searchers. A mf range of 1-10 mf/ml was cited but often proved inconvenient for analytical studies as not many cases fell into this category. Comparing the 60 µl F-P method and 1 ml nuclepore filtration method, 1-20 mf/ml was defined as low-density microfilaremia (l.d.m.) for studies in Samoa [36]. The prevalence of l.d.m. in all mf positives was 23.6% (90/381), which led to an estimate of 1,700 low-density mf carriers in 1979 throughout the country. L.d.m was proportionally more frequent in villages with lower mf prevalences and in people < 20 years of age. By sex, there was no difference.

Since low-density mf carriers were recognized to occupy a substantial proportion of all mf carriers in Samoa, and *Ae. polynesiensis* were known to ingest more mf than estimated (up to 4.7 times) while having a blood meal on low-density mf carriers [37], the significance of l.d.m. as a source of transmission was seriously discussed, especially because it was suspected that annual single-dose DEC would not exert a sufficient adulticidal effect and therefore produce more low-density carriers than multi-dose treatment.

d-2. *Significance of low-density mf carriers in filariasis transmission: Quantitative assessment*

Based on the mosquito "infectivity index" concept proposed by Sasa [33], the significance of l.d.m. in transmission can be determined by estimating the proportion of mosquito infectivity produced by the l.d.m. group as compared to the total mosquito infectivity produced by all levels of mf carriers in an endemic community. Assuming that all people are exposed evenly to mosquito bites, the latter can be computed as follows:

Total mosquito infectivity = Σ (infection rate when mosquitoes feed on a carrier with mf density of k) x (No. of carriers with mf density of k in a community)

where k is from 1 to the maximum mf density (/1 ml of venous blood) observed in the community. Mosquito infectivity produced by low-density mf carriers will be obtained using the same formula with k value from 1 to 20.

To study the rate of mosquito infection, Samarawickrema et al. (1985) [37] conducted a detailed transmission experiment using *Ae. polynesiensis*, a main vector in Samoa, and 14 mf carriers with different mf densities ranging from 0 to 5,290 mf/ml. The results showed that the percentage of mosquitoes infected and the average number of larvae found in each infected mosquito were directly proportional to the mf densities in the carrier at the time of feeding. Based on the data from this transmission experiment, it was possible to obtain theoretical mosquito infection rates when mf carriers with different mf densities were blood sucked by mosquitoes [36]. To estimated the numbers of mf carriers with certain mf densities, a negative binomial distribution was fitted following the method of Pichon et al. (1980) [38] to the 1979 mf data from Samoa (n = 358 mf positives) [25].

The computation of mosquito infectivity is shown in Table 2. The infectivity produced by l.d.m. was 251.2 (B) and that by all levels of mf density was 11645.8 (A), and the contribution of l.d.m. to the total (B)/(A) was 0.0216 or only 2.16%. This would suggest a minor role of l.d.m. in the transmission of filariasis in Samoa [36].

E. Re-evaluation of low-dose DEC treatment, introduction of new drugs and their combination therapy

e-1. *New evidences supporting the efficacy of low-dose DEC treatments*

After around 1990, a variety of low-dose DEC treatments were tested in various countries where *W. bancrofti* is endemic. In Tahiti, a single dose of 3 mg/kg was reported effective in reducing geometric mean mf count by 95% when assessed 180 days after treatment [39]; in Papua New Guinea, 2 annual single doses at 6 mg/kg reduced the mf rate from 41% to 17%, and mf density from 71 mf/20 µl to 20 [40]; in Brazil, the efficacy of 6 mg/kg given only once was reported equally effective to 12 daily doses at 6 mg/kg when measured 12 months after treatment, although the single dose was significantly less effective than the multi doses during the first 6 months [41]; and in Tanzania, two 6-monthly treatments at 6 mg/kg reduced the geometric mean mf intensity by 92.2%, while 12 daily doses at 6 mg/kg reduced it by 98.6%, when assessed 1 year after the start of treatment. The former regimen was considered more suitable for MDAs than the standard 12-dose treatment [42]. The single dose treatment with DEC at 6 mg/kg was also applied to *Brugia malayi* infections. In Kerala, India, 2 annual mass treatments reduced the mf prevalence from 4.9% to 1.2%, and the mean mf count by 81%. In addition, clinical benefits such as a reduction in acute manifestations and recent edema cases were reported [43].

A single dose DEC at 6 mg/kg reduced not only the mf level by 90.5% but circulating filarial antigen by 39.7% 18 months after treatment, suggesting that the treatment was effective against adult parasites [44]. Brazilian researchers successfully studied the adulticidal effect of low-dose DEC treatment by direct observation of live adults using ultrasonography. The adults live in a dilated lymph vessel in the scrotal area, making a "nest" and moving actively. Amaral et al. (1994) [45] named the movement "filaria dance sign" ([46] for video image). Norões et al. (1997) [47] reported

Table 2. Mosquito infectivity produced by the low-density microfilaria (mf) carriers (B) and all levels of mf carriers (A)

Microfilaria density (mf/ml)	Theoretical % of infected fed mosquitoes (I)*	Theoretical No. of mf positive persons in each mf density group (II)	(I) x (II)	
1	0.492	13.214	6.5	
2	1.152	8.584	9.9	
-4	2.102	11.999	25.2	
-6	2.842	8.774	24.9	
-8	3.473	7.074	24.6	
-10	4.032	6.000	24.2	
-20	6.254	21.729	135.9	
		Subtotal	251.2	...(B)
-30	7.985	15.076	120.4	
-40	9.458	11.854	112.1	
-50	10.766	9.892	106.5	
-60	11.954	8.550	102.2	
-70	13.052	7.565	98.7	
-80	14.078	6.805	95.8	
-90	15.045	6.198	93.2	
-100	15.963	5.700	91.0	
-200	23.456	41.077	963.5	
-300	29.292	26.579	778.6	
-400	34.259	19.642	672.9	
-500	38.666	15.443	597.1	
-600	42.673	12.589	537.2	
-700	46.374	10.511	487.4	
-800	49.834	8.925	444.8	
-900	53.094	7.675	407.5	
-1000	56.187	6.666	374.5	
-2000	81.449	36.479	2971.2	
-3000	100 (101.123)	13.242	1324.2	
-4000	100 (117.869)	5.524	552.4	
≥4001	100 (--)	4.634	463.4	
		Subtotal	11394.6	
Total		358.000	11645.8	...(A)

* Estimated from $\log (Y + 1) = 0.5278 \log X + 0.1739$, where Y = % infected, X = mf density of a carrier.
{Source: Adopted from Ref. [36]}

that within a week after treatment at 6 mg/kg single dose, the dance sign became undetectable in 7 of 14 nests, and scrotal nodules became palpable at each site of the 7 nests. Biopsy specimens from these nodules revealed "nests" of degenerating adults, confirming the adulticidal effect. A separate histopathological study with DEC-induced nodules reported that even a single dose of 1 mg/kg could damage adult worms. However, it should be noted that, even after repeated high dose DEC treatments, some worms in the same nest remained intact [47, 48].

Fortunately, no drug resistance has been reported so far with DEC.

e-2. *Ivermectin as a new drug against lymphatic filariae*

On the other hand, ivermectin, the drug of choice for onchocerciasis that is also effective against intestinal helminths and ectoparasites such as lice, was reported in 1988 [49] to be effective against *W. bancrofti*. Since it is effective with a single oral dose at 25-200 μg/kg, ivermectin was cited as a candidate to replace DEC. The efficacy was further confirmed: when assessed at 6 months, a single dose at 21.3 μg/kg and 126 μg/kg reduced mf to 18.3% and 19.5% of the original levels, respectively, while 13 daily DEC treatments (one 3 mg/kg dose, followed by 12 daily doses at 6 mg/kg) reduced the level to 6.0% [50]. With a

higher dosage of 420 μg/kg (20 μg/kg at day 1 plus 400 μg/kg at day 5), the geometric mean mf density was reduced to 0.9% of the pretreatment level 1 year after treatment, while DEC at 6 or 7 mg/kg reduced the mean to 9.3% ($P < 0.006$) [51]. Another study with 420 μg/kg of ivermectin reported a mean mf reduction of 86.3% after 18 months, while it was 90.5% with a single dose of DEC at 6 mg/kg ($P > 0.05$). In this study, the efficacy of ivermectin was much stronger than DEC in the first 30 days after treatment, but by 18 months, the latter took over the lead and resulted in a slightly higher reduction [44]. The stronger effect of DEC as opposed to ivermectin in the second year was also reported in Brazil [52]. To study adulticidal effect of ivermectin, Dreyer et al. (1995) [53] treated 15 *W. bancrofti*-infected Brazilian men with a single dose of ivermectin at 400 μg/kg and observed the filaria dance sign by ultrasound for 3-9 months. Contrary to all expectations, there was no observable change in the dance sign, and it was concluded that ivermectin had no effect on adult worms.

e-3. *Combination chemotherapies*

Having two potent anti-filarial drugs, DEC and ivermectin, researchers tested the efficacy of their use in combination. More recently, the effectiveness of albendazole, an established antiparasitic agent, against filarial parasites was reported, and various combinations of these 3 drugs have been evaluated.

A possible additive or synergistic effect of DEC and ivermectin was reported in Haiti based on a finding that 20 μg/kg ivermectin given as a clearing dose at day 1, followed by a single 6 mg/kg dose of DEC at day 5 resulted in higher efficacy in reducing mf density than DEC alone 1 year after treatment [50]. The same regimen tested in Brazil produced the best results among different combinations of the 2 drugs: reduction of microfilaremia to 2.4% of the pretreatment level (100%) at 2 years [51]. In Tahiti, 2 annual single dose MDAs were conducted using 4 different regimens: (i) ivermectin 400 μg/kg plus DEC 6 mg/kg, (ii) ivermectin 400 μg/kg alone, (iii) DEC 6 mg/kg alone, and (iv) ivermectin 400 μg/kg plus DEC 3 mg/kg [54]. After 1 year, regimens (i) and (iv) resulted in the same 32% reduction in mf prevalence, while the reduction was only 11-14% using regimens (ii) and (iii). As for mf density, the former 2 regimens brought about a 95-96% reduction from the pretreatment level, and the latter 2 regimens 80-82%. To clarify the effect of combination therapy on adult worms, a single dose of DEC at 6 mg/kg was co-administered with either 200 μg/kg or 400 μg/kg of ivermectin, and filaria dance sign was observed with ultrasonography [55]. Interestingly, the dance sign was evident in all of the 30 nests studied, suggesting that the co-administration interfered with the already established adulticidal effect of DEC.

Other studies have looked at the combination of albendazole and DEC or ivermectin. A review by Ottesen et al. (1999) [56] summarized the role of albendazole for lymphatic filariasis elimination. Ismail et al. (1998) [57], working with *W. bancrofti*, showed more reduction in geometric mean mf density with a combined single dose of albendazole (600 mg) plus ivermectin (400 μg/kg) than with a combination of albendazole (600 mg) plus DEC (6 mg/kg) up to 12 months after treatment. At 15 months, however, there was no significant difference between the two (reduction of > 98% in both regimens). As for circulating filarial antigen, the latter combination resulted in significantly more reduction (77%) than the former at 15 months. A combined single dose of albendazole (400 mg) plus DEC (6 mg/kg) reduced mean mf density by 85.7-99.6% 12-24 months after treatment [58-61]. On the other hand, several studies could not confirm the benefit of albendazole in various combinations with ivermectin/DEC [62-65]. Dreyer et al. (2006) [66] compared the adulticidal effect of DEC (6 mg/kg) alone and DEC (6 mg/kg) plus albendazole (400 mg), and reported that the combination resulted in a much lower effect on filaria dance sign. The authors concluded that co-administration appeared to reduce the adulticidal effect of DEC. Further studies are necessary to confirm or refute anti-filarial effects of albendazole in combined use with the other anti-filarials [67].

The role of albendazole in the global program for filariasis elimination has to be emphasized for its "beyond-filariasis" benefits [55]. The drug is very effective against intestinal helminths such as *Ascaris*, *Trichuris* and hookworm, which have inflicted a tremendous burden on the health of poor people in developing countries. In Haiti, 2 annual single-dose MDAs with DEC (6 mg/kg) and albendazole (400 mg) reduced *Ascaris*, *Trichuris* and hookworm prevalences from 20.9% to 14.1%, 34.0% to 14.6%, and 11.2% to 2.0%, respectively, 9 months after the second MDA [68]. In India, the same treatment reduced the prevalence of *Ascaris* by 83%, *Trichuris* by 63% and hookworm by 69%, 11 months after the second MDA [69]. These "beyond-filariasis" benefits will not only give the anti-filariasis campaign a broader public health significance but also help to improve compliance among people and sustain the elimination program. The combination of drugs is also said to be effective in preventing the acquirement of drug resistance by filarial parasites.

F. Global Programme to Eliminate Lymphatic Filariasis (GPELF)

f-1. *Strategies/activities*

The World Health Assembly made a resolution in 1997 to eliminate lymphatic filariasis from the world as a public health problem by 2020. To execute the resolution, the GPELF was organized with a main strategy of conducting annual single-dose MDA for 4-6 years. Under MDA, all people living in endemic areas with or without filarial infection are expected to be treated, meaning that the number in 83 endemic countries will reach some 13 billion people. The drugs used for MDA are the combination of DEC (6 mg/kg) and albendazole (400 mg) in onchocerciasis-free areas, and ivermectin (200-400 μg/kg) and albendazole (400 mg) in onchocerciasis-endemic areas of Africa. The reason for the use of 2 separate regimens is that DEC could cause severe reactions if administered to *Onchocerca*-infected individuals. The biggest barrier to financing the drug supply was removed by the donation of albendazole and ivermectin by the 2 pharmaceutical giants. The GPELF has also put particular emphasis on the care of existing clinical cases of lymphedema/elephantiasis and hydrocele. Simple procedures for lymphedema management have been established [70], in which the cure and prevention of bacterial/fungal infections by maintaining good hygiene of the affected skin is the basic concept. Daily washing with soap and water, together with exercise and elevation of the affected limb to drain accumulated lymph fluid, is the most essential part of the "care" for which family members and volunteers have been trained [71].

In the beginning, many researchers/clinicians were suspicious about the success of such a huge program. Some rejected the idea outright because they considered filariasis a low-priority disease. However, a tectonic shift was already underway: the idea of DALY had changed disease priority in favor of filariasis that produces permanent or long-lasting disability, and non-profit activities for the underprivileged by a variety of voluntary groups/citizens had matured. People suffering from lymphatic filariasis, one of the world's most neglected diseases, have gained global attention for the first time in history.

f-2. *Achievements*

Ottesen et al. (2008) [72] described in detail the results of 8 years of global effort (2000-2007). More than 740 million albendazole tablets and 590 million ivermectin tablets were donated by the partner drug companies of the GAELF, while 4.7 billion DEC tablets were purchased by endemic countries. A minimum of 570 million individuals were treated in 48 of the 83 endemic countries. In 68 pre-fixed sentinel sites to monitor treatment effects, 5 rounds of MDA reduced mf prevalence by ca. 85% and cleared mf in 63% of the sites. The WHO report in 2008 [8] listed 5 countries which no longer have active transmission foci, and 2 countries (China and Korea) where the elimination of filariasis was declared.

The benefits of the MDAs conducted in 2000-2007 include the following: 6.6 million newborns were protected from filarial infection, of whom 1.4 million and 0.8 million individuals will escape hydrocele and lymphedema, respectively, in their lifetimes; 9.5 million asymptomatic parasite carriers were protected from developing hydrocele (6.0 mil.) or lymphedema (3.5 mil.). The DALYs averted in 8 years were estimated to reach 32 million, for which US$ 190 million was spent to cover MDA-related costs. Thus, the cost per DALY averted was US$ 5.90 (excluding donated drugs), which is one of the most cost effective programs in the world [73].

In addition to the benefits of the 2000-07 MDAs relating to filariasis, 56.6 million children and 44.5 million women of childbearing age were treated with albendazole for intestinal parasitic infections, and in onchocerciasis endemic countries of Africa, more than 45 million were treated with ivermectin for various skin diseases caused by *Onchocerca volvulus*, scabies mites and lice, although DALYs averted by these treatments were not quantified. It can readily be said that the global filariasis program has already established new precedents: collaboration in combating neglected diseases, single-dose treatment for different diseases, and confidence of local people in maintaining a public health program, all on a global scale.

f-3. *The future*

The progress made by the GPELF has been remarkable so far. However, the elimination program is not necessarily proceeding satisfactorily in all endemic countries. Problems arise when compliance to MDAs is not sufficient, pretreatment endemicity levels are high, the species of vector mosquito is an efficient transmitter, MDA drug dosage is not sufficient (particularly with ivermectin), etc. [74]. A more serious question will be the endpoint for the elimination program in each endemic country. The variability of biological, human-behavioral and socio-economic factors make it difficult to clarify a threshold at which filariasis transmission disappears spontaneously. With strong continuous global cooperation as a precondition, each endemic country needs to carry out well-organized and effective MDAs. The drug administration may have to be repeated if necessary. Vector control measures may become an essential part of the program in some areas [75]. And it is expected that mathematical models will play a more important

role in planning future operations [76].

G. Expansion of intervention activities by people: Community-directed treatment (ComDT)

In many endemic areas where health manpower is running short, it is difficult to carry out a large scale MDA. In Okinawa, Japan, in the 1960s, senior high school graduates living in the endemic areas were trained as "ad hoc" laboratory technicians [77]. In Sri Lanka, the MDA in 2003 achieved 80% drug coverage of 9.8 million endemic population, and more than half of the drug coverage (55.2%) was executed by volunteers making door-to-door visits [78].

In West Africa, when the Onchocerciasis Control Programme started employing annual mass treatment with ivermectin, they faced the same problem of manpower shortage. Then in 1995, WHO/TDR conducted a landmark study in 5 African countries to clarify how well local people can plan and execute the distribution of ivermectin by themselves [79]. In the study, 2 different treatment schemes were compared: community-designed treatment and program-designed treatment. In the former, after a minimal essential health education/information session, the community was invited to decide who in the community would be drug distributers and when and how the drugs would be distributed, while in the latter, experienced program staff pre-designed the criteria for selecting drug distributers and detailed procedures for drug distribution. The results were rather unexpected: the former achieved as good a treatment coverage as the latter. This was a clear indication that local people can be a reliable player in public health activities. A similar study with lymphatic filariasis followed in Ghana and Kenya [80], where community-directed treatment with some input from health services (ComDT/HS) was compared with the treatment planned and executed by the regular care system (HST). The results: ComDT/HS achieved a high treatment coverage of 75.7% - 88.0%, whereas HST obtained only 43.6% - 46.5%.

The African Programme for Onchocerciasis Control (APOC), which was set up in 1995 covering 16 countries, adopted the idea of community-designed treatment from the above 1995 TDR study and implemented the community-directed treatment with ivermectin (CDTI). The program was so successful in reducing the burden of onchocerciasis that, in the year 2007, close to 1 million DALYs could be averted [81]. For CDTI, "community drug distributers" (CDDs) play an essential role. After training, they take a census, distribute drugs, monitor adverse reactions and keep records. In 2006, there were 429,385 trained CDDs in APOC countries. These batteries of manpower with health care knowledge have become involved as a matter of course in other intervention programs. In Nigeria, successful integration of insecticide-treated bed net distribution for malaria control with lymphatic filariasis/onchocerciasis MDA was reported [82]. WHO/TDR, in 2005, launched a study to investigate whether the concept and experience of CDTI can be applied to other interventions such as delivering vitamin A supplement, insecticide treated nets, DOTS treatment for tuberculosis, and home management of malaria. The results showed that all 5 interventions (including CDTI) could be done simultaneously by the community [83].

Community-directed treatment, which was invented as a measure necessary to conduct a large scale MDA for onchocerciasis in areas where health infrastructures were poor, has been transformed into a new sustainable way of delivering therapeutic and preventive measures for rural people suffering from a variety of neglected diseases. The MDAs for lymphatic filariasis, which have been conducted side by side with APOC, can be expected to strengthen the new development synergistically.

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Longistatin, a novel EF-hand protein from the ixodid tick *Haemaphysalis longicornis*, is required for acquisition of host blood-meals ☆

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ABSTRACT

Calcium and the EF-hand Ca^{++}-binding proteins have been undisputedly recognised as the key players in almost all aspect of cell functions, starting from the cell's birth, during mitosis to its end with apoptosis. But in a few exceptional cases the EF-hand proteins are secreted from the cells and play their crucial roles extracellularly. Here, to our knowledge for the first time, we have identified and characterised an EF-hand Ca^{++}-binding protein from the salivary glands of the ixodid tick, *Haemaphysalis longicornis*, herein called longistatin. Longistatin possesses two EF-hand domains which conserve canonical structure and bind with Ca^{++}. Both the recombinant and endogenous proteins were stained with Rutheninum red. Reverse-transcription PCR data showed that longistatin-specific transcript was expressed in all life-cycle stages of *H. longicornis* and was up-regulated only in blood-fed ticks. Organ-specific transcription analysis revealed a salivary gland-specific expression of the gene which peaked at 96–120 h of feeding when ticks acquired full blood-meals and become engorged but its expression declined sharply as they detached and dropped off the host. Consistently, endogenous protein was localised in the salivary glands of adult ticks and in the lumen of the functional acini of the salivary glands. Furthermore, longistatin was detected in feeding lesions at the site of attachment of ticks on the host. These results suggest that longistatin is synthesised in, and is secreted from, the salivary glands and may have functional roles in the feeding process of ixodid ticks.

1. Introduction

Ticks are obligate haematophagous ectoparasites and all of their motile life-cycle stages are exclusively dependent on the blood-meals from host animals. Ticks are broadly classified into three families such as Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae. *Haemaphysalis longicornis* Neumann 1901, commonly known as Bush Tick or New Zealand Cattle Tick, is a hard tick and is widely distributed in many countries from the Far East to Australia (Fujisaki et al., 1994; Hoogstraal et al., 1968). In addition to direct severe adverse effects on health and productivity of infested animals, *H. longicornis* acts as vector of many bacterial, viral, protozoan and rickettsial diseases (Hoogstraal et al., 1968). Among these diseases, babesiosis, anaplasmosis, theileriosis and Q fever have both veterinary and medical importance. These diseases are associated with human suffering, and are a major constraint to animal production as they may cause morbidity or mortality in affected animals (Fujisaki et al., 1994; Ho et al., 1995).

Control of ticks may be an effective strategy to eradicate tick-borne diseases. But tick control is a herculean task as they have a wide host range. In addition, ticks are able to spend about 95% of their life-time away from the host, especially during the period of starvation (Needham and Teel, 1991). Even in this post-genomic era, various controversial methods are being used to control ticks, including the application of chemical acaricides which are very hazardous for human and animal health as they have direct toxic effects. Due to their residual effects, the acaricides are not environmentally friendly and furthermore, repeated usages of acaricides lead to the development of resistance in ticks (Zaim and Guillet, 2002). These obstacles in controlling ticks necessitate the

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development of sustainable therapeutic interventions. Therefore, identification and characterisation of potential vaccine candidate and drug target bioactive molecules from the blood-feeding ixodid ticks are of considerable interest.

EF-hand proteins have been reported from a variety of sources such as bacteria, protozoa, helminths, arthropods and mammals including humans (Nelson and Chazin, 1998; Kawasaki et al., 1998). Calcium and the EF-hand Ca^{++}-binding proteins have been undisputedly recognised as the key players in almost all aspects of cell functions, starting from the cell's birth, through mitosis to its end with apoptosis (Berridge et al., 1998; Kahl and Means, 2003). EF-hand Ca^{++}-binding proteins modulate various biochemical reactions within the cell and in a few exceptional cases they are secreted from the cells and play their crucial roles extracellularly. For example, Osteonectin, a glycoprotein with EF-hand Ca^{++}-binding domains, is secreted from the cell and exerts versatile functions in the extracellular matrix including, a vital role as a morpho-regulatory element (Pottgiesser et al., 1994). Similarly, acetylcholinesterase, having two EF-hand domains, functions extracellularly in cell surface associations (Tsigelny et al., 2000).

Here, we have identified and cloned a full-length cDNA from the salivary glands of the ixodid tick, *H. longicornis*, which encodes a protein (longistatin) containing two functional EF-hand Ca^{++}-binding domains and is able to bind calcium. We also show that longistatin is secreted from the salivary glands and may function in the feeding process of the blood-meals from host animals by ixodid ticks. To our knowledge, longistatin is the first characterised EF-hand protein isolated from the salivary glands of ticks.

2. Materials and methods

2.1. Ticks

Parthenogenetic Okayama strains of *H. longicornis* were propagated at the Laboratory of Parasitic Diseases, National Institute of Animal Health (NIAH), Tsukuba, Japan, by feeding on the ear of specific pathogen-free (SPF) Japanese White rabbits according to the methods described previously (Alim et al., 2007) to obtain different life-cycle stages of ticks at different feeding intervals. Briefly, the ears of rabbits were cleaned by hair clipping; ticks were attached and given support with ear bags and an Elizabethan collar. Ear bags were changed at 24 h intervals. Ticks were collected after detachment following full engorgement or after the indicated period of attachment. Rabbits used in these experiments were acclimatised to the experimental laboratory conditions for 2 weeks prior to the commencement of the experiment. Animal care was conducted according to the protocols approved by the Animal Care and Use Committee, NIAH (Approval Nos. 441, 508, 578).

2.2. Cloning and sequencing of longistatin cDNA

The gene coding for longistatin was identified from the expressed sequence tags (ESTs) constructed from the salivary gland cDNA libraries of *H. longicornis* following the methods described previously (Boldbaatar et al., 2006). Briefly, the plasmid containing the gene coding for longistatin was extracted using a Qiagen DNA Purification kit (QIAGEN Science, Germantown, MA, USA). The nucleotide sequences of the cDNA were determined using the big dye terminator method on an ABI PRISM 3100 automated sequencer (Applied Biosystem, Foster City, CA, USA). The GENETYX-WIN DNA analysis software system (Software Inc.) was used to deduce the amino acid sequence of longistatin. Sequence similarity searches were performed using the BLAST programme (Altschul et al., 1997). Alignment with the previously reported similar protein sequences, available in GenBank (Benson et al., 2002), were done using CLUSTALW. The putative signal sequence was analysed using the prediction server SignalP V2.0.b2 (http://www.cbs.dtu.dk/services/SignalP) (Nielsen et al., 1997). Theoretical mol. wt and pI were determined using PeptideMass (http://us.expasy.org/tools/peptidemass.html) (Wilkins et al., 1997). Domains were searched using ExPASy-PROSITE (http://au.expasy.org/prosite/). N-linked glycosylation sites were searched using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc).

2.3. Expression of longistatin

The open reading frame (ORF) of longistatin was amplified by PCR from PBS/longistatin using a set of primers, 5′ CCG CTC GAG CGG GCA GGC CGG CGA CCA GCA G 3′ and 5′ GGA ATT CCC TAA ATT TGG TTG GTC AGG TC 3′, which contained XhoI and EcoRI restriction sites, respectively. PCR was performed for 3 min at 95 °C followed by 35 cycles of 30 s at 95 °C, for 30 s at 57 °C and 1.5 min at 72 °C with a final elongation at 72 °C for 5 min. Both the PCR product and the vector pTrcHisB (Invitrogen, Carlsbad, CA, USA) were digested by XhoI and EcoRI restriction enzymes. The purified PCR product was inserted into the XhoI and EcoRI sites of the vector pTrcHisB (Invitrogen). The resultant plasmid was transformed into competent cells of *Escherichia coli* Top10F′strain (Invitrogen) following the conventional method. The expression of longistatin in *E. coli* with a Polyhistidine-tag was performed according to the procedure described by Tsuji et al. (2001). Briefly, the transfected cells were allowed to grow in SOB medium (Tryptone 20.0 g, Yeast extract 5.0 g, Sodium chloride 0.5 g, Magnesium sulphate anhydrous 2.4 g and Potassium chloride 0.186 g per litre) containing 50 μg ampicillin/ml at 37 °C under vigorous shaking (200 rpm) until the OD of 1 at 600 nm (OD_{600}) was achieved. To induce recombinant protein expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM concentration and the culture was grown for an additional 4 h at 37 °C on a shaking incubator. The culture was then centrifuged at 10,000*g* and 4 °C for 20 min and the pellet was resuspended in 10 ml of lysis buffer (20 mM sodium phosphate and 500 mM sodium chloride, pH 7.8). Egg white lysozyme (100 μg/ml) was added to the cell suspension and incubated in ice for 15 min. The suspension was sonicated for 2 min on ice with an ultrasonic processor (VP-15S, TAITEC, Japan) followed by immediate freezing at −80 °C and then thawing at 37 °C for 15 min in each case. After three cycles of sonication, freezing and thawing, the *E. coli* lysate was centrifuged at 23,900*g* and 4 °C for 30 min and supernatant was collected. The recombinant protein was purified using ProBond™ resin (Invitrogen) under native conditions as described by the manufacturer and subsequently eluted with a stepwise gradient of imidazole (25–500 mM). The eluted recombinant protein was concentrated using Centrisart® (Sartorius, Goettingen, Germany) having a mol. wt cut-off of 10 kDa. The concentrated protein was dialysed extensively at 4 °C with successive changes of 20 mM Tris–HCl (pH 7) and a decreasing concentration of NaCl (500–250 mM) using a Slide-A-Lyser Dialysis Cassette (Pierce, Rockford, IL, USA) with a mol. wt cut-off of 10 kDa. Purified recombinant longistatin was electrophoresed on 12.5% SDS–PAGE gel under reducing conditions. The gel was treated with 50% methanol in 10% acetic acid for 10 min at room temperature. The protein was subjected to silver staining (Daiichi Pure Chemicals, Tokyo, Japan) following the manufacturer's instructions. Finally, protein concentration was determined using micro-BCA reagent (Pierce) and stored at −20 °C until further use.

2.4. Western blot analysis

Crude *E. coli* lysate was electrophoresed through 12.5% SDS–PAGE gel under reducing conditions and the proteins were trans-

ferred onto nitrocellulose membrane. The membrane was incubated with 5% skim milk for 30 min at room temperature. Polyhistidine-tagged longistatin was probed with the anti-His monoclonal antibody (1:1000) (Anti-6 X His (Mouse IgG1-K), Monoclonal (HI192) AS, Nacalai Tesque,Inc., Kyoto, Japan). The membrane was washed with Tris-buffered saline-Tween 20 (TBS-T) and incubated with alkaline phosphate conjugated goat anti-mouse IgG (H + L) (ZYMED, San Francisco, CA, USA) as a secondary antibody for 1 h. The membrane was washed again with TBS-T and the bound protein was visualised with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT, Promega, Medison, WI, USA).

2.5. N-terminal sequencing of longistatin

Purified recombinant protein was subjected to electrophoresis on a 12.5% SDS–PAGE gel under reduced conditions. After electrophoresis, the protein was electro-blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 11) containing 10% methanol. The blotted protein was stained with coomassie blue, followed by destaining with 50% methanol. The target protein band was excised from the blot and was subjected to N-terminal sequencing using the Procise 494 cLC Protein Sequencing System (Applied Biosystem) (Furuse et al., 1998).

2.6. Generation of mouse polyclonal antibody

BALB/c mice were immunized by s.c. injection of 50 μg of recombinant protein emulsified with FCA (DIFCO, Detroit, MI, USA) followed by booster immunizations 2 weeks apart using the same route. The immunized mice were sacrificed 1 week after the last booster and sera were collected and stored at −20 °C for further use.

2.7. Reverse transcription-PCR (RT-PCR) analysis

Unfed and fed adult ticks (24 h, 48 h, 72 h, 96 h and engorged (120 h)), and post-engorged ticks (day 0, day 1 and day 2) were washed with 70% ethanol and rinsed in PBS. Then ticks were dissected under a dissecting microscope (Olympus, SZ7, Japan) using ice-cool PBS and different organs were collected. Shortly after collection, the tissues were submerged in RNA*later*, a RNA Stabilization Reagent (QIAGEN). The total RNA was isolated by using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. Total RNA was also collected from all life-cycle stages (egg, larva and nymph) of *H. longicornis*. Five hundred nanograms of total RNA was used for RT before PCR. Single stranded cDNA was prepared by using a Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) following the manufacturer's instructions. A series of PCRs were carried out using 500 ng of cDNA from each sample and longistatin-specific oligonucleotides (5′ GCT ATC TCG GCT CCT GTG TC 3′ and 5′ ATC TTC GCC AGG TCC TTC TT 3′) or oligonucleotides specific for a control cDNA encoding β-actin in a final volume of 20 μl. PCR was performed for 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 54 °C and 1.5 min at 72 °C and finally elongation at 72 °C for 5 min. The PCR product was subjected to electrophoresis in a 1% agarose gel.

2.8. Immunohistochemistry

Immunohistochemistry was performed as previously described (You et al., 2001). Briefly, ticks were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and embedded in paraffin. Thin transverse sections were cut from paraffin-embedded well-fixed ticks. Sections were fixed on glass slides and deparaffinized with xylene. The sections were rehydrated with graded series of alcohol washes, followed by inactivation of endogenous peroxidase with 1% H_2O_2 in PBS containing 10% ethanol for 1 h and blocked with 10% goat serum (Wako, Osaka, Japan) in PBS. They were then incubated overnight at 4 °C with mouse anti-longistatin serum (1: 200). Sections treated with pre-immune mouse sera (1: 200) were used as a control. Slides were rinsed thoroughly with ice-cool PBS by placing those on a rotator (40 rpm). The sections were then reacted with peroxidase-labelled anti-mouse IgG secondary antibody and the substrate 3′,3′-diaminobenzidine tetrahydrochloride (Fast™ DAB set, Sigma, St. Luis, Mo, USA). After colour development, the slides were dehydrated with graded series of alcohol washes, cleared in xylene and then covered with cover slips and examined under a microscope (Leica Microsystem, Wetzlar, Germany).

2.9. Cell separation

Salivary gland cells were collected from partially fed (96 h) adult ticks by teasing the freshly obtained salivary glands through a stainless steel mesh in PBS containing complete protease inhibitors. The cells were washed in PBS to remove cellular debris and centrifuged at 4400*g* for 10 min at room temperature. Finally, 200 μl PBS were added and sediments were resuspended. A thin smear was prepared on a glass slide using 10 μl of cell-suspension. Cells were then fixed with 4% paraformaldehyde in PBS (0.1 M, pH 7.4) for 20 min at room temperature and were treated with 0.1% Triton X-100 in PBS for 20 min under the same conditions. The slides were washed with PBS and blocked with 10% goat serum (Wako) for 30 min at room temperature and then incubated overnight with mouse anti-longistatin serum (1: 100) at 4 °C. The cells were washed in PBS and reacted with green fluorescent-labelled secondary antibody (Alexa Flour® 488 goat anti-mouse IgG (H + L), Invitrogen) for 1 h at room temperature. The slides were then washed thoroughly with PBS and mounted with VECTASHIELD® mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and examined under a fluorescent microscope (Leica).

2.10. Detection of longistatin in secretory pathways of salivary glands and in host tissues

Immunofluorescent staining was performed using thin sections from partially fed (72 h) adult ticks following the methods described previously (Miyoshi et al., 2007). Sections were treated with mouse anti-longistatin sera and bound antibodies were detected using green fluorescent-labelled secondary antibody (Alexa Flour® 488 goat anti-mouse IgG (H + L), Invitrogen). Slides were mounted with VECTASHIELD® mounting medium containing DAPI (Vector Laboratories) and examined under a fluorescent microscope (Leica). We also employed immunofluorescent staining for the detection of longistatin in the feeding lesions of the host. Thin sections were prepared from tissue samples collected from the feeding lesions developed on a rabbit ear at the site of attachment of the tick, as previously described (Islam et al., 2009). Tissues were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Immunofluorescent staining was performed using mouse anti-longistatin sera as described above.

2.11. Ca^{++}-binding assays

Purified longistatin was electrophoresed using 10% SDS–PAGE gel in the presence of 3 mM $CaCl_2$ or 3 mM EDTA. BSA was used as a control. Additionally, both the endogenous and recombinant proteins were transferred onto nitrocellulose membranes and were stained with 25 mg/L Ruthenium red (Calbiochem) in 60 mM KCl,

5 mM $MgCl_2$ and 10 mM Tris–HCl (pH 7) for 15 min at room temperature.

3. Results

3.1. Molecular characterisation of longistatin

Sequence analysis revealed that the full-length longistatin cDNA consisted of 750 bases which included the complete coding region together with 5′ and 3′ non-coding regions. The in-frame start codon is located at nucleotides 133–135 and the stop codon is at 601–603. The ORF consisting of 471 nucleotides, extends from residues 133–603 which codes for a protein of 156 amino acid (aa) residues having a calculated molecular mass of 17,788 Da and a pI of 4.84. From the analysis of the N-terminus of the polypeptide with the SignalP programme (http://www.cbs.dtu.dk/services/SignalP), it was determined that the molecule has a signal peptide and is predicted to be cleaved at Ala^{21}–Gln^{22}. The mature protein has a predicted molecular mass of 15,541 Da with theoretical pI of 4.59. No potential site for N-glycosylation was predicted in the putative polypeptide encoded by the longistatin cDNA. Longistatin consists of two EF-hand Ca^{++}-binding domains (http://au.expasy.org/prosite/) at residues 83–94 and 135–146 (Fig. 1A). Alignment of longistatin EF-hand domains-I and -II (LongEFd-I and -II) with the EF-hand domains from diversified sources revealed that LongEFd-I and -II has conserved canonical structures. The loop region of each domain consists of 12 aa residues and is arranged in the pattern of X · Y · Z · −Y · −X · −Z, where X, Y, Z, −X, −Y and −Z are the Ca^{++} coordinating ligands and the dot represents the intervening residues. In the case of both domains, LongEFd-I and -II, *X* and −Z positions are occupied by aspartic acid and glutamic acid, respectively (Fig. 1B). Despite a few radical departures from the general liganding rule, the loop region of the most common canonical EF-hands usually consists of 12 aa residues which starts with aspartic acid and ends with glutamic acid (Kretsinger and Nockolds, 1973). These negatively charged aas play important roles in the binding of positively charged Ca^{++}. In conjunction, the bidentate glutamic acid at the −Z position is indispensible for the formation of pentagonal bipyramid coordination geometry (Grabarek, 2006). Furthermore, LongEFd-I and LongEFd-II contain several bulky hydrophobic residues (Fig. 1B), and are predicted to form a hydrophobic core, which is a salient characteristic of helix-loop-helix proteins (Gariépy and Hodges, 1983). The aa sequence of LongEFd-II represents the conserved glycine residue at position 6 in the loop, which plays an important role in the proper folding of ligands around the metal ion (Gariépy et al., 1983). In the LongEFd-I, the glycine residue has been substituted by asparagine. The substitution of glycine at position 6 with other aa residues is not unexpected and has been detected in some well-recognised Ca^{++}-binding proteins like, carp parvalbumin, rabbit parvalbumin, S-100a, S-100b, porcine intestinal Ca^{++}-binding protein (PICBP), bovine intestinal Ca^{++}-binding protein (BICBP) and rabbit skeletal muscle alkali light chain (Gariépy and Hodges, 1983). Results of BLAST (NCBI, National Institute of Animal Health, http://www.ncbi.nlm.nih.gov/BLAST) searches revealed that longistatin has the highest identity with multiple coagulation factor deficiency 2-like protein of *Ixodes scapularis* (51% identity).

3.2. Production of recombinant longistatin

To determine the biochemical properties, longistatin was expressed in *E. coli*, strain TOP10F′, using a pTrcHisB™ vector and was harvested as soluble protein from bacterial cultures. Translation of recombinant longistatin was detected with anti-His monoclonal antibody (Nacalai Tesque, Inc.) (Fig. 2B). One litre of bacterial culture, on average, yielded 3.15 mg purified longistatin. The purity of recombinant longistatin was confirmed by SDS–PAGE analysis followed by silver staining, and a single band was detected (Fig. 2A). The calculated molecular mass of longistatin is 15.54 kDa (excluding signal peptide). Longistatin migrated on SDS–PAGE gels as a 24 kDa fusion protein with a polyhistidine tag (Fig. 2A and B). This unexpected mobility of prokariotically expressed longistatin to 24 kDa instead of its calculated molecular mass of 15.54 kDa on SDS–PAGE analysis was not due to N-linked glycosylation, as longistatin does not contain any potential N-glycosylation sites. However, this type of mobility of the recombinant protein over the expected size has been observed in other cases. Thrombostasin, a thrombin inhibitor isolated from horn fly saliva, showed a significantly higher molecular mass (16.7 kDa) on SDS–PAGE instead of its calculated molecular mass of 9.2 kDa, which was predicted to be due to its acidic characteristics (Zhang et al., 2002). Longistatin is also acidic in nature (pI 4.59) and it is assumed that the higher molecular mass band of longistatin on SDS–PAGE is due to its acidic characteristics. Sequence analysis of the purified recombinant longistatin (24 kDa protein) was performed. The representative first 10 aa residues were QAGDQQQMQP which exactly corresponds to the 22–31 aa residues of longistatin (GenBank Accession No. AB519820), confirming that the 24 kDa protein is true longistatin.

3.3. Transcription profiling of longistatin

For transcription profiling of longistatin, total RNA was extracted from the unfed and fed different life-cycle stages and organs of ticks and was subjected to RT-PCR analysis. Longistatin-specific transcripts were expressed at a level which corresponded well to its expected size (246 bp). The transcript was detected in all life-cycle stages including eggs but its expression was highly up-regulated after feeding (Fig. 3A). Furthermore, the gene was specifically expressed in salivary glands and completely absent in other organs (Fig. 3B), suggesting its salivary gland specificity. RT-PCR data also revealed that the gene was dramatically up-regulated with the feeding process of ticks. The highest expression was detected at 96 h of feeding and in fully engorged (120 h) adult ticks (Fig. 3C). Expression of the longistatin-specific gene decreased abruptly after detachment from the host following full engorgement (Fig. 3D), implying that longistatin may have some potential roles in the blood-feeding process of hard ticks.

3.4. Detection of endogenous longistatin in salivary glands and in the blood pool in hosts' tissues

To detect the endogenous form of longistatin, both immunohistochemistry and immunofluorescent staining were performed using thin sections of partially fed (72 h) adult *H. longicornis*. Immunohistochemical examination showed that the endogenous longistatin, which reacted with mouse anti-longistatin sera, was localised only in the salivary glands, further indicating its salivary gland-specific expression. No positive reaction was detected in either the internal or external tissues of ticks treated with pre-immune mouse sera (Fig. 4A). Using immunofluorescent staining, longistatin was detected within the cells as well as in the lumen of the functional acini of salivary glands (Fig. 4A, arrow), suggesting that longistatin is synthesised and secreted by the salivary cells.

To determine the intracellular localisation of the endogenous protein, salivary gland cells from partially fed (96 h) adult ticks were isolated and were subjected to immunofluorescent staining. Cells treated with mouse anti-longistatin sera showed intense reactivity with the entire cytoplasm, however, the longistatin-specific reaction was slightly more intense near the periphery of the

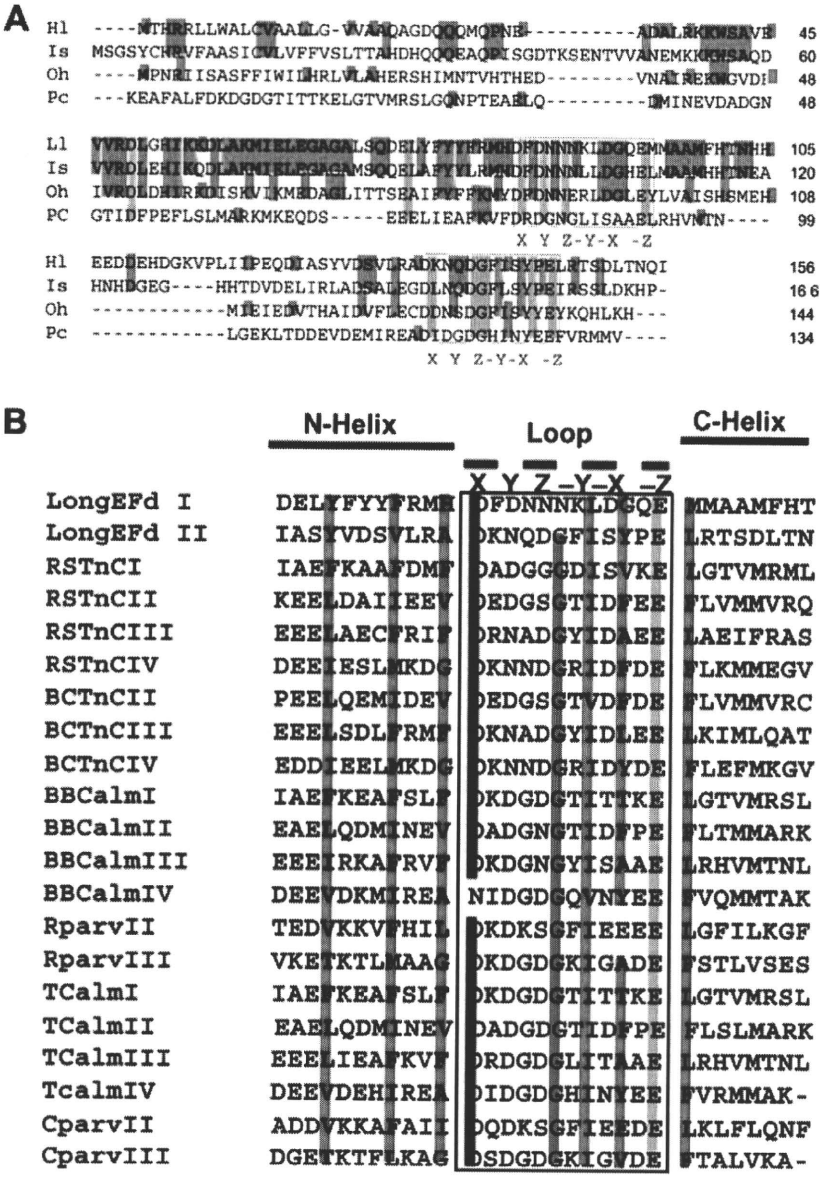

Fig. 1. Sequence alignment of longistatin. (A) Comparison of the deduced amino acid (aa) sequence of longistatin (Hl) with the aa sequences of homologous molecules. Sequences were selected from (GenBank Accession Nos. are indicated in parentheses): Is, *Ixodes scapularis* (AAY66924), Oh, *Ornithoctonus huwena* (ACH48184) and Pc, *Paramacium* calmodulin (Guy et al., 2008). Loop regions of EF-hand domains are boxed. Amino acid residues in yellow are conserved in all sequences and those in green, are identical to longistatin only. *X*, *Y*, *Z*, *–Y*, *–X* and *–Z* represent calcium coordinating positions of the loop. (B) Alignment of longistatin EF-hand domains-I and -II with EF-hand domains of proteins with known structures such as rabbit skeletal troponin C (RSTnC I, II, III and IV), bovine cardiac troponin C (BCTnC II, III and IV), bovine brain calmodulin (BBCalm I, II, III and IV), rabbit parvalbumin (Rparv II and III), *Tetrahymena* calmodulin (TCalm I, II, III and IV) and carp parvalbumin (Cparv II and III) (Gariépy and Hodges, 1983). Yellow denotes the omnipresence of glutamic acid in *–Z* position and dark red indicates commonly occurring aspartic acid in the *X* position of canonical EF-hands. Amino acid residues in green are involved in the formation of the hydrophobic core of the EF-hand domain. Violet indicates the frequently occurring glycine residue at position 6 of the loop region of standard EF-hands.

cell (Fig. 4A). These results suggest that endogenous longistatin is distributed throughout the cytoplasm of the salivary gland cells.

To ensure the presence of longistatin in the feeding lesions, thin histological sections prepared from rabbit ear tissues containing blood pools following a successful blood-feeding by adult ticks were also subjected to immunofluorescent staining. Intense longistatin-specific reactions were detected by using mouse anti-longistatin sera (1:100) in the feeding lesions (Fig. 4Bc), especially in areas where massive haemorrhages were observed by H&E staining (Fig. 4Bb), implying that longistatin is secreted and is injected through saliva into the blood pools.

3.5. Ca^{++}-binding affinity of longistatin

To verify the Ca^{++}-binding affinity of longistatin, a gel mobility shift assay was performed using 10% SDS–PAGE gel under reducing condition in the presence of 3 mM Ca^{++} or 3 mM EDTA. Data showed that migration of longistatin was relatively faster in the presence of 3 mM Ca^{++} than in the presence of a metal chelator, EDTA, under the same conditions, suggesting that longistatin is capable of binding with Ca^{++}. Longistatin appeared at the level of 21 kDa on SDS–PAGE containing 3 mM Ca^{++}. But the longistatin-specific band was observed at the level of about 24 kDa in the presence of 3 mM EDTA while the control protein BSA appeared at the

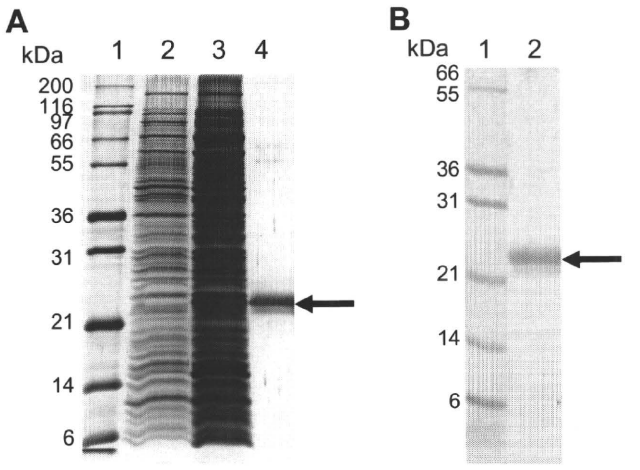

Fig. 2. Detection and purification of recombinant longistatin. (A) Detection of purity of longistatin by SDS–PAGE analysis. Purified longistatin together with *Escherichia coli* lysate was electrophoresed on 12.5% SDS–PAGE under reducing conditions. The gel was stained with silver stain. Lane 1, molecular weight marker; lane 2, *E. coli* lysate before induction; lane 3, *E. coli* lysate 3 h after induction with isopropyl-β-D-thiogalactopyranoside (IPTG) and lane 4, purified longistatin. (B) Western blot analysis to detect the His-tagged recombinant longistatin using anti-His monoclonal antibody. Crude *E. coli* lysate was electrophoresed on 12.5% SDS–PAGE gel under reducing conditions and proteins were transferred onto nitrocellulose membrane. The membrane was probed with anti-His monoclonal antibody as described in the Materials and methods section. Lane 1, molecular weight marker and lane 2, longistatin.

same level in both of the gels (Fig. 5A). We hypothesised that the binding of Ca^{++} with longistatin induces some significant conformational changes rendering the molecule to migrate rapidly during electrophoresis. A similar pattern of electrophoretic mobility was observed in several other well-studied Ca^{++}-binding proteins such as Calmodulin, Calcineurin B, Troponin C (Klee et al., 1979). For further confirmation of Ca^{++}-binding affinity, both endogenous and recombinant proteins were electrophoretically transferred onto nitrocellulose membranes and stained with Ruthenium red (Fig. 5B), which is a specific stain for Ca^{++}-binding proteins. Ruthenium red inhibits the binding of Ca^{++} with the Ca^{++}-binding proteins and specifically stains them. The interaction between Ruthenium red and Ca^{++}-binding sites is extremely specific and sensitive (Charuk et al., 1990). It is therefore widely used for the detection of Ca^{++}-binding proteins and in this case suggests the specific interaction with the Ca++ binding sites of longistatin.

4. Discussion

Tick saliva contains a wide array of pharmacologically active bio-molecules having diversified functions which help ticks to feed persistently (Nuttall et al., 2006; Islam et al., 2009), modify host defense mechanisms (Brossard and Wikel, 2004), inhibit angiogenesis (Francischetti et al., 2005; Islam et al., 2009) or modulate coagulation cascades by altering one or more of the steps of coagulation pathways (Maritz-Olivier et al., 2007). Here, we describe that longistatin, a novel EF-hand protein isolated from the salivary glands of *H. longicornis*, binds with calcium and is thought to be closely linked to the feeding process of ticks.

Sequence alignment reveals that both of the Ca^{++}-binding sites of longistatin conserve the general rules of arrangement of ligands of standard EF-hand domains with the most noteworthy, at the Ca^{++}-binding loop region at positions 8 and 10, occupied by hydrophobic aas. This ligand pattern is identical to the classical arrangement of aas in the historical consensus sequence of EF-hand first described by Kretsinger and Nockolds (1973), which is also seen in the Ca^{++}-binding sites of other well-known standard EF-hand proteins such as bovine brain calmodulin (Watterson et al., 1980), *Tetrahymena* calmodulin (Yazawa et al., 1981) and in rabbit skeletal troponin C (van Eerd and Takahashi, 1975). In the LongEFd-I, positions 8 and 10 of the loop contain leucine and glycine, respectively, but in the LongEFd-II leucine has been replaced with isoleucine, and glycine with tyrosine. Although position 8 and to a lesser extent position 10 of the EF-hand loop region are conserved hydrophobic sites, but more precisely, position 8 is most frequently occupied by the residues isoleucine, leucine or valine (Grabarek, 2006). These two well-conserved sites of the loop region, together with the C- and N-terminal hydrophobic surface, are involved in the metal dehydration process (Gariépy and Hodges, 1983).

Localisation studies strongly support the salivary gland-specific expression of longistatin. It is suggested that longistatin is

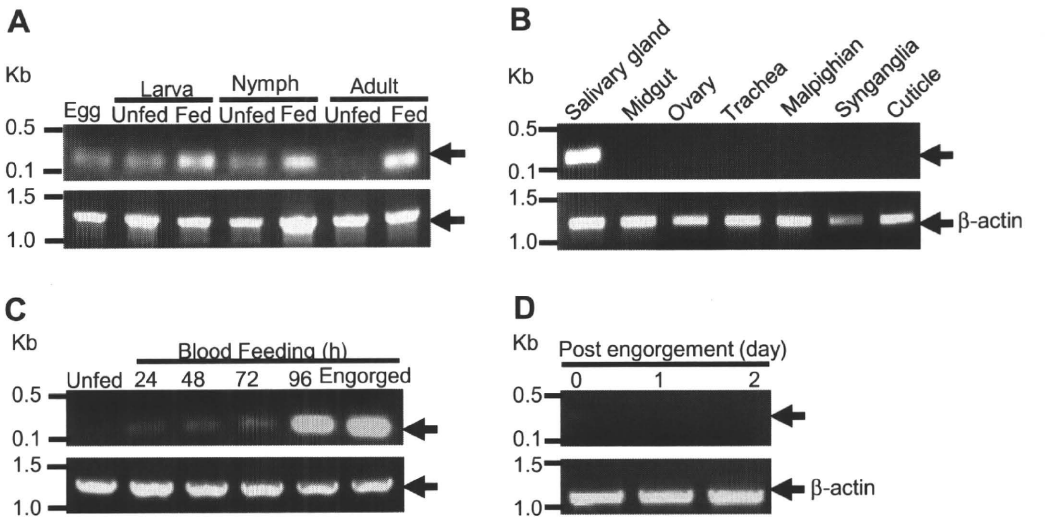

Fig. 3. Transcription profiling of longistatin in *Haemaphysalis longicornis*. (A) Expression of the longistatin-specific gene in all life-cycle stages of *H. longicornis* (arrow). Reverse transcription-PCR (RT-PCR) was performed using total mRNA extracted from all developmental stages, including eggs. (B) Organ-specific expression of longistatin gene (arrow). Total mRNA was collected from various organs of partially fed (72 h) adult ticks and subjected to RT-PCR analysis. (C) Expression profile of the gene in salivary glands of adult ticks at different blood-feeding stages (arrow). RT-PCR analysis was performed using total mRNA collected from salivary glands of adult *H. longicornis* at different blood-feeding stages. (D) Expression of the longistatin-specific gene in the post-engorgement period (arrow). Total mRNA was collected from salivary glands of adult ticks in the post-engorgement period (day 0, day 1 and day 2) and was subjected to RT-PCR analysis. Actin is shown as an internal control.

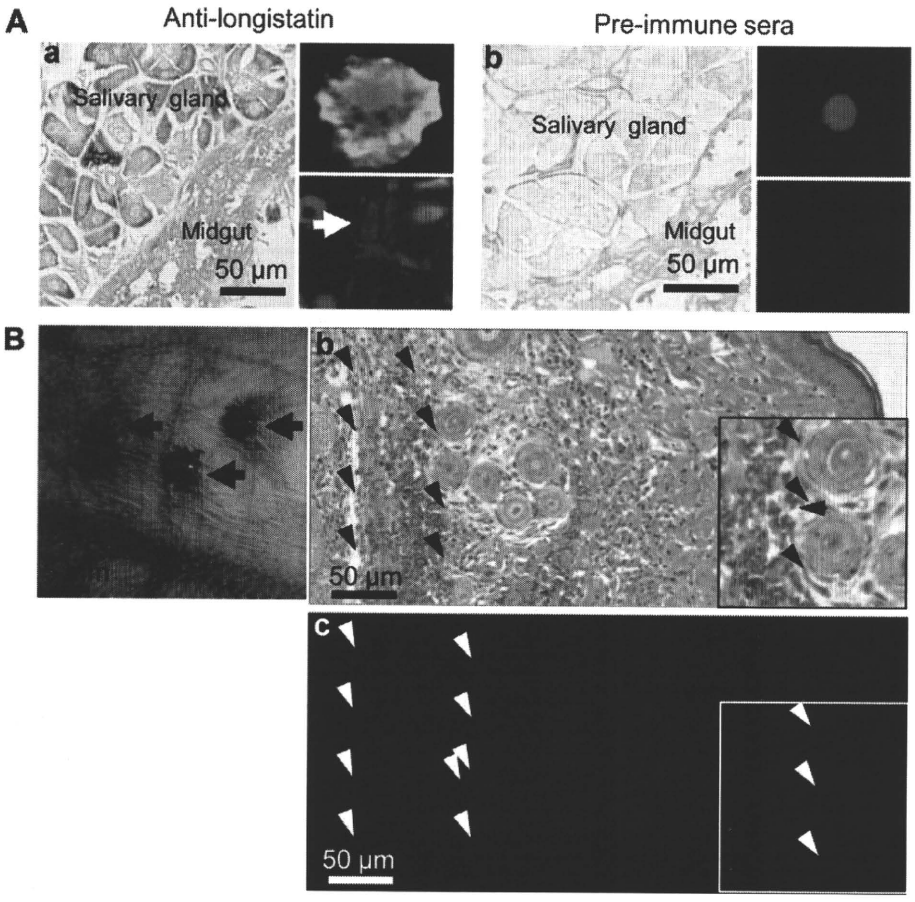

Fig. 4. Endogenous longistatin detected in the tick, *Haemaphysalis Longicornis*, and in host tissues. (A) Endogenous localisation of longistatin in partially fed (72 h) adult ticks. Ticks were fixed in paraformaldehyde and embedded in paraffin. Sections were treated with either immune (1: 100, Aa) or pre-immune mouse serum (1: 100, Ab). The longistatin-specific reaction was detected in the salivary glands and in the lumen of the functional acini (arrow). Salivary gland cells were collected from partially fed (96 h) adult ticks and reacted with mouse anti-longistatin (1: 100, Aa)/pre-immune sera (1: 100, Ab) followed by the treatment with green fluorescent-labelled secondary antibody (Alexa Flour® 488 goat anti-mouse IgG (H + L), Invitrogen). (B) Longistatin detected in the feeding lesions in vivo. A rabbit's ear showing blood pools (arrows) at the site of attachment of ticks (Ba). The area of haemorrhage in the blood pool (arrowheads), H&E staining (Bb, 10× objective). Detection of longistatin in the feeding lesions of a rabbit's ear (arrowheads; Bc, 10× objective). Rabbit's ear tissues were collected from a euthanised rabbit as described in the Materials and methods section and were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were treated with mouse anti-longistatin sera (1:100) followed by the treatment with green fluorescent-labelled secondary antibody (Alexa Flour® 488 goat anti-mouse IgG (H + L), Invitrogen). The area marked by the square is at 40× objective.

synthesised and accumulated in the cytoplasm, finally secreted out of the salivary gland cells and takes part in the feeding process after reaching the blood pools, the feeding lesions on the host. Usually EF-hand Ca^{++}-binding domains are prevalent among the cytoplasmic proteins (Tsigelny et al., 2000) and act as a calcium buffer in the cytosol of the cell (Lewit-Bentley and Réty, 2000) engaging in various biological functions such as ciliary beating, muscle contraction, membrane excitability, cell proliferation and cell death through signalling of multiple physiobiochemical pathways (Skelton et al., 1994; Berridge et al., 1998). In some rare cases EF-hand Ca^{++}-binding proteins are secreted from the cells and exert their function extracellularly (Pottgiesser et al., 1994; Tsigelny et al., 2000). RT–PCR data also demonstrated that the gene coding for longistatin is expressed in all life-cycle stages, including eggs. Notably, the longistatin-specific gene is dramatically up-regulated in pre-engorged (96 h) and engorged (120 h) adult ticks but declines sharply in the post-feeding period, suggesting its vital roles in the blood-feeding processes of ixodid ticks. Feeding patterns of ixodid ticks are not like the vessel feeder haematophagous insects that suck blood directly and rapidly from the blood vessel. Ixodid ticks usually take blood over a long period of time extending from 4 to 10 days depending on the species (Nuttall, 1998), making a large blood pool under the skin. During the process of piercing the skin of mammalian hosts, ticks tear the dermis, lacerate the surrounding tissues and disrupt the continuity of the smaller blood vessels with their specialised mouth parts. Thus, they produce a blood pool under the skin from which they suck blood and fluid that are drained into the resulting wound (Maritz-Olivier et al., 2007). Tick-feeding lesions on a non-immune host are characterised by cavities filled with unclotted blood (Binnington and Kemp, 1980). While feeding, ixodid ticks inject large amounts of fluid into the host at the site of attachment which is a mixture of salivary cocktail and the regurgitated fluid component of the ingested blood. In ixodid ticks, the total blood-feeding period is distinctly divided into two phases such as the slow feeding period (up to 3 days post-attachment) and the rapid feeding period (further 2–3 days). During the slow feeding period, the size of the blood pool is relatively smaller, contains scanty blood and exudates and the tick imbibes very little blood. On the contrary, during the rapid feeding period, the blood pool becomes voluminous, contains a considerable amount of blood and exudates and the tick takes the blood-meal very rapidly (Kemp et al., 1982). Therefore, the high level of expression of the longistatin-specific gene in the pre-engorged (96 h) and engorged ticks together with the sharp declination of expression after detachment of ticks following full engorgement, clearly indicates that longistatin modulates the feeding process of host blood.

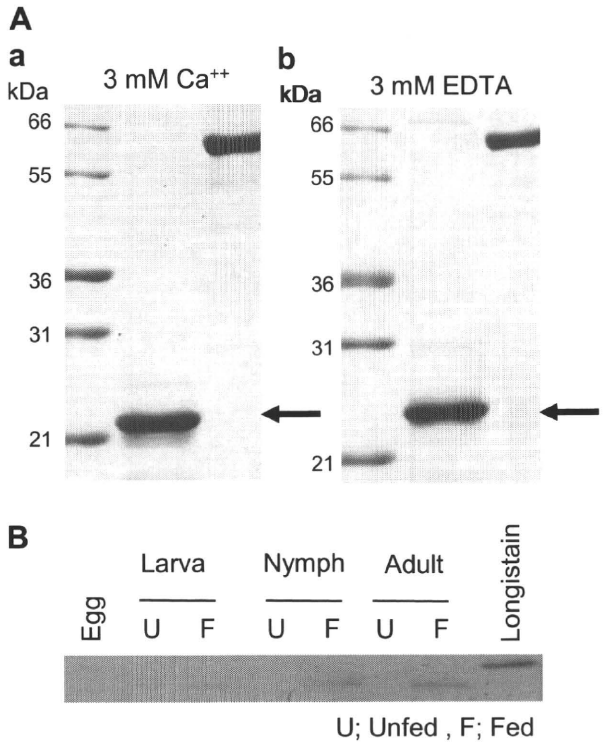

Fig. 5. Ca^{++}-binding specificity of longistatin. (A) Gel mobility shift assay. Longistatin was electrophoresed in the presence of 3 mM Ca^{++} (Aa) or 3 mM EDTA (Ab) through a 10% SDS–PAGE gel. Lane 1, molecular weight marker; lane 2, longistatin (arrow); lane 3, BSA (control). Longistatin migrated rapidly in the presence of Ca^{++}. (B) Ruthenium red staining of longistatin. Purified recombinant and endogenous forms of longistatin at different life-cycle stages were subjected to SDS–PAGE analysis followed by blotting onto nitrocellulose membranes. Nitrocellulose membranes were then stained with Ruthenium red for 15 min at room temperature. U, unfed; F, fed.

In conclusion, longistatin, an EF-hand protein, is synthesised in the salivary glands of *H. longicornis*. Our data show that longistatin is secreted with the saliva into the blood pool of hosts' tissues and is able to bind with calcium. Furthermore, our transcriptional data strongly suggest that longistatin contributes to the feeding success of blood-meals from host animals by ixodid ticks.

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Detection of Early and Single Infections of *Schistosoma japonicum* in the Intermediate Host Snail, *Oncomelania hupensis*, by PCR and Loop-Mediated Isothermal Amplification (LAMP) Assay

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Abstract. Polymerase chain reaction (PCR) with the specific primer set amplifying 28S ribosomal DNA (rDNA) of *Schistosoma japonicum* was able to detect genomic DNA of *S. japonicum*, but not *S. mansoni*, at 100 fg. This procedure enabled us to detect the DNA from a single miracidium and a snail infected with one miracidium at just 1 day after infection. We compared these results with those from loop-mediated isothermal amplification (LAMP) targeting 28S rDNA and found similar results. The LAMP could amplify the specific DNA from a group of 100 normal snails mixed with one infected snail A PCR screening of infected snails from endemic regions in Anhui Province revealed schistosomal DNA even in snails found negative by microscopy. PCR and LAMP show promise for monitoring the early infection rate in snails, and they may be useful for predicting the risk of infection in the endemic places.

INTRODUCTION

Schistosomiasis japonica is a relatively neglected tropical disease, and it is a chronic zoonotic parasitic disease in China, the Philippines, and small pockets of Indonesia.[1] In China, the causative organism, *Schistosoma japonicum*, and its intermediate snail host, *Oncomelania hupensis*, are distributed along the Yangtze River valley and recently, in the hilly and mountainous regions of Sichuan Province.[2] Since the mid-1950s, the People's Republic of China has markedly decreased the prevalence of schistosomiasis through mass-chemotherapeutic treatment and the control of the intermediate snails.[3,4] However, a complete eradication of this disease is difficult in endemic areas. The estimated prevalence in the provinces of Hunan, Hubei, Jiangxi, Anhui, Yunnan, Sichuan, and Jiangsu was 4.2%, 3.8%, 3.1%, 2.2%, 1.7%, 0.9%, and 0.3%, respectively, in 2004.[5] A total of 564, 207, 83, and 57 acute cases of *S. japonicum* infection were reported nationwide in 2005, 2006, 2007, and 2008, respectively.[6] These findings suggest that control measures must be improved among at-risk populations, especially in lake and marshland regions. A new integrated strategy was tested for the control of schistosomiasis in China.[7,8] It involved the reduction of infectious sources by the replacement of water buffaloes with tractors for agricultural work, improved access to clean water and general sanitation, better livestock management through fencing to isolate schistosomal egg sites, and better feces management using newly constructed latrines on-shore. These strategies markedly reduced the infection rate in both humans and intermediate snails in the pilot areas. Remarkably, the prevalence of infected snails reportedly decreased to almost 0% in some areas.[8] To maintain these successes, it may be useful to use new snail-monitoring systems in such areas.

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Molecular tools such as conventional polymerase chain reaction (PCR) and improved DNA amplification methods have been shown capable of detecting schistosome DNA in a variety of samples. A highly repetitive, 121-base pair (bp) sequence has been used to detect DNA from *S. mansoni* and *S. haematobium* in stool, serum, urine, and plankton samples.[9–13] Because no similar repetitive sequence has been found in the *S. japonicum* genome, the repetitive non-long terminal repeat (LTR) retrotransposon SjR2[14] was used for DNA detection as a target sequence.[15] In an experimental rabbit model, the SjR2 sequence was detected in serum (1 week after infection) and stool samples using a PCR assay, and the 230-bp band of SjR2 was absent at 10 weeks after treatment with praziquantel,[16] and real-time PCR was applied to the detection of SjR2 gene from cercaria in an environmental water sample.[17] Alternatively, real-time PCR was also applied to the detection of a mitochondrial nicotinamide adenine dinucleotide (NADH) dehydrogenase I gene at low intensity in an infected pig model.[18] Another highly repeated sequence, 28S ribosomal DNA (rDNA), was used for multiplex PCR to detect a distinct *Schistosoma* sp. from human urine samples.[19]

Loop-mediated isothermal amplification (LAMP) is a simple, sensitive, and rapid DNA detection method.[20] The LAMP reaction requires only a single enzyme, *Bst* DNA polymerase, that can synthesize a new strand of DNA while simultaneously displacing the former complementary strand, thereby enabling DNA amplification at a single temperature. The LAMP reaction can be achieved using four primers (FIP, BIP, F3, and B3), two of which (F3 and B3) contribute to the formation of a stem-loop structure, whereas the other two (FIP and BIP), designed complementary to the inner sequence of the stem-loop structure, are used for amplification of the target sequence. This provides a higher specificity to the reaction than conventional PCR methods.[20] The LAMP assay has been widely applied for diagnosis and detection against several infectious diseases, including *Plasmodium*,[21] *Trypanosoma*,[22] *Leishmania*,[23] and *Taenia*.[24] In the present application, LAMP targeting to SjR2 for detecting the DNA from *S. japonicum* was also reported.[25]

In the present study, we evaluated the performance of the PCR method by comparing SjR2 and 28S rDNA from

S. japonicum. Next, we detected the schistosomal DNA from experimentally infected snails at 1 day after infection and detected schistosomal DNA from wild snails collected from endemic areas of Anhui Province in China. We also applied a LAMP assay to detect infected snails on-site in endemic local areas. Finally, we developed a simple, rapid, and safe screening method for determining the infection rate of snails in endemic areas after implementation of the above-described integrated strategy and detected infections using the LAMP assay with DNA extracted from a large number of snails.

MATERIALS AND METHODS

Parasites and snails. *S. japonicum* was maintained using ICR mice as a final host and *O. hupensis nosophora* from a non-endemic area (Yamanashi strain) as an intermediate host. The livers from infected mice were digested with 1 mg/mL collagenase and 0.5 mg/mL actinase, and then, purified eggs were put into water to hatch the miracidia. The collected miracidia were experimentally infected to each snail in a 96-well plate. Wild snails from endemic areas in China (*O. hupensis hupensis*) were collected from three places in Anhui Province as follows: (1) Shankou-city (30.52° N, 116.93° E) in marshland regions of Anquine county, (2) Shun'an town (30.56° N, 117.54° E) in the sand regions of the Yangtze River in Tongling county, and (3) Guanghui City (30.56° N, 117.45° E) in the marshland regions of the Yangtze River in Tongling county. Figure 1 presents detailed locations about each area. The snails were picked up in Anquine in March 2007 and in Tongling in September 2007. The collected snails were crushed and checked for infection under microscopy before preparation for DNA extraction.

DNA extraction. To detect schistosomal DNA by PCR and LAMP assay, we applied the DNA extraction method using heated NaOH.[26] Briefly, the counted miracidium was put into a 200-μL volume of 50 mM NaOH and heated at 95°C for 30 minutes. After centrifugation, the 50-μL supernatant was recovered and then mixed to an equal volume of 1 M Tris-HCl (pH 8.0). This solution was directly used as a template (1 μL) for the PCR and LAMP methods. For direct extraction from a single infected snail (non-endemic area), each snail was also put into a distinct tube, and 200 μL of 50 mM NaOH solution was added to the tube. After crushing the snail with tweezers, the DNA was extracted using the above procedures. A large-scale DNA extraction from different numbers (100, 50, 25, 10, 5, and 1) of snails from non-endemic area was also performed with 10 mL of 50 mM NaOH in a 50-mL tube that was heated at 95°C for 60 minutes. After neutralization with 1 M Tris-HCL (pH 8.0), 1 μL of the solutions was directly used as a template. Genomic DNA of *S. japonicum* was purified from adult worms using the Get-pure DNA Kit (Dojindo, Kumamoto, Japan), and the concentration of DNA was measured with a spectrometer.

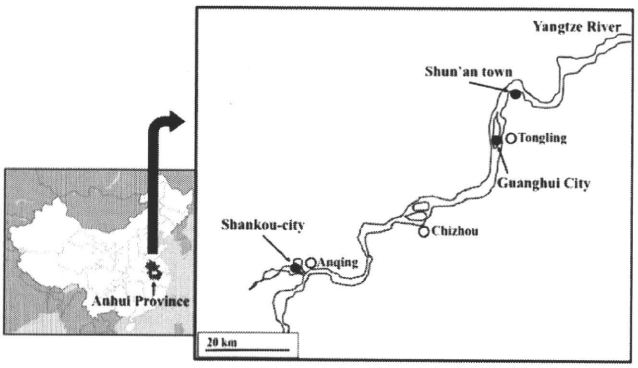

FIGURE 1. Schema of the selective areas for snail sampling in Anhui Province of China. Three points located along the Yangtze River in Anhui Province are shown as closed circles, and the capital of each country is shown as an open circle. Shankou-city in Anquine county and Guanghui City in Tongling county were marshland regions, and Shun'an town in Tongling county was in the sand regions.

Primer sets. To amplify the specific DNA of *S. japonicum*, the 28S rDNA gene (GenBank Accession No. Z46504) was selected as a target sequence. For the conventional PCR and LAMP methods, we designed specific primer sets (Table 1). As in the previous report, SjR2 (GenBank Accession No. AF412221) primers were generated for conventional PCR[16] and the LAMP assay[25] (Table 1). The LAMP primer sets were prepared to be high performance liquid chromatography (HPLC) purification grade.

PCR and LAMP assay. The PCR solution (20 μL) was prepared with a standard procedure using Top polymerase (BIONEER, Daejeon, Korea). The reaction consisted of 35 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The PCR products were resolved by agarose gel electrophoresis and stained in ethidium bromide. The LAMP method was performed according to the manufacturer's instructions (Eiken Sci, Tokyo, Japan), except for use of the 20-μL total reaction mixture. The LAMP reaction was performed at a constant 65°C. The amplification of the target gene was confirmed based on the turbidity of magnesium pyrophosphate and by gel electrophoresis.

RESULTS

Sensitivity and specificity of PCR and LAMP assay. To determine the sensitivity of the PCR and LAMP methods, we performed the reactions using *S. japonicum* genomic DNA from 10 pg to 10 fg, respectively, by serial dilution. As shown in Figure 2, PCR using specific primers amplified the band of 405 bp from 28S rDNA, and the PCR method was able to detect more than 100 fg of genomic DNA (Figure 2A). The LAMP assay had the same level of sensitivity as the conventional PCR assay (Figure 2B). Furthermore, both methods amplified only DNA from *S. japonicum* and none from *S. mansoni*. Thus, our methods distinguished the *S. japonicum* species from others. However, PCR using SjR2 primers detected DNA at the level of 1 pg (Figure 2A), whereas LAMP did not detect the SjR2 gene at all, contrary to a recent report[25] (data not shown). Taken together with these results, we performed the following experiments using 28S rDNA primers as the appropriate targeting genes because of higher sensitivity.

Detection of the schistosomal DNA from miracidia and infected snails. To confirm whether a single miracidium DNA could be detected by the PCR and LAMP assay using 28S rDNA primers, we extracted DNA using the heated NaOH method from one miracidium and performed both methods with 10 independent samples. The PCR and LAMP detected the DNA from one miracidium in all samples (Figure 3A and B), indicating that the total DNA included in a single miracidium was enough to be amplified by both the PCR and LAMP methods. Furthermore, we performed the infection experiment with the intermediate snail with a different number

TABLE 1
Specific primer sets used in this study

PCR	Sj28S
	Forward primer; 5′-GGTTTGACTATTATTGTTGAGC-3′
	Reverse primer; 5′-TCTCACCTTAGTTCGGACTGA-3′
	SjR2[16]
	Forward primer; 5′-TCTAATGCTATTGGTTTGAGT-3′
	Reverse primer; 5′-TTCCTTATTTTCACAAGGTGA-3′
LAMP	Sj28S
	F3 primer; 5′-GCTTTGTCCTTCGGGCATTA-3′
	B3 primer; 5′-GGTTTCGTAACGCCCAATGA-3′
	FIP primer; 5′-ACGCAACTGCCAACGTGACATACTGGTCGGCTTGTTACTAGC-3′
	BIP primer; 5′-TGGTAGACGATCCACCTGACCCCTCGCGCACATGTTAAACTC-3′
	SjR2[25]
	F3 primer; 5′-GCCGGTTCCTTATTTTCACAAGG-3′
	B3 primer; 5′-CTAACATAATTTTATCGCCTTGCG-3′
	FIP primer; 5′-CTACGACTCTAGAATCCCGCTCCGCGAATGACTGTGCTTGGATC-3′
	BIP primer; 5′-CCTACTTGATATAACGTTCGAACGTATTGGTTTGAGTTCACGAAACGT-3′

of miracidia and extracted total DNA from each snail at 1 day after the infection. As a result, we found four positive samples out of a total of five samples infected with one miracidium, although all samples were positive in the five samples infected with 5 or 10 miracidia, respectively (Figure 3C). We considered that one negative snail was not penetrated by a miracidium, because not all miracidia could enter the snail. These results showed that the PCR detected the schistosome-specific band

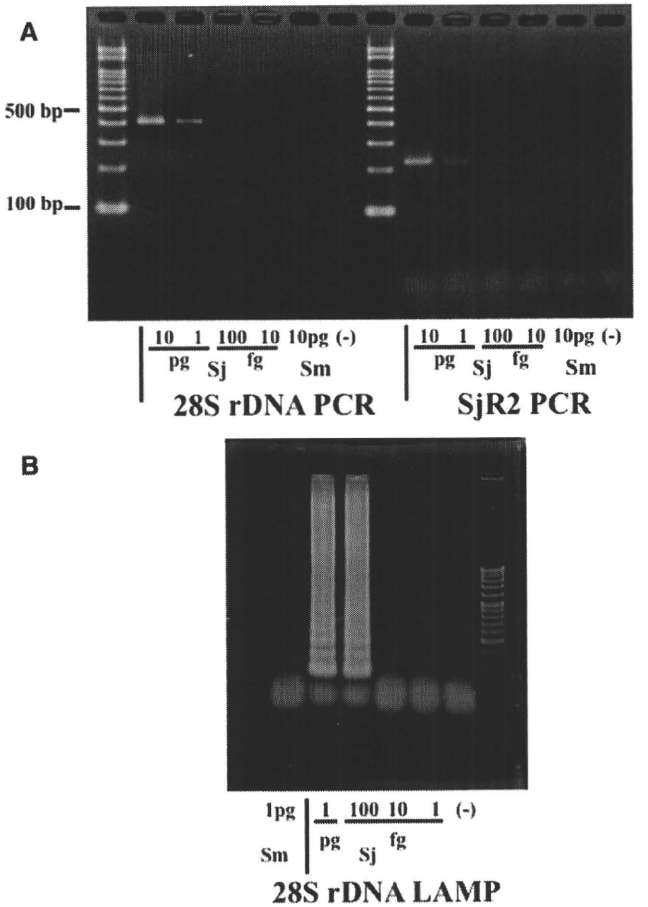

FIGURE 2. Sensitivity of the PCR and LAMP methods using genomic schistosomal DNA comparing 28S rDNA with SjR2 primers. (**A**) PCR was performed with different weights of genomic DNA, and the 28S rDNA primer set was able to detect 100 fg of DNA from *S. japonicum* but none from *S. mansoni*; the SjR2 primer set was able to detect just 1 pg of DNA. (**B**) The LAMP assay method showed the same sensitivity (100 fg) as the PCR method. Neither method reacted to DNA from *S. mansoni*, and no template (-) was the negative control.

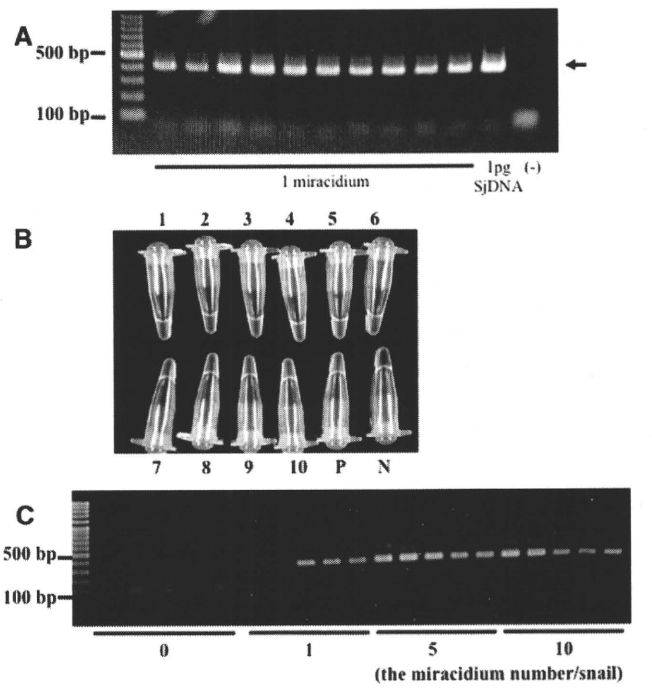

FIGURE 3. Detection of a schistosome-specific band in genomic DNA extracted from naked miracidia and the experimentally infected snail by PCR and LAMP. (**A**) The DNA extracted from one miracidium was amplified by PCR. PCR detected the specific band (arrow) in each of 10 samples extracted distinctly from one miracidium but not the no-template sample (-). Genomic DNA (1 pg) of *S. japonicum* was used for the positive control. (**B**) The DNA extracted from one miracidium was amplified by LAMP assay. LAMP showed the positive results as the white turbidity of magnesium pyrophosphate in all 10 samples extracted distinctly from one miracidium (1–10) and Sj DNA (1 pg) as positive control (P) but not the no-template sample (N). (**C**) Each snail from the non-endemic area was experimentally infected with a different number of miracidia (0, 1, 5, and 10 miracidia/snail), and genomic DNA was extracted from each snail at 1 day after infection. The PCR method detected the schistosome-specific band in DNA from a snail infected with just one miracidium without amplifying DNA from non-infected snails. Each lane represents a distinct snail infected with the same number of miracidia.