 2003. Factors related to infestation level and resistance to acaricides in *Boophilus microplus* tick populations in New Caledonia. Vet. Parasitol. 112, 75–89.

Boldbaatar, D., Sikasunge, C.S., Battsetseg, B., Xuan, X., Fujisaki, K., 2006. Molecular cloning and functional characterization of an aspartic protease from the hard tick *Haemaphysalis longicornis*. Insect Biochem. Mol. Biol. 36, 25–36.

Cheon, H.M., Shin, S.W., Bian, G., Park, J.H., Raikhel, A.S., 2006. Regulation of lipid metabolism genes, lipid carrier protein lipophorin, and its receptor during immune challenge in the mosquito *Aedes aegypti*. J. Biol. Chem. 281, 8426–8435.

Chilton, N.B., 1992. An index to assess the reproductive fitness of female ticks. Int. J. Parasitol. 22, 109–111.

Coons, L.B., Rosell-Davis, R., Tarnowski, B.I., 1986. Blood meal digestion in ticks. In: Sauer, J.R., Hair, J.A. (Eds.), Morphology, Physiology and Behavioural biology of Ticks. Ellis Harwood/John Wiley, New York, pp. 248–279.

de la Fuente, J., Almazan, C., Blouin, E.F., Naranjo, V., Kocan, K.M., 2005. RNA interference screening in ticks for identification of protective antigens. Parasitol. Res. 96, 137–141.

de la Fuente, J., Almazan, C., Blas-Machado, U., Naranjo, V., Mangold, A.J., Blouin, E.F., Kocan, K.M., 2006. The tick protective antigen, 4D8, is a conserved protein involved in modulation of tick blood digestion and reproduction. Vaccine 24, 4082–4095.

Drummond, R.O., Whetstone, T.M., 1970. Oviposition of the Gulf Coast tick. Ann. Entomol. Soc. Am. 63, 1547–1551.

Fire, A., Xu, S., Montogomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

Francischetti, I.M.B., Mather, T.N., Ribeiro, J.M.C., 2005. Tick saliva is a potent inhibitor of endothelial cell proliferation and angiogenesis. Thromb. Haemost. 94, 167–174.

Fujisaki, K., Kawazu, S., Kamio, T., 1994. The taxonomy of the bovine *Theileria* spp. Parasitol. Today 10, 31–33.

Grandjean, O., 1984. Blood digestion in *Ornithodoros moubata* Murray sensu stricto Walton (Ixodoidea: Argasidae) females: I Biochemical changes in the midgut lumen and ultrastructure of the midgut cells, related to intracellular digestion. Acarologia 25, 147–165.

Grubhoffer, L., Golovchenko, M., Vancova, M., Zacharovova-Slavickova, K., Rudenko, N., Oliver Jr., J.H., 2005. Lyme borreliosis: insights into tick-/host-borrelia relations. Folia Parasitol. 52, 279–294.

Hansen, I.A., Attardo, G.M., Park, J.H., Peng, Q., Raikhel, A.S., 2004. Target of rapamycin-mediated amino acid signaling in mosquito anautogeny. Proc. Natl. Acad. Sci. USA 101, 10626–10631.

Hatta, T., Kazama, K., Miyoshi, T., Umemiya, R., Liao, M., Inoue, N., Xuan, X., Tsuji, N., Fujisaki, K., 2006. Identification and characterization of a leucine aminopeptidase from the hard tick *Haemaphysalis longicornis*. Int. J. Parasitol. 36, 1123–1132.

Hatta, T., Umemiya, R., Liao, M., Gong, H., Harnnoi, T., Tanaka, M., Miyoshi, T., Boldbaatar, D., Battsetseg, B., Zhou, J., Xuan, X., Tsuji, N., Taylor, D., Fujisaki, K., 2007. RNA interference of cytosolic leucine aminopeptidase reduces fecundity in the hard tick, *Haemaphysalis longicornis*. Parasitol. Res. 100, 847–854.

Hoogstraal, H., Roberts, F.H., Kohls, G.M., Tipton, V.J., 1968. Review of *Haemaphysalis* (Kaiseriana) *longicornis* Neumann (resurrected) of Australia, New Zealand, New Caledonia, Fiji, Japan, Korea, and northeastern China and USSR, and its parthenogenetic and bisexual populations (Ixodoidea, Ixodidae). J. Parasitol. 54, 1197–1213.

Ho, T., Htwe, K.K., Yamasaki, N., Zhang, G.Q., Ogawa, M., Yamaguchi, T., Fukushi, H., Hirai, K., 1995. Isolation of *Coxiella burnetii* from dairy cattle and ticks, and some characteristics of the isolates in Japan. Microbiol. Immunol. 39, 663–671.

Huang, X., Tsuji, N., Miyoshi, T., Motobu, M., Islam, M.K., Alim, M.A., Fujisaki, K., 2007. Characterization of glutamine: fructose-6-phosphate aminotransferase from the ixodid tick, *Haemaphysalis longicornis*, and its critical role in host blood feeding. Int. J. Parasitol. 37, 383–392.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., Linsley, P.S., 2003. Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. 21, 635–637.

Karim, S., Miller, N.J., Valenzuela, J., Sauer, J.R., Mather, T.N., 2005. RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding. Biochem. Biophys. Res. Commun. 334, 1336–1342.

Klompen, H., 2005. Ticks, the Ixodida. In: Marquardt, W.C., Black, W.C., IV, Preier, J.E., Hagedorn, H.H., Hemingway, J., Higgs, S., James, A.A., Kondratieff, B., Moore, C.G. (Eds.), Biology of Disease Vectors, second ed. Elsevier Academic Press, New York, pp. 45–55.

Kocan, K.M., de la Fuente, J., Blouin, E.F., Garcia-Garcia, J.C., 2004. *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. Parasitology 129, S285–S300.

Luo, Y., Zhou, H., Krueger, J., Kaplan, C., Lee, S.H., Dolman, C., Markowitz, D., Wu, W., Liu, C., Reisfeld, R.A., Xiang, R., 2006. Targeting tumor-associated macrophages as a novel strategy against breast cancer. J. Clin. Invest. 116, 2132–2141.

Mendiola, J., Alonso, M., Marquetti, M.C., Finlay, C., 1996. *Boophilus microplus*: multiple proteolytic activities in the midgut. Exp. Parasitol. 82, 27–33.

Miyoshi, T., Tsuji, N., Islam, M.K., Kamio, T., Fujisaki, K., 2004. Cloning and molecular characterization of a cubilin-related serine proteinase from the hard tick *Haemaphysalis longicornis*. Insect Biochem. Mol. Biol. 34, 799–808.

Morita, Y., Araki, H., Sugimoto, T., Takeuchi, K., Yamane, T., Maeda, T., Yamamoto, Y., Nishi, K., Asano, M., Shirahama-Noda, K., Nishimura, M., Uzu, T., Hara-Nishimura, I., Koya, D., Kashiwagi, A., Ohkubo, I., 2007. Legumain/asparaginyl endopeptidase controls extracellular matrix remodeling through the degradation of fibronectin in mouse renal proximal tubular cells. FEBS Lett. 581, 1417–1424.

Narasimhan, S., Montgomery, R.R., DePonte, K., Tschudi, C., Marcantonio, N., Anderson, J.F., Sauer, J.R., Cappello, M., Kantor, F.S., Fikrig, E., 2004. Disruption of *Ixodes scapularis* anticoagulation by using RNA interference. Proc. Natl. Acad. Sci. USA 101, 1141–1146.

Nijhof, A.M., Taoufik, A., de la Fuente, J., Kocan, K.M., de Vries, E., Jongejan, F., 2007. Gene silencing of the tick protective antigens, *Bm86*, *Bm91* and *subolesin*, in the one-host tick *Boophilus microplus* by RNA interference. Int. J. Parasitol. 37, 653–662.

Oliveira, M.C., Oliveira-Sequeira, T.C., Araujo Jr., J.P., Amarante, A.F., Oliveira, H.N., 2005. *Babesia* spp. infection in *Boophilus microplus* engorged females and eggs in Sao Paulo State, Brazil. Vet. Parasitol. 130, 61–67.

Ostfeld, R.S., Price, A., Hornbostel, V.L., Benjamin, M.A., Keesing, F., 2006. Controlling ticks and tick-borne zoonoses with biological and chemical agents. Bioscience 56, 383–394.

Pal, U., Li, X., Wang, T., Montgomery, R.R., Ramamoorthi, N., deSilva, A.M., Bao, F., Yang, X., Pypaert, M., Pradhan, D., Kantor, F.S., Telford, S., Anderson, J.F., Fikrig, E., 2004. TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. Cell 119, 457–468.

Parrish, S., Fleenor, J., Xu, S., Mello, C., Fire, A., 2000. Functional anatomy of a dsRNA trigger differential requirements for the two trigger strands in RNA interference. Mol. Cell 6, 1077–1087.

Ramamoorthi, N., Narasimhan, S., Pal, U., Bao, F., Yang, X.F., Fish, D., Anguita, J., Norgard, M.V., Kantor, F.S., Anderson, J.F., Koski, R.A., Fikrig, E., 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. Nature 436, 573–577.

Renard, G., Garcia, J.F., Cardoso, F.C., Richter, M.F., Sakarani, J.A., Ozaki, L.S., Termignoni, C., Masuda, A., 2000. Cloning and functional expression of a *Boophilus microplus* cathepsin L-like enzyme. Insect Biochem. Mol. Biol. 30, 1017–1026.

Sojka, D., Franta, Z., Horn, M., Hajdusek, O., Caffrey, C.R., Mares, M., Kopacek, P., 2008. Profiling of proteolytic enzymes in the gut of the tick *Ixodes ricinus* reveals an evolutionarily conserved network of aspartic and cysteine peptidases. Parasit. Vectors 1, 7. doi:10.1186/1756-3305-1-.

Sojka, D., Hajdusek, O., Dvorak, J., Sajid, M., Franta, Z., Schneider, E.L., Craik, C.S., Vancova, M., Buresova, V., Bogyo, M., Sexton, K.B., McKerrow, J.H., Caffrey, C.R., Kopacek, P., 2007. IrAE – an asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. Int. J. Parasitol. 37, 713–724.

Sonenshine, D.E., Lane, R.S., Nicholson, W.L., 2002. Ticks (Ixodida). In: Mullen, G., Durden, L. (Eds.), Medical and Veterinary Entomology. Academic Press, San Diego, CA, pp. 530–552.

Soulsby, E.J.L., 1986. Helminths, Arthropods and Protozoa of Domesticated Animals, seventh ed. Balliere Tindall, London. pp. 469–495.

Tarnowski, B.I., Coons, L.B., 1989. Ultrastructure of the midgut and blood meal digestion in the adult tick *Dermacentor variabilis*. Exp. Appl. Acarol. 6, 263–289.

Taylor, D., Chinzei, Y., 2002. Vitellogenesis in ticks. In: Adiyodi, K.G., Adiyoi, R.G., Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, vol. XII. Science Publishers, Enfield, NH, USA, pp. 175–199.

Tsuji, N., Battsetseg, B., Boldbaatar, D., Miyoshi, T., Xuan, X., Oliver Jr., J.H., Fujisaki, K., 2007. Babesial vector tick defensin against *Babesia* sp. parasites. Infect. Immun. 75, 3633–3640.

Walker, A.R., Fletcher, J.D., 1987. Histology of digestion in nymphs of *Rhipicephalus appendiculatus* fed on rabbits and cattle naive and resistant to the ticks. Int. J. Parasitol. 17, 1393–1411.

Zaim, M., Guillet, P., 2002. Alternative insecticides: an urgent need. Trends Parasitol. 18, 161–163.

NOTE *Parasitology*

Hepatic Lesions Caused by Migrating Larvae of *Ascaris suum* in chickens

Shinobu YOSHIHARA[1)], Jyunko HATTORI[1)], Kazuya NISHIZONO[1)], Ayano KAWAMURA[1)], Kana SHIMOZAKI[1)], Yumi NISHIDA[1)], Kenji ODA[1)], Naotoshi TSUJI[2)]* and Norio HIRAYAMA[1)]

[1)]*Research Institute for Animal Science in Biochemistry and Toxicology, 3–7–11, Hashimotodai, Sagamihara, Kanagawa, 229–1132, and* [2)]*Laboratory of Parasitic Diseases, National Institute of Animal Health, National Agricultural and Food Research Organization, 3–1–5 Kannondai, Tsukuba, Ibaraki 305–0856, Japan*

(Received 8 November 2007/Accepted 3 June 2008)

ABSTRACT. Group A consisted of chickens infected with a single dose of *Ascaris suum* and group B of chickens infected with two successive doses. At days 1, 3, 7, 14 and 21 after the first or second infection dose, six chickens from each group were sacrificed. In both groups, larvae were recovered from the livers on days 1, 3, and 7 and lungs on days 3 and 7. No larvae were detected in chickens on day 14. Clear white lesions were noticed only on the livers from chickens of group B at day 7 but had disappeared at day 14. A comparison with group B showed mild histological changes that developed relative to the livers from group A.

KEY WORDS: *Ascaris suum*, chickens, visceral larval migrans.

J. Vet. Med. Sci. 70(10): 1129–1131, 2008

Similar to the visceral larval migrans (VLM) of *Toxocara canis (T. canis)* infection [3], *Ascaris suum (A. suum)* infection in humans has been reported in Japan [2, 8, 10, 11, 15]. It was considered that at least three human cases were caused by eating fresh raw meat and liver from cattle or chickens [2, 8, 10]. These reports suggest that chickens may play an important role in the zoonotic transmission of *A. suum*. To clarify the VLM of *A. suum* larvae in infected chickens, an investigation was carried out on the distribution of larvae and the pathological changes in the livers.

Fresh *A. suum* eggs were obtained from female worms at a local abattoir. Embryonation of the eggs was performed as described by Tsuji *et al.* [16]. A total number of 65 male broiler chickens aged about 20-days old were used. Initially, groups A (chickens infected with a single dose of *A. suum*) and B (chicken infected two successive dose) of 30 chickens each received 10,000 eggs and were kept in cages. The same number of eggs were given again to chickens of group B 17 days after the first dose. On days 1, 3, 7, 14, and 21 after the first or second infection, six chickens from each group were sacrificed by cervical dislocation. Larvae were collected from the liver (*lobus dexter*), lung, pectoral muscle (*musculus pectoralis profurdus*; about 30 g), and the duodenum (without contents and mucus) using the Baerman method. The contents and mucus of the duodenum were fixed separately in 5% formalin solution and examined microscopically. A video micrometer was used for the measurement of the length of larvae. Statisitical analysis of differences in larval counts between both groups on the same day after first or second infection was done using Statisitica (Stat Soft, Tulsa, OK, U.S.A., $P<0.05$). The liver (*lobus sinister*) was investigated macroscopically and fixed in 10% buffered formalin solution. Paraffin sections were stained with hematoxylin and eosin (HE) or with Azan. To examine the lesion in repeatedly infected animals, remaining 5 chickens (group C) were infected with 10,000 eggs twice weekly for five weeks and sacrificed 7 days after the final infection.

No clinical signs were observed in any of the infected chickens during the study period. Table 1 shows the distribution of *A. suum* larvae in chickens of groups A and B. Larvae were recovered from the livers on days 1, 3, and 7 and from the lungs on days 3 and 7 after the first and second infections. We identified morphorogically the larvae with *A. suum*. About half of the larvae (1-day old larvae) that were collected on day 1 (Fig. 1, A) moved extremely vigorously and ranged from 234 μm to 396 μm in length. No larvae were detected in chickens on day 14. The pattern of larval distribution recovered from the livers and lungs of group B was similar to that for group A. There was no significant difference in the number of larvae detected in the livers and lungs between both groups on the same day after the first or second infection for groups A and B. A large larva was noticed in each three samples of the liver containing small numbers of the worms on day 3 and 7 and the most developed nematode reached 1,385 μm in length. No larvae were collected from the duodenum or pectoral muscle.

Macroscopically, the livers were normal in color, and several clear white lesions ranging from 0.5 mm to 1.0 mm in diameter were recognized only on the liver surface from chickens of group B on day 7 (Fig. 1, B) but not on 14 after secondary infection. The lesions were nodular but not accompanied with fibrous changes. A few white lesions ranging from 2.0 mm to 2.5 mm in diameter were seen distinctly on the livers from chickens of group C. No the changes were observed distinctly in the livers of the animals from group A. Microscopically, larvae surrounded by eosinophilic substances were found occasionally in the lesions with granulocytes, fibroblasts, and machrophages in the livers from group B on day 3 after secondary infection (Fig. 1, C). Sometimes, boring focus, granuloma, infiltration of eosinophils and lymphocytes, and lymphofollicular hyper-

* CORRESPONDENCE TO: TSUJI, N., National Institute of Animal Health, 3–1–5 Kannondai, Tsukuba, Ibaraki 305–0856, Japan.
e-mail: tsujin@affrc.go.jp

Table 1. Migration of *A. suum* larvae in chickens from groups A and B

Group	Sample	Number of larvae recovered: Days after the first or second infection with the eggs				
		1	3	7	14	21
A	Liver	10, 12, 8, 20, 8, 22 12.3[b]	5[a], 10, 6, 171, 52, 6 16.6[b]	1[a], 0, 0, 0, 0, 0 1.1[b]	0	0
	Lung	0	0, 1, 0, 10, 10, 1 1.8[b]	3, 2, 7, 1, 16, 1 4.3[b]	0	0
	Muscle	0	0	0	0	0
	Duodenum	0	0	0	0	0
B	Liver	6, 180, 16, 20, 11, 0 13.3[b]	10, 40, 30, 41, 27, 79 33.1[b]	0, 0, 0, 0, 2[a], 0 1.2[b]	0	0
	Lung	0	1, 1, 0, 0, 1, 1 1.6[b]	0, 0, 2, 0, 0, 3 1.3[b]	0	0
	Muscle	0	0	0	0	0
	Duodenum	0	0	0	0	0

a) Sample contained a large larva in length
b) Number shows geometric mean value.

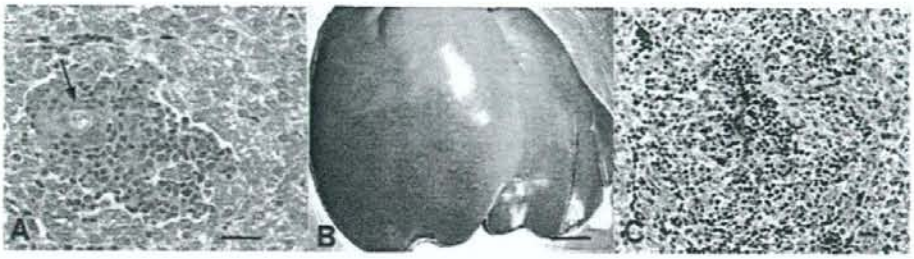

Fig. 1. Pathological observations of the livers from chickens of group B. (A) one-day old larva (arrow) in a lesion. Bar=20 μm, (B) Macroscopic finding of the surface of the liver on 7 days. Bar=1 cm. (C) Three-day old larva (arrow) in a lesion. Bar=20 μm.

plasia were seen on 3 and 7 days in this group. These histological changes without lymphofollicular hyperplasia were also found in the livers from group A. The changes in the livers from group A was less marked than those from group B. No distinct hyperplasia of interlobular connective tissue was recognized by Azan staining in groups A, B or C.

The VLM with *A. suum* was clarified pathologically as well as parasitologically in this investigation. The findings on the distribution of larvae suggest that the larvae may be eliminated from chickens within 14 days after infection. It is of interest to note that few large larvae of 1,000 μm or longer were recovered on day 3 and 7 from the livers of chickens. The length of larvae on day 4 in the livers of experimentally infected pigs was 533 μm to 619 μm [6] and on day 3 in mice was 386.7 ± 121.1 μm [7]. Permin *et al.* [13] investigated the VLM in chickens infected with *A. suum* and reported that no liver lesions were found macroscopically on the organs on day 7 after first infection. As no the lesions were observed on the liver from chickens of group A in this study, our results may agreed with their finding. Based on the findings in chickens of groups B and C, it seems likely that multiple infections with *A. suum* might be involved in the appearance or enhancement of liver lesions similar to the findings in pigs experimentally infected with *A. suum* [5, 17]. No typical 'milk spot' lesions with fibrous changes [5] as observed in the livers of *Ascaris* infected pigs, were found macroscopically in the present study. The typical lesions did not also develop in experimentally infected lambs [4]. Histopathologically, this change is a focal increase in the width of the interlobular connective tissue [12]. An inflammatory response with infiltration of lymphocytes and eosinophils occurred in the liver of the lambs infected with *A. suum* eggs, but because of the absence of the interlobular connective tissue, the reaction was mild and confined to areas of larval migration in the adjacent portal triads [4, 5]. The difference in the intensity of the lesions was probably due to anatomical differences of the interlobular connective tissue in the livers between lambs and pigs [4]. The tissue in the liver of chicken is also indistinct [9].

The raw pectoral muscle of the chickens is traditionally eaten as white meat in some areas of Japan, and the raw liver of local chickens features the menus of Japanese-style grill restaurants. In the case of *T. canis*, it is estimated that the

larvae can migrate by a hepatopulmonary route in chickens, which reinforces the possibility that chickens harboring migrating *T. canis* larvae may pose a zoonotic risk, especially if the liver is consumed [14]. Permin *et al.* [13] also demonstrated *A. suum* larvae in the lungs of piglets fed the livers and lungs from chickens infected with the nematode and suggested that there was a possibility for zoonotic transmission of *A. suum* if raw chicken livers were consumed by humans. Fortunately, an inspection system for chickens has been established in Japan, and the liver from local chicken once examined closely may be offered to consumers at grill restaurants. With such precautions in place it seems unlikely that consumers may eat contaminated fresh raw livers infected with *A. suum*. However, considering the possibility of parasitism by other parasites [1] in the livers from chickens it would be a sensible precaution for humans to avoid eating them if possible.

ACKNOWLEDGMENTS. The authors express their thanks to Dr. M. Yamaguchi of the Research Institute for Animal Science in Biochemistry and Toxicology for help in preparing paraffin sections. This study was supported in part by a grant from the Zoonosis Control Project (ZCP22) of the Ministry of Agriculture, Forestry and Fishery of Japan.

REFERENCES

1. Akao, N. and Ohta, N. 2007. Toxocariasis in Japan. *Parasitol. Int.* **56**: 87–93.
2. Arimura, Y., Mukae, H., Yanagi, S., Sano, A., Matsumoto, K., Ihiboshi, H., Matsumoto, N., Shiomi, K., Matsukura, S. and Matsuzaki, Y. 2001. Two cases of visceral larva migrans due to *Ascaris suum* showing a migratory nodular shadow. *Nihon Kokyuki Gakkai Zasshi* **39**: 716–720 (in Japanese with English summary).
3. Beaver, P. C. 1959. Visceral and cutaneous larva migrans. *Public Health Rep.* **74**: 328–332.
4. Brown, M., Hinton, M. and Wright, I. 1984. Parasitic liver damage in lambs with particular reference to the migrating larvae of *Ascaris suum*. *Vet. Rec.* **22**: 300–303.
5. Copeman, D. B. and Gaafar, S. M. 1972. Sequential development of hepatic lesions of ascaridosis in colostrum-deprived pigs. *Aust. Vet. J.* **48**: 263–268.
6. Douvres, F. W., Tromba, F. G. and Malakatis, G. M. 1969. Morphogenesis and migration of *Ascaris suum* larvae developing to fourth stage in swine. *J. Parasitol.* **55**: 689–712.
7. Geenen, P. L., Bresciani, J., Boes, J., Pedersen, A., Eriksen, L., Fagerholm, H.P. and Nansen, P. 1999. The morphogenesis of *Ascaris suum* to the infective third-stage larvae within the egg. *J. Parasitol.* **85**: 616–622.
8. Ito, K., Sakai, K., Okajima, T., Ouchi, K., Funakoshi, A., Nishimura, J., Ibayashi, H. and Tsuji, M. 1986. Three cases of visceral larva migrans due to ingestion of raw chicken or cow liver. *Nihon Naika Gakkai Zasshi* **75**: 39–46 (in Japanese).
9. Kato, Y. 1963. pp. 244–245. *In*: Kachiku Hikaku Kaibougaku Zusetsu (Kato, Y. ed.), Youkenndou, Tokyo (in Japanese).
10. Matsushita, R., Tahara, Y., Yamamoto, S., Nagata, K., Komada, N., Hori, T., Ido, A., Hirono, S., Hayashi, K., Nawa, Y. and Tsuboi, H. 1997. A case of visceral larva migrans (VLM) with multiple intra-hepatic nodular lesions due to *Ascaris suum*. *Kanzo* **38**: 730–734 (in Japanese).
11. Marayama, H., Nawa, Y., Noda, S., Mimori, T. and Choi, W. Y. 1999. An outbreak of visceral larva migrans due to *Ascaris suum* in Kyushu, Japan. *Lancet* **347**: 1766–1767.
12. Nakagawa, M., Yoshihara, S., Suda, H. and Ikeda, K. 1983. Pathological studies on white spots of the liver in fattening pigs. *Natl. Inst. Anim. Health Q. (Jpn.)*. **23**: 138–149.
13. Permin, A., Henningse, E., Murrell, K. D., Roepstorff, A. and Nansen P. 2000. Pigs become infected after ingestion of livers and lungs from chickens infected with *Ascaris* of pig origin. *Int. J. Parasitol.* **30**: 867–868.
14. Taira, K., Permin, A. and Kaple, K. D. 2003. Establishment and migration pattern of *Toxocara canis* larvae in chickens. *Parasitol. Res.* **90**: 521–523.
15. Tokojima, M., Ashitani, J. I. and Nakazato, M. 2004. A case of eosinophilic pneumonia caused by visceral larva migrans due to *Ascaris suum*. *Kansenshogaku Zasshi* **78**: 1036–1040 (in Japanese with English summary).
16. Tsuji, N., Suzuki, K., Kasuga-Aoki, H., Isobe, T., Arakawa, T. and Matsumoto, Y. 2003. Mice intranasally immunized with a recombinant 16-kilodalton antigen from roundworm *Ascaris* parasites are protected against larval migration of *Ascaris suum*. *Infect. Immun.* **71**: 5314–5323.
17. Yoshihara, S., Nakagawa, M., Suda, H., Ikeda, K. and Hanashiro, K. 1983. White spots of the liver in pigs experimentally infected with *Ascaris suum*. *Natl. Inst. Anim. Health Q. (Jpn.)* **23**: 127–137.

Molecular & Biochemical Parasitology 164 (2009) 26–31

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Peroxiredoxin-1 from *Schistosoma japonicum* functions as a scavenger against hydrogen peroxide but not nitric oxide

Takashi Kumagai[a,b,*], Yoshio Osada[b], Nobuo Ohta[a], Tamotsu Kanazawa[b]

[a] Section of Environmental Parasitology, Department of International Health Development, Division of Public Health, Tokyo Medical and Dental University Graduate School, Japan
[b] Department of Immunology and Parasitology, School of Medicine, University of Occupational and Environmental Health, Kitakyusyu, Japan

ARTICLE INFO

Article history:
Received 8 April 2008
Received in revised form 4 November 2008
Accepted 4 November 2008
Available online 12 November 2008

Keywords:
Schistosoma japonicum
Peroxiredoxin
RNA interference
Reactive oxygen species
Nitric oxide

ABSTRACT

Three peroxiredoxins (Prxs) are expressed during most of the developmental stages in the schistosome. Prx-1 is localized on the surface of the schistosomula and adults of *Schistosoma japonicum*, while Prx-2 is localized in the sub-tegumental tissues, parenchyma, vitelline glands, and gut epithelium, but not on the surface of the worms. We applied RNA interference techniques to suppress the specific genes of *S. japonicum* Prxs. Schistosomula of *S. japonicum* were cultured together with long-dsRNA encoding Prx-1 and Prx-2 of *S. japonicum* (the soaking method). The transcription level of each Prx gene was reduced by an RNA interference (RNAi)-mediated effect specifically. Although neither Prx was the essential protein for survival of *S. japonicum* schistosomula, Prx-1 dsRNA-treated larvae were susceptible to hydrogen peroxide. Moreover, these larvae were also susceptible to *t*-butyl hydroperoxide and cumene-hydroperoxide. However, the knockdown of neither Prx-1 nor Prx-2 influenced the resistance against nitric oxide generated from DETA/NO. Prx-1 may work as a scavenger against reactive oxygen species (ROS) generated outside of the schistosomes to prevent the oxidation of the bodies and/or the attack by immune cells producing the ROS. These findings suggest that Prx-1 may become a novel target of drugs and vaccines for schistosomiasis.

1. Introduction

Schistosoma japonicum, which is a species of Asian schistosome, has a complex life cycle, surviving in the veins of the final host without destruction from the immune system [1]. The worms are exposed to oxidative stresses due to reactive oxygen species (ROS), which are released from host effector cells adhering to the antibody-coated worms. However, several studies have reported that the antioxidants from schistosomes might allow the worms to survive in the bloodstream by removing the ROS generated in the veins and those released from host immune cells [2–4].

Peroxiredoxin (Prx) is a family of novel antioxidant proteins containing two conserved Cys residues, which are essential for the enzymatic scavenging of hydrogen peroxide [5]. The molecular cloning and characterization of Prxs from *S. mansoni* have been reported [6–9]. Recently, Prx-1 and Prx-2 were also found in the sporocysts and in excretory-secretory proteins from sporocysts of *S. mansoni* [10]. We previously characterized three Prxs: Prx-1, Prx-2, and Prx-3, from *S. japonicum*, as well as the expression of each Prx throughout the life cycle [11]. Because Prx-3 alone contained the mitochondria targeting sequences, it was thought to work as a scavenger against ROS in mitochondria. We found that Prx-1 was not only expressed in the tegument, but was also present in the excretory/secretory products of the schistosomula and adult worms. Moreover, Prx-2 was mainly detected in the sub-tegumental tissues, parenchyma, vitelline glands, and gut epithelium of the adult worms, but was not detected in the tegument of adults or in the schistosomula. These data suggest that Prx-1 acts to protect the parasite against the ROS generated within the host vein.

RNA interference (RNAi) has been applied to the study of a wide range of organisms, including schistosomes. This method makes it possible for the targeted organisms to down-regulate their gene expressions specifically. Several studies have reported gene suppression in the schistosomula and the sporocyst of *S. mansoni* by RNAi [12–15]. One recent study reported that the down-regulation of Prx-1 by RNAi in *S. mansoni* was lethal, because it increased its sensitivity to hydrogen peroxide [16].

In the present study, we performed RNAi against the skin-penetrated schistosomula of *S. japonicum* to suppress the gene

Abbreviations: Cys, cysteine; DETA/NO, diethylenetramine with nitric oxide; dsRNA, double-stranded RNA; MBP, maltose-binding protein; NO, nitric oxide; Prx, peroxiredoxin; RNAi, RNA interference; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; TPI, triose-phosphate isomerase; Trx, thioredoxin.

* Corresponding author at: Section of Environmental Parasitology, Department of International Health Development, Division of Public Health, Tokyo Medical & Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Tel.: +81 3 5803 5195; fax: +81 3 5684 2849.
E-mail address: tkuma.vip@tmd.ac.jp (T. Kumagai).

0166-6851/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2008.11.002

expression of Prx-1 and Prx-2. After the treatment was applied to the schistosomula with the double-stranded RNA (dsRNA) coding each Prx, larvae were subjected a killing assay with several ROS and reactive nitrogen species (RNS) to confirm the essential functions of Prxs in this worm.

2. Materials and methods

2.1. Preparation of the parasite at each stage

S. japonicum, isolated at Yamanashi, Japan, was maintained in the laboratory, using female ICR mice (SLC, Hamamatsu, Japan) and its snail host, *Oncomelania hupensis nosophora*. Cercariae were shed from the crushed snails, collected and used for infection and other experiments, as previously described [17]. Schistosomula were prepared by the previously described method [11]. Briefly, anesthetized ICR mice were percutaneously infected with more than 1000 cercariae through shaved abdominal skin. The mice were then killed, and the skin regions containing the schistosomula were peeled off and cultured in RPMI1640 medium supplemented with 5 mM L-glutamate, 200 U/ml penicillin, and 200 μg/ml streptomycin at 37 °C in 5% CO_2 overnight. The schistosomula were released into the medium (skin-penetrated schistosomula), following which the skin was removed. The schistosomula were used in RNAi experiments. For the killing assay with NO, another batch of schistosomula was prepared by shearing off their tails mechanically with repeated passages through a 20-gauge needle. These transformed schistosomula were incubated in RPMI1640 medium at 37 °C in 5% CO_2 for 3 h (3 h m-schistosomula), and 24 h (24 h m-schistosomula), respectively. The animal experiments were performed under the control of the Ethics Committee of Animal Care and Experimentation in accordance with The Guiding of Principles for Animal Care Experimentation, University of Occupational and Environmental Health, Japan, and the Japanese Law for Animal Welfare and Care (No. 221).

2.2. Preparation of dsRNA

Double-stranded RNA molecules were prepared using the Hiscribe™ RNAi transcription kit (NEB, Ipswich, USA). Each complete coding region from the cloned pCR2.1-Prx-1 vector and pCR2.1-Prx-2 vector was amplified by PCR using primer sets with the addition of BamHI and HindIII restriction sites at the 5′ and 3′-ends, respectively. After checking the sequences, each fragment coding Prx-1 or Prx-2 was sub-cloned into the pLITMUS™ vector, containing the T7 RNA polymerase dual promoter sequence at each end. The PCR reaction with T7 primer was performed using the purified sub-cloned constructs as a template. To synthesize dsRNA, *in vitro* transcription by T7 RNA polymerase (Invitrogen, San Diego, USA) was performed using the amplified products as a template at 42 °C for 6 h. The dsRNAs were treated with DNase and purified by ethanol precipitation. After checking the bands corresponding to each dsRNA by gel electrophoresis, their concentrations were determined by measurement of OD at 260 nm using a spectrometer. Using the same procedure, dsRNA was synthesized from the construct of the maltose-binding protein (MBP) from *E. coli* as a negative control.

2.3. RNA interference (RNAi)

The skin-penetrated schistosomula were washed with medium and approximately thirty larvae in 100 μl of medium were put into each well of a 96-well plate. Each dsRNA solution adjusted to the 200 nM concentration was added to each well at a final volume of 200 μl (final concentration was 100 nM). The treated schistosomula were cultured at 37 °C in 5% CO_2 for 6 days without a medium change. At the same time, to examine the influence of RNAi on worm survival, approximately 50 larvae treated or non-treated by RNAi were incubated for 14 days, with 100 μl of fresh medium being added every 6 days. The number of surviving larvae was checked under microscopy daily and each experiment was performed in triplicate twice.

2.4. Preparation of total RNA and RT-PCR

Three days after RNAi treatment, total RNA was isolated from each treated group of the schistosomula using TRIzol reagent (Invitrogen). Complementary DNAs were synthesized with reverse transcriptase (Invitrogen) in a 20-μl reaction mixture using random hexamer oligonucleotides as primers. The resulted solutions were adjusted to 11 larvae per 20 μl with TE buffer. As a template, 1 μl of cDNA solution was applied to PCR in a 20-μl reaction mixture. The specific primer sets for each gene were as follows: Prx-1 (5′-TGGATTGGGTGACATGAGAA-3′, 5′-CGAATTGTACACTGCCATTCA-3′), Prx-2 (5′-TTCCTCGATTTCAAGTCAGTCA-3′, 5′-CCTAATCCACCAGCTTTTCG-3′), and triose-phosphate isomerase (TPI) (5′-ATGGCAGTAGAGCCGACAAC-3′, 5′-AACGCTTAGACCTCCTGCAA-3′). The last one was used as an internal control [18]. The PCR consisted of 30 cycles each at 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR products were resolved by agarose gel electrophoresis, fand stained in ethidium bromide.

2.5. Schistosomula killing assay

Following schistosomula treatment with dsRNA, cell-free killing was performed by incubating the dsRNA-treated schistosomula or 3 h schistosomula with hydrogen peroxide (10 μM), *t*-butyl hydroperoxide (10 μM), cumene-hydroperoxide (1 μM), and DETA/NO (the adduct of diethylenetramine with nitric oxide; 0.5 mM), which is the chemical donor for generating NO in aqueous solution [19]. Trypan blue was added to the killing assay culture at a final concentration of 0.01%. After 48 h, the schistosomula mortality was assessed by counting the non-stained worms. All experiments were performed in triplicate three times.

2.6. Statistical analysis

The significance of the decrease in worm survival rate was determined by two-tailed Student's *t*-test.

3. Results

3.1. Specific suppression of expression from Prx-1 and Prx-2 by dsRNA treatment

To determine the function of each Prx from *S. japonicum*, we performed RNAi using the entire coding region of Prx-1 and Prx-2. Each dsRNA was prepared from the Prx-coding vector by *in vitro* transcription, and the purified dsRNA was checked by gel-electrophoresis. After performing the RNAi (soaking method) against the skin-penetrated schistosomula using each dsRNA, total RNA was recovered from each treated group. By RT-PCR, we confirmed that the gene expression was specifically suppressed depending on the dsRNA employed (Fig. 1). However, the dsRNA coding maltose-binding protein from *E. coli* as a negative control did not affect the Prx expressions. Furthermore, the treatment of the dsRNA rarely influenced the expression of TPI, which is expressed constitutively throughout developmental stages. Taken together, these results suggest that the specific RNAi was successful against the schistosomula using Prxs–dsRNA.