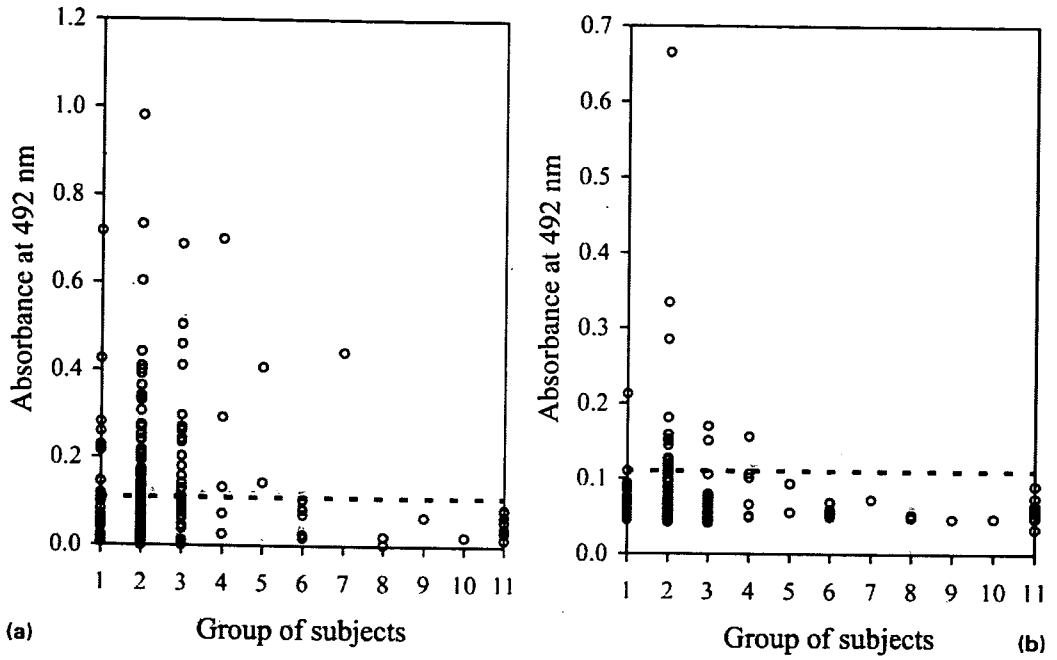

FIG. 2. The absorbances recorded in the ELISA used to measure urine levels of IgG (a) and IgG_4 (b) to *Opisthorchis viverrini*. Group 1 contained the 32 subjects who appeared parasite-free despite having no history of anthelmintic use. Groups 2 (98 subjects), 3 (32 subjects) and 4 (six subjects) contained the subjects who were only found positive for *O. viverrini*, excreting, respectively, 1–100, 101–1000 and >1000 eggs of this parasite/g faeces. Group 5 contained two subjects found positive only for *Strongyloides* larvae, Group 6 held five subjects found positive only for hookworm eggs, and Group 7 consisted of a single subject who was found positive only for the eggs of 'minute intestinal flukes'. Also included in the comparison were two subjects positive only for *Giardia* cysts (Group 8), one found positive only for *Taenia* eggs (Group 9), one positive only for *Sarcocystis* eggs (Group 10) and, as controls, 11 subjects from an area of Central Thailand where *O. viverrini* is unknown (Group 11). In each plot, the dotted line indicates the threshold for positivity in the ELISA.

specific IgG and IgG_4 in the serum gave good sensitivities (99.2% and 93.0%, respectively) but poor specificities (23.1% and 29.6%, respectively). In comparison, the ELISA based on the urine levels of specific IgG and IgG4 gave much better specificity (64.5% and 67.2%, respectively) but much lower sensitivity (43.0% and 45.9%, respectively). The sensitivity of assays based on IgG_4 in the urine appears much lower for *O. viverrini* infection (45.9%; present study) than for *W. bancrofti* infection (95.6%; Itoh *et al.*, 2001). There is no evidence that the addition of sodium azide to urine samples, as a preservative, affects IgG affinity (Itoh *et al.*, 2001).

As indicators of the intensity of *O. viverrini* infection, serum levels of specific IgG_4 appeared much better than urine levels of IgG_4, perhaps indicating that little IgG_4 is excreted in the urine or that the antigenic extract used in the present study was not good enough. In the search for a better diagnostic test based on urine samples, it would appear worthwhile to investigate urine samples for subclasses of specific IgG other than IgG_4.

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REFERENCES

Akai, P. S., Pungpak, S., Chaicumpa, W., Viroj, K., Bunnag, D. & Befus, A. D. (1994). Serum antibody

response to *Opisthorchis viverrini* antigen as a marker for opisthorchiasis-associated cholangiocarcinoma. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **88**, 471–474.

Gryseels, B., Stelma, F., Talla, I., Polman, K, van Dam, G., Sow, S., Diaw, M., Sturrock, R. F., Decam, C., Niang, M. & Deelder, A. M. (1995). Immuno–epidemiology of *Schistosoma mansoni* infections in a recently exposed community in Senegal. *Memórias do Instituto Oswaldo Cruz*, **90**, 271–276.

Itoh, M., Pairojkul, C., Thamawit, W., Sithithaworn, P., Tiwawech, D., Uttaravicien, T., Shirai, T. & Ito, N. (1994). Association of antibodies to *Opisthorchis viverrini* with hepatobiliary disease in northeastern Thailand. *American Journal of Tropical Medicine and Hygiene*, **51**, 424–429.

Itoh, M., Weerasooriya, M. V., Qiu, X.-G., Gunawardena, N. K., Anantaphruti, M. T., Tesana, S., Rattanaxay, P., Fujimaki, Y. & Kimura, E. (2001). Sensitive and specific enzyme-linked immunosorbent assay for the diagnosis of *Wuchereria bancrofti* infection in urine samples. *American Journal of Tropical Medicine and Hygiene*, **65**, 362–365.

Jongsuksuntigul, P., Chaeychomsri, W., Techamontrikul, P., Jeradit, P. & Suratanavanith, P. (1992). Study on prevalence and intensity of intestinal helminthiasis and opisthorchiasis in Thailand. *Journal of Tropical Medicine and Parasitology*, **19**, 80–95.

Le, T. H., De, N. V., Blair, D., Sithithaworn, P. & McManus, D. P. (2006). *Clonorchis sinensis* and *Opisthorchis viverrini*: development of a mitochondrial-based multiplex PCR for their identification and discrimination. *Experimental Parasitology*, **112**, 109–114.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randal, R. J. (1951). Protein measurement with the folin–phenol reagent. *Journal of Biological Chemistry*, **123**, 265–275.

Pospisilova, Z., Ditrich, O., Stankova, M. & Kodym, P. (1997). Parasitic opportunistic infections in Czech HIV-infected patients—a prospective study. *Central European Journal of Public Health*, **5**, 208–213.

Radomyos, P., Radomyos, B. & Tungtrongchitr, A. (1994). Multi-infection with helminths in adults from northeast Thailand as determined by post-treatment fecal examination of adult worms. *Journal of Tropical Medicine and Parasitology*, **45**, 133–135.

Radomyos, B., Wongsaroj, T., Wilairatana, P., Radomyos, P., Praevanich, R., Meesomboon, V. & Jongsuksuntikul, P. (1998). Opisthorchiasis and intestinal fluke infections in northern Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, **29**, 123–127.

Ramzy, R. M., Faris, R., Bahgat, M., Helmy, H., Franklin, C. & McKerrow, J. H. (1997). Evaluation of a stage-specific proteolytic enzyme of *Schistosoma mansoni* as a marker of exposure. *American Journal of Tropical Medicine and Hygiene*, **56**, 668–673.

Snyman, J. R., de Sommers, K., Steinmann, M. A. & Lizamore, D. J. (1997). Effects of calcitriol on eosinophil activity and antibody responses in patients with schistosomiasis. *European Journal of Clinical Pharmacology*, **52**, 277–280.

Sriamporn, S., Pisani, P., Pipitgool, V., Suwanrungruang, K., Kamsa-ard, S. & Parkin, D. M. (2004). Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, northeast Thailand. *Tropical Medicine and International Health*, **9**, 588–594.

Sripa, B.& Kaewkes, S. (2000). Relationship between parasite-specific antibody responses and intensity of *Opisthorchis viverrini* infection in hamsters. *Parasite Immunology*, **22**, 139–145.

Tesana, S. (2005). Opisthorchiasis in Thailand. In *Asian Parasitology, Volume 1: Food-borne Helminthiasis in Asia*, eds Arizono, N., Chai, J.-Y., Nawa, Y. & Takahashi, Y. pp. 113–124. Chiba, Japan: FAP Journal.

Tesana, S., Kaewkes, S. & Phinlaor, S. (1986). Infectivity and survivorship of *Opisthorchis viverrini* metacercariae in fermented fish. *Journal of Parasitology of the Tropical Medicine Association of Thailand*, **9**, 21–29.

Upatham, E. S., Viyanant, V., Kurathong, S., Brockelman, W. Y., Menaruchi, A., Saowakontha, S., Intarakhao, C., Vajrasthira, S. & Warren, K. S. (1982). Morbidity in relation to intensity of infection in *Opisthorchiasis viverrini*: study of a community in Khon Kaen, Thailand. *American Journal of Tropical Medicine and Hygiene*, **31**, 1156–1163.

Upatham, E. S., Viyanant, V., Kurathong, S., Rojborwonwitaya, J., Brockelman, W. Y., Ardsungnoen, S., Lee, P. & Vajrasthira, S. (1984). Relationship between prevalence and intensity of *Opisthorchis viverrini* infection, and clinical symptoms and signs in a rural community in north–east Thailand. *Bulletin of the World Health Organization*, **62**, 451–461.

Vatanasapt, V., Tangvoraphonkchai, V., Titapant, V., Pipitgool, V., Viriyapap, D.& Sriamporn, S. (1990). A high incidence of liver cancer in Khon Kaen province, Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, **21**, 489–494.

World Health Organization (1995). *Control of Foodborne Trematode Infections*. Technical Report Series No. 849. Geneva: WHO.

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Babesial Vector Tick Defensin against *Babesia* sp. Parasites▽†

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Antimicrobial peptides are major components of host innate immunity, a well-conserved, evolutionarily ancient defensive mechanism. Infectious disease-bearing vector ticks are thought to possess specific defense molecules against the transmitted pathogens that have been acquired during their evolution. We found in the tick *Haemaphysalis longicornis* a novel parasiticidal peptide named longicin that may have evolved from a common ancestral peptide resembling spider and scorpion toxins. *H. longicornis* is the primary vector for *Babesia* sp. parasites in Japan. Longicin also displayed bactericidal and fungicidal properties that resemble those of defensin homologues from invertebrates and vertebrates. Longicin showed a remarkable ability to inhibit the proliferation of merozoites, an erythrocyte blood stage of equine *Babesia equi*, by killing the parasites. Longicin was localized at the surface of the *Babesia* sp. parasites, as demonstrated by confocal microscopic analysis. In an in vivo experiment, longicin induced significant reduction of parasitemia in animals infected with the zoonotic and murine *B. microti*. Moreover, RNA interference data demonstrated that endogenous longicin is able to directly kill the canine *B. gibsoni*, thus indicating that it may play a role in regulating the vectorial capacity in the vector tick *H. longicornis*. Theoretically, longicin may serve as a model for the development of chemotherapeutic compounds against tick-borne disease organisms.

Antimicrobial peptides are major defensive molecules of the innate immune system in animals (43). They appear to be well-conserved, evolutionally ancient molecules useful for the survival of vertebrates and invertebrates (23, 24, 46, 50). These peptides respond differently from those of antibiotics and currently used chemotherapeutic drugs. They are less toxic and more effective against multidrug-resistant bacteria, and it is hoped that they might be better choices for control of some bacterial and fungal infectious diseases (30).

Parasite disease-bearing vectors may require an extensive spectrum of innate immunity mechanisms, as evidenced by their diverse protective strategies. The presence of antibacterial and antiparasitic peptides is observed for mosquitoes (12, 49). Expression of defensin-like proteins has also been claimed for several ticks (9, 15, 24, 27, 34, 42, 44); however, their parasiticidal mechanisms in insect and tick vectors still remain unclear. Molecules operating in the innate immune system of numerous vectors (4, 11, 14, 21, 23, 41) may be candidates for the development of a chemotherapeutic effective against arthropod-borne diseases.

Babesiosis is a well-recognized malaria-like disease that occurs in animals and people worldwide and has recently gained increased attention as an emerging zoonosis (25, 47). The suffering and financial cost associated with this disease demand a search for new methods of control. *Babesia* species undergo a complex developmental cycle in the vertebrate host and tick vector somewhat analogous to that of the malaria parasite and mosquito vector (32). The major tick vectors of *Babesia* globally are *Boophilus* species and *Haemaphysalis longicornis* (40). The ixodid tick *H. longicornis*, one of the most important tick species in Asia and Australia, is a natural vector of the pathogens causing babesiosis of humans and domestic animals (16, 28). We hypothesize that *H. longicornis* possesses a specific gene product(s) that mediates partial protective responses against *Babesia*.

Here we report a defensin peptide, longicin, from the tick *H. longicornis* that exerts a babesiacidal effect. Longicin inhibited the growth of *Babesia* in vitro and in vivo. The babesiacidal effect was demonstrated at the merozoite stage, which causes babesiosis and *Babesia*-associated pathology, and was induced by the specific adherence of longicin to the parasite membrane. Interestingly, functional analysis by our validated double-stranded RNA (dsRNA) knockdown procedure revealed that longicin is involved in *Babesia* killing in *H. longicornis*. Our findings suggest that longicin might be useful in designing new chemotherapeutic agents against human and animal babesiosis. Vector ticks possess specific defense molecules probably acquired in the process of their evolution. Elucidation of the relationship between longicin and the *Babesia* parasite may thus help us to better understand the origin of differences between ticks that do and those that do not transmit pathogens.

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MATERIALS AND METHODS

Ticks. *H. longicornis* ticks were obtained from the parthenogenetic Okayama strain maintained at the National Research Center for Protozoan Diseases (NRCPD), Obihiro University, Obihiro, Hokkaido, Japan, and were fed on rabbits (16).

***Babesia* sp. parasites.** The *Babesia* sp. parasites used in this study were as follows: the horse parasite *Babesia equi* (22), the dog parasite *B. gibsoni* (17), and the mouse parasite *B. microti* (37). The U.S. Department of Agriculture strain of *B. equi* and the NRCPD strain of *B. microti* were maintained in in vitro culture at NRCPD. The NRCPD strain of *B. gibsoni* was maintained in chronically infected dogs at NRCPD.

Animals. All animals used this study were acclimatized to these conditions for 2 week prior to the experiment. Animal experiments at the National Institute of Animal Health (NIAH) were conducted in accordance with the protocols approved by the NIAH Animal Care and Use Committee (approval nos. 441, 508, and 578). Animal experiments at Obihiro University were conducted in accordance with the guiding principles for Care and Use of Research Animals promulgated by Obihiro University (approval nos. 6 to 42, C-2).

Cloning of longicin cDNA. Longicin was identified from expressed sequence tags constructed from the midgut cDNA libraries of *H. longicornis* as described previously (6). The plasmids containing longicin-encoding gene inserts were extracted using a QIAGEN DNA purification kit (QIAGEN, Hilden, Germany). The nucleotide sequences of the cDNAs were determined by the BigDye Terminator method on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster City, CA). The GENETYX-WIN DNA analysis software system (Software Inc., Tokyo, Japan) was used to deduce the amino acid sequence of longicin (3) and the BLAST program (2) for alignment was used to compare this sequence with previously reported sequences available in GenBank (5). The putative signal sequence was analyzed using the prediction server SignalP V2.0.b2 (http://www.cbs.dtu.dk/services/SignalP) (36). Analysis of the secondary structure was done using the PSIPRED (http://bioinf.cs.ucl.ac.uk) and *SSThread* (http://www.ddbj.nig.ac.jp) programs.

Recombinant longicin. The entire coding region for longicin except the signal sequence was subcloned into a plasmid expression vector, pTrcHisB (Invitrogen, Carlsbad, CA), as described previously (10). The plasmid was transformed into *Escherichia coli* strain TOP10F' (Invitrogen) and the purification process was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a T7 Taqâ monoclonal antibody (Takara, Otsu, Japan). The recombinant protein was purified using AKTA equipped with a HiTrap chelating HP column (Amersham Pharmacia Biotech, Piscataway, NJ), and the recombinant longicin band resolved by SDS-PAGE was excised (10). The purified protein was cleaved with EK max (Invitrogen), and the digested proteins corresponding to longicin were purified by SDS-PAGE gels with a zinc stain kit (Bio-Rad Laboratories, Hercules, CA). EK-digested longicin proteins were dialyzed against 20 mM Tris-HCl (pH 7.5), 150 mM NaCl by use of a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL). Protein concentrations were measured using a Micro BCA protein assay reagent (Pierce). The results of matrix-assisted laser desorption ionization–time of flight (mass spectrometry) [MALDI-TOF (MS)] analysis of longicin agreed with its expected mass as determined using a Kratos Axima CFR (Shimadzu, Kyoto, Japan).

Synthetic peptides. Peptides were synthesized using a Perkin-Elmer Applied Biosystems 431 A synthesizer by use of prederivatized polyethylene glycol polystyrene arginine resin, FastMoc chemistry, and double coupling for residues. The reduced peptides were purified using reversed-phase high-performance liquid chromatography. The partial peptides were as follows: P1 (residues 23 to 37), P2 (33 to 45), P3 (42 to 57), and P4 (53 to 73). Peptide purity and integrity were assessed by MALDI-TOF (MS) (Kratos Axima CFR).

Production of an antibody against longicin. A mouse polyclonal antibody was generated against a peptide consisting of the N-terminal 20 amino acids of mature longicin. The animals were immunized with 50 μg of bovine serum albumin-conjugated peptide by use of TiterMax Gold (Syntex, Norcross, GA) and were boosted two more times with the bovine serum albumin-conjugated peptide as described previously (10).

Immunoblot analysis. Immunoblot analysis was performed as previously described (51). Adult female ticks were homogenized under liquid nitrogen. Antigens separated by one- or two-dimensional gel electrophoresis were transferred onto nitrocellulose membranes. For detection of endogenous longicin, the membranes were incubated with mouse anti-longicin serum followed by alkaline-phosphatase-conjugated goat anti-mouse immunoglobulin G (ICN Pharmaceutical, Irvine, CA) secondary antibody. The membranes were washed and visualized with the alkaline-phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium (Promega, Madison, WI).

Tick immunohistochemistry. The adult female ticks were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and processed for immunohistochemistry using a mouse anti-longicin serum as described previously (51). The color was developed by incubation with 3,3'-diaminobenzidine (Sigma, St. Louis, MO) solution containing 0.03% H_2O_2. After dehydration and clearance, the sections were observed under an Axiophot instrument (Carl Zeiss, Jena, Germany). Preimmune mouse serum was used as a negative control.

Bactericidal and fungicidal assay. Bactericidal activity was determined by a CFU assay (19). The following bacteria used in these assays were a gift from the NIAH, Tsukuba, Japan: the human pathogenic bacteria *Escherichia coli* O-157 (ATCC 35150), *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, derived from bovine mastitis, and plasmid-dependent multidrug-resistant *Salmonella enterica* serovar Typhimurium. Overnight bacterial culture was subcultured in 3% trypticase soy broth (TSB) with shaking at 37°C to obtain log-phase bacterial cells. Bacterial cells were then washed and diluted to 1×10^6/ml in 10 mM sodium phosphate buffer, pH 7.4, in 1% TSB. The bacterial cell suspensions (90 μl) were mixed with 10 μl of longicin (0, 10, 50, 100, 200 μmol) or synthetic peptide stock (0, 10, 20, 100 μmol) solutions and incubated at 37°C for 2 h. Samples were then diluted 100-fold in 1% TSB and spread on TSB agar plates with a spiral plater. Plates were incubated for 16 h at 37°C, colonies were counted, and CFU per ml were calculated. Fungicidal activity was also determined by a CFU assay (19). The yeast *Pichia pastoris* (GS115) was obtained from Invitrogen. Fungal cell suspensions (90 μl of 1×10^5/ml) were mixed with 10 μl of longicin or synthetic peptide stock solutions (0, 20, 100, 200, 400 μmol) and incubated in 10 mM sodium phosphate buffer, pH 7.0, at 37°C for 2 h. Samples were then diluted 10-fold in yeast-tryptone broth (YTB) and spread on YTB agar plates.

Hemoparasiticidal assay. Hemoparasiticidal activity was determined against *B. equi* in an in vitro culture system (22). *B. equi* merozoites were grown in horse erythrocytes in vitro and incubated in the presence of longicin or various synthetic peptides at different concentrations. Parasitemia was assessed every day using Giemsa-stained medium smears and microscopic observation (24).

Confocal fluorescence microscopy. The target peptide P4 was labeled with fluorescein isothiocyanate (FITC) according to the manufacturer's protocol (Pierce). The conjugate was purified by reversed-phase high-performance liquid chromatography (Shimadzu CLASS-VP; Shimadzu) and resuspended in distilled water. The labeled peptide was added to culture medium and washed several times with phosphate-buffered saline (PBS). Cells were smeared and fixed in 1% paraformaldehyde. Specimens were imaged using a confocal laser scanning microscope (Leica TCS-NT; Leica Microsystems, Wetzlar, Germany) with excitation of FITC. Images were collected by using Leica confocal software.

In vivo parasiticidal assay. Parasiticidal activity of longicin was examined in a *B. microti*-BALB/c mouse infection system (37). Mice that were intravenously inoculated with 1×10^7 *B. microti*-infected erythrocytes were simultaneously treated with various doses of longicin (0 to 3 mg/kg). Parasitemia was assessed every day by microscopic observation of Giemsa-stained blood smears and the results were expressed as mean values ± standard deviations from five mice per dosage. The survival of the mice was monitored for the next 8 weeks.

RNAi. The RNA interference (RNAi) procedure in ticks was carried out using dsRNA as described previously (33, 35). The coding sequence of mature longicin was cloned into pBluescript II SK+ plasmid, and the inserted sequence was amplified by PCR using the oligonucleotides T7 (5'-GTAATACGACTCACTA TAGGGC-3') and CMo422 (5'-GCGTAATACGACTCACTATAGGGAACA AAAGCTGGAGCT-3') to attach T7 promoter recognition sites at both the 5' and 3' ends. The PCR products were purified by use of a gel extraction kit (QIAGEN). dsRNA complementary to the DNA insert was synthesized by in vitro transcription using the T7 RNA polymerase (Promega, Madison, WI) according to the manufacturer's protocol. Two micrograms of double-stranded DNA was used as a template, and 50 to 100 μg of dsRNA was synthesized. We injected 1 μg of *longicin* dsRNA in 0.5 μl of PBS into the hemocoel through the fourth coxae of unfed adult *H. longicornis* females fixed on a glass slide with adhesive tape. The injections were carried out by using 50-μl microcapillaries (MICROCAP; Drummond Scientific, Broomall, PA) drawn in to fine-point needles by heating. The needles were connected to an air compressor. Control ticks were injected with 0.5 μl PBS alone. The ticks were allowed to rest for 1 day at 25°C. No mortality resulted from the injection alone, as both control and *longicin* dsRNA-treated ticks survived after injection while being held in an incubator prior to placement on the host.

Infestation by RNAi-treated ticks of dogs infected with canine *Babesia* sp. parasite. The dsRNA-injected ticks were placed on the ears of 8-month-old female beagles preinfected with *B. gibsoni* (18). During attachment, the dogs kept 12.5% intraerythrocytic parasitemia in the peripheral blood. The pattern of the control ticks injected with the buffer alone was comparable to that for

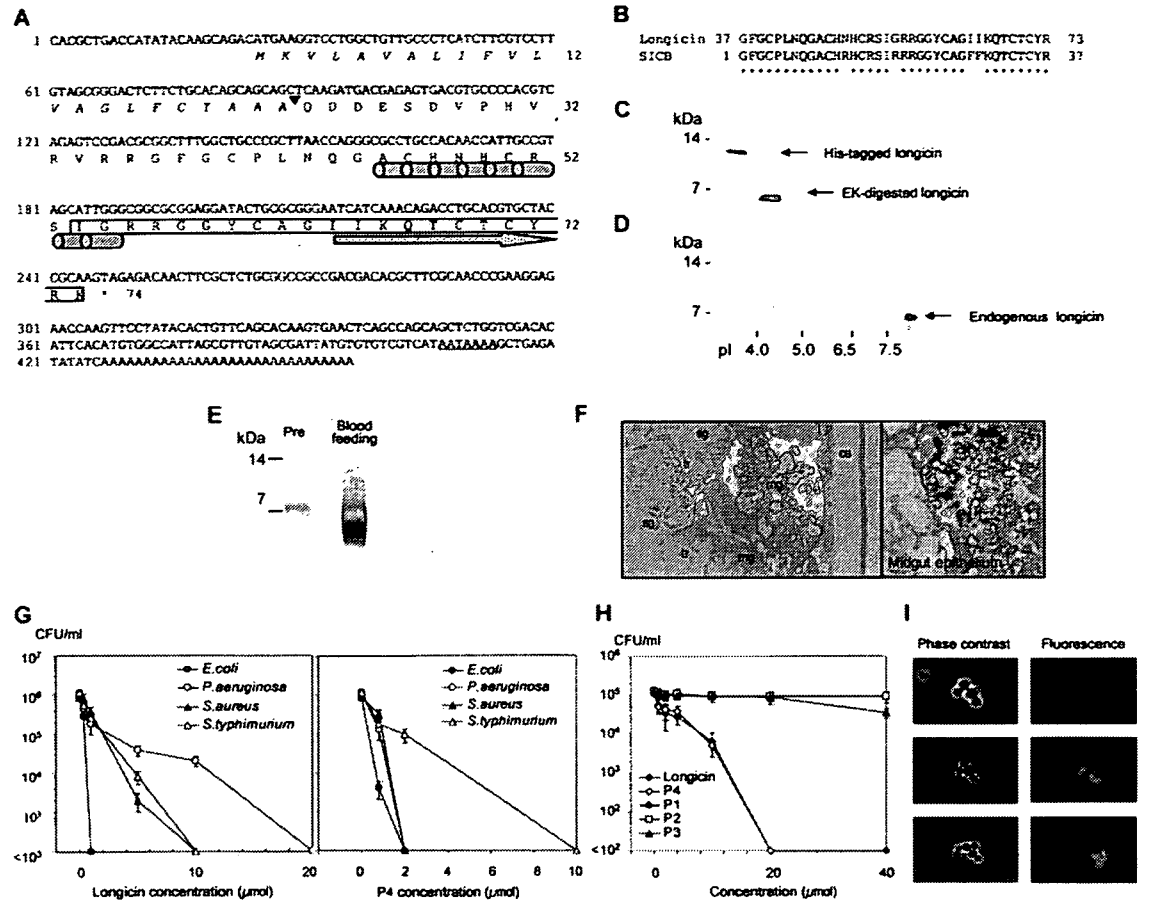

FIG. 1. Molecular and functional characterization of longicin. (A) cDNA and deduced amino acid sequences and charge density of longicin. Locations of secondary structure elements are shown in the form of cylinders (α-helixes) and an arrow (β-strand). Italics indicate a putative signal sequence, the triangle indicates the mature peptide, the box indicates P4 peptide, and a bold underline marks the polyadenylation signal. (B) Alignment of longicin and the scorpion ion channel blocker (SICB) from Sahara scorpion. Asterisks indicate conserved residues. (C) Coomassie blue-stained recombinant longicins. (D) Endogenous longicin in tick protein extract. Detection was performed by two-dimensional immunoblot analysis with anti-longicin antibody. (E) Longicin expression induced by blood feeding. An equal amount of protein (three ticks) was loaded in each lane. Detection was performed by immunoblot analysis with anti-longicin antibody. Pre, prefeeding. (F) Immunohistochemical localization of endogenous longicin. Note the strong staining of the midgut epithelium (high magnification). Abbreviations: cu, cuticle; mg, midgut; sg, salivary gland; tr, trachea. (G) Bactericidal activity of longicin. Bacterial cells were exposed to recombinant longicin or synthetic peptides for 2 h at 37°C, and then the incubation mixture was spread on TSB agar plates. The results shown are from four plates from different batches. (H) Fungal cells were exposed to recombinant longicin or P4 peptide for 2 h at 37°C, and then the incubation mixture was spread on YTB agar plates. (I) Morphological changes in *P. pastoris* induced by longicin.

uninjected ticks infested simultaneously on the same host. On day 6, ticks were recovered from the dogs. After dissection of the ticks, individual organs were removed and the midgut contents were opened under a microscope. To verify gene silencing of *longicin* dsRNA, reverse transcription-PCR was performed as described previously (51). Total mRNA was isolated using a QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech) as described in the protocols. cDNA was then synthesized with 30 μg of mRNA using an RNA PCR kit (AVM) Ver.3.0 (Takara) following the manufacturer's instructions. PCR was performed using *longicin*-specific oligonucleotides and β-actin-specific oligonucleotides for *H. longicornis* with 500 ng of cDNA as the template in a final volume of 50 μl. PCR products were resolved by agarose gel electrophoresis.

Immunofluorescence microscopy. We examined endogenous longicin expression and localization of *B. gibsoni* in dissected tick organs. Tick immunofluorescent analysis was performed as described previously (26). Bound mouse anti-longicin or mouse anti-*B. gibsoni* antibodies (17) were detected using anti-mouse immunoglobulin G Alexa 488 (Invitrogen). The sections were mounted in Vectashield (Vector, Burlingame, CA) with 4′,6′-diamino-2-phenylindole (DAPI) and photographed with a fluorescence microscope (Leica) using appropriate filter sets. Images were collected by using Leica FW4000 software.

Real-time PCR assay for quantifying *B. gibsoni* infection. The numbers of *B. gibsoni* organisms and the intensity of *B. gibsoni* infection in the dissected organs were evaluated using a real-time quantitative PCR assay. Initially, we standardized the PCR protocol by use of *B. gibsoni* P18 gene-specific primers (D3, 5′-TCCGTTCCCACAACACCAGC-3′; D4, 5′-TCCTCCTCATCATCC TCATTCG-3′) and purified *B. gibsoni* genomic DNA. *B. gibsoni* P18, encoding a major surface protein, is a well-known gene, and its use as a diagnostic tool for dog *B. gibsoni* infection has been demonstrated (18). PCR was performed using a LightCycler 1.5 (Roche Diagnostics GmbH, Nonnenwald, Germany) and DNA master SYBR green I (Roche) with 4 mM $MgCl_2$. Standard curves used to quantify relative gene concentrations were made from a 10-fold serial dilution (3×10^0 to 3×10^3) of the *B. gibsoni* parasites with the following setting: 95°C for 600 s (denaturing step), 45 cycles of 95°C for 15 s, 55°C for 10 s, and 72°C for 15 s under the fit point method in LightCycler software, version 3.5.3. The protocol was observed to be highly specific for *B. gibsoni* P18, with no amplification of dog, tick, or a range of other *B. gibsoni* DNA. The standard plot is shown elsewhere (see Fig. S1 in the supplemental material). Evaluation of the number of *B. gibsoni* parasites from excised organs was determined on the basis of the standard plot. DNA extraction and concentration were determined as described previously (51).

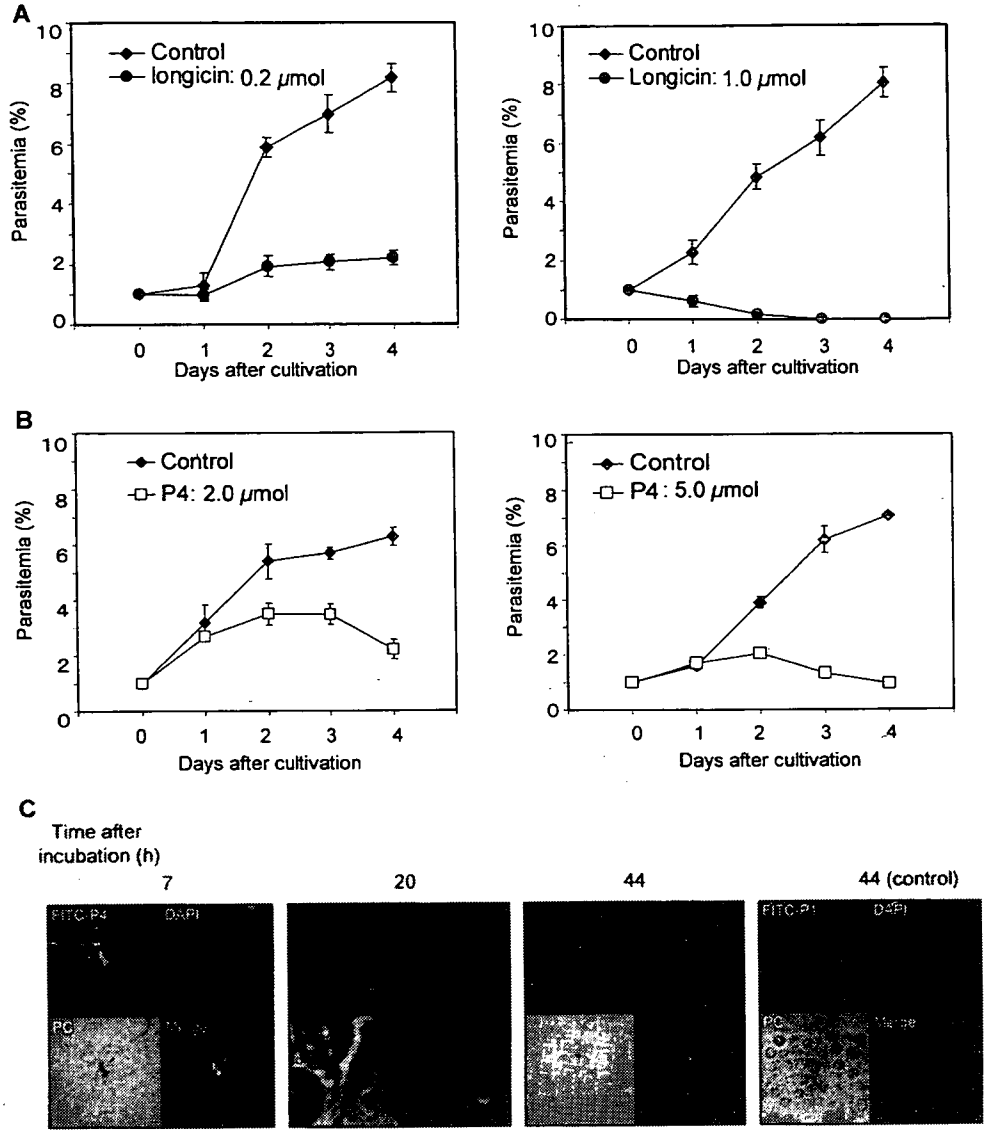

FIG. 2. Babesiacidal activity of longicin. Longicin or synthetic peptides were incubated with *B. equi*-infected erythrocytes (1% parasitemia) in culture medium. Parasite-infected erythrocytes were counted as percentages of total erythrocytes. (A) Parasiticidal effect in the presence of longicin. *Babesia*-infected cells had almost disappeared on day 2 of 1.0 μmol treatment. (B) Parasiticidal effect in the presence of synthetic peptides. The error bars indicate standard errors of the mean (SEM). (C) Detection of longicin at the surface of *B. equi* merozoites. FITC-conjugated longicin P4 synthetic peptide was used for localization by fluorescent confocal microscopy. The control panel shows that FITC-labeled P1 did not bind to any cells. PC, phase contrast.

Statistical analysis. Statistically significant analysis was performed by using Student's *t* test.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have the DDBJ/EMBL/GenBank accession no. AB105544.

RESULTS

Molecular and functional characterization of longicin. The composite full-length *longicin* cDNA sequence was 456 nucleotides long and contained a single open reading frame of 216 bases. The open reading frame coded for a protein of 74 amino acids, including a signal peptide of 20 residues (Fig. 1A). The putative mature protein has a molecular mass of 5,820 Da and a pI of 8.3, including six cysteine residues. The predicted secondary structure of longicin showed a well-defined β-sheet at the C terminus. The greatest amino acid sequence similarity (86%) found for longicin was with the scorpion ion channel blocker (13) (Sahara scorpion, accession no. P56686; Fig. 1B). Longicin also shares great similarity with defensins from ixodids and argasids (hard and soft ticks) (9, 15, 27, 34, 42, 44). The cDNA corresponding to the deduced premature protein was subcloned into a plasmid expression vector to produce a recombinant fusion longicin (Fig. 1C). To characterize the expression of endogenous longicin, we generated a specific polyclonal antibody. The antibody bound to a single spot in two-dimensional immunoblots (Fig. 1D), indicating that the peptide with a molecular mass of 6.4 kDa and pI of 8.5 from adult *H. longicornis* extract was the endogenous form of longicin. We confirmed by MALDI-TOF (MS) analysis that this

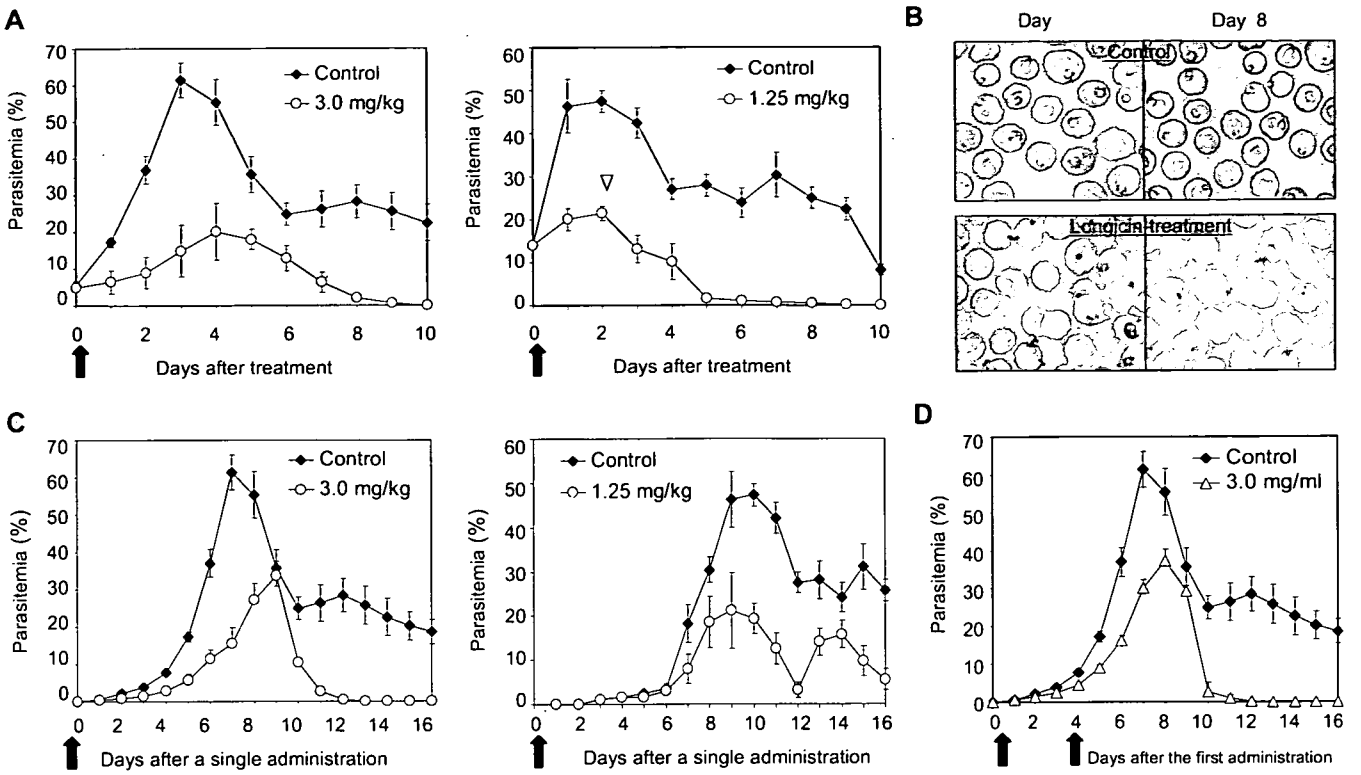

FIG. 3. In vivo babesiacidal activity of longicin. (A) Therapeutic effect of a single treatment dose. Arrows and an arrowhead indicate administration of longicin and 60% reduction of parasitemia, respectively. (B) Representative images of the parasites from mice treated with longicin (3.0 mg/kg). Longicin blocked parasite invasion but not host erythrocyte rupture. (C) Growth inhibition was dependent on the dose of longicin. (D) Growth inhibition by a double treatment with longicin. The error bars indicate SEM.

protein was consistent with the polypeptide predicted from the cDNA (data not shown). Constitutive expression of longicin was detected throughout the normal life cycle, including larval, nymphal, and adult stages, and the level was clearly increased after blood feeding (Fig. 1E). This pattern of endogenous expression resembled that of defensin homologues from other bloodsucking arthropods (11). However, endogenous longicin was produced mainly in the midgut epithelium (Fig. 1F), suggesting that longicin is secreted into the lumen, unlike mosquito defensin (1).

Recent studies indicate that some ixodid tick defensins possess bactericidal activity against several bacterial pathogens (15, 27, 42). To assess the functional properties of longicin, we measured the antimicrobial activity against several human and animal pathogenic bacteria. Recombinant longicin and synthetic peptides consisting of 16 to 22 residues were also prepared based on the deduced amino acid sequences. The bactericidal activity of longicin was tested against a variety of gram-positive and gram-negative bacteria, including multidrug-resistant strains from human and animal patients (Fig. 1G). Although the synthetic peptides P1, P2, and P3 did not exert a bactericidal effect against any of the screened bacterial strains, P4 consistently caused concentration-dependent killing of the four species tested at concentrations of 2.0 to 10.0 μmol (Fig. 1G). It also possessed fungicidal activity. Proliferation of the yeast *P. pastoris* was completely inhibited by P4 at 20.0 μmol (Fig. 1H). When *P. pastoris* was incubated with FITC-labeled P4, the cell membrane was clearly fluorescent (Fig. 1I).

Babesiacidal activity of longicin. To further assess the properties of longicin, we incubated the equine *Babesia* sp. parasite, *B. equi*, in medium supplemented with longicin. Interestingly, recombinant longicin completely inhibited merozoite proliferation at a concentration of 1.0 μmol (Fig. 2A). The inhibitory effects of longicin were concentration dependent. Next, we attempted to identify the babesiacidal active site of longicin against *B. equi* by using the four types of synthetic peptides (P1 to P4) described above. The merozoite-infected erythrocytes were maintained for 4 days in the presence of the peptides at a dose of 0 to 5.0 μmol. Similarly to the full-length longicin, P4 induced complete protection against the infection of new erythrocytes (Fig. 2B), and the parasiticidal effects were concentration dependent. No synergistic effects were induced by treatment with any two of the peptides. Neither longicin nor the four types of partial peptides caused hemolysis at any concentration that exerted a parasiticidal effect.

To explore how longicin interacted with the parasites, we used fluorescence confocal microscopy to examine *B. equi* at the merozoite stage. *B. equi* was incubated with FITC-labeled P4 peptide in culture medium. Fluorescent reactions were seen at the surface of the merozoites, but morphological changes did not occur at 7 h of incubation (Fig. 2C). At 44 h, longicin clearly caused lysis of the parasites. Quantitative assays showed that the residual peptide was decreased in the presence of

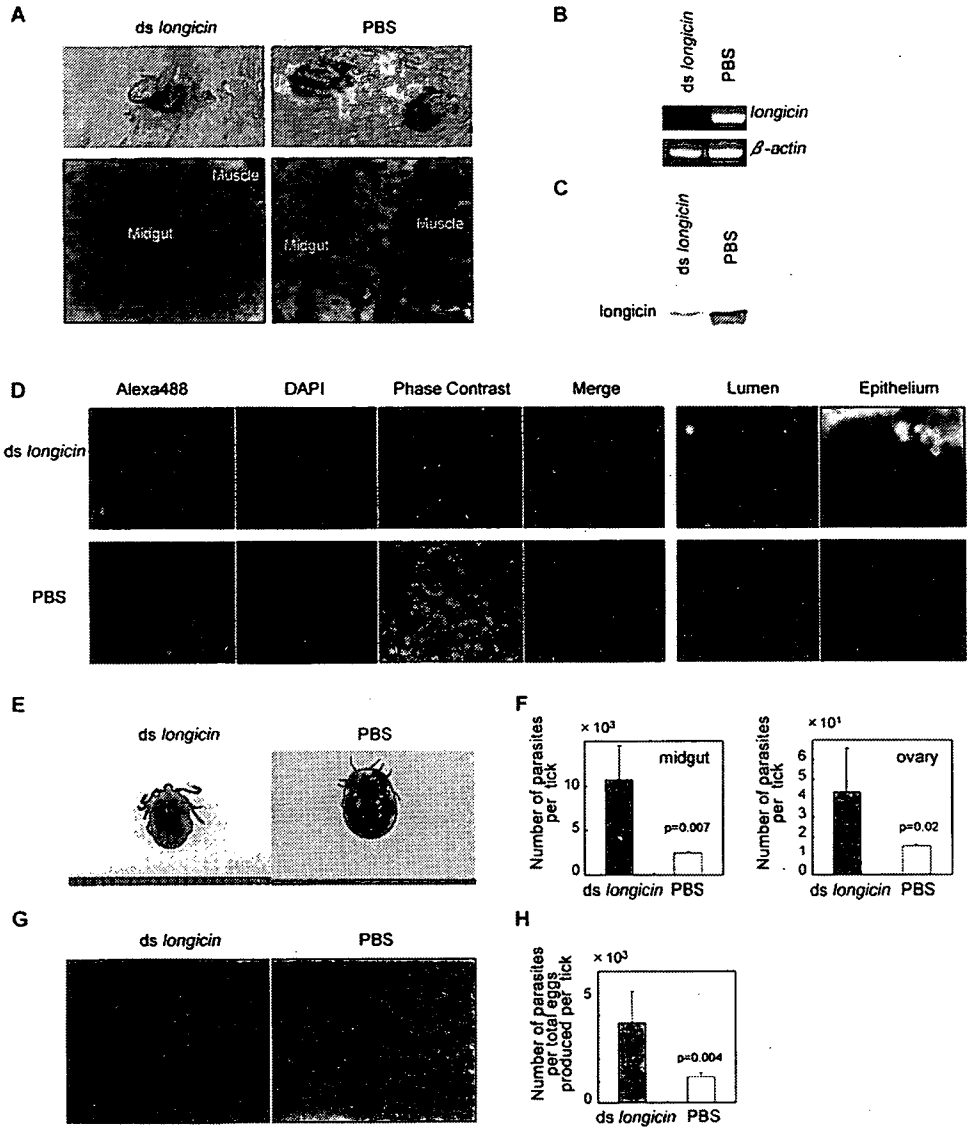

FIG. 4. Knockdown of *longicin* by RNAi facilitates transmission of *Babesia* parasite through the vector ticks. Unfed adult female ticks were injected with *longicin* dsRNA (ds *longicin*) into the hemocoel through the fourth coxae by use of fine-point glass needles. Control ticks were injected with PBS alone. Ticks were collected from the ear of a *Babesia gibsoni*-infected dog on day 6 after attachment. (A) Microinjection-treated ticks at day 3 on the ear of a dog. Injection of *longicin* dsRNA inhibited endogenous longicin expression (green) in the midgut. Figures are representative of three independent RNAi experiments. (B) RT-PCR analysis (day 3). Note the reduced expression of *longicin* mRNA in *longicin* dsRNA-injected ticks. (C) Immunoblot analysis of endogenous longicin expression (day 3). Results showed the absence of longicin expression, indicating that the effective knockdown of *longicin* mRNA was achieved by dsRNA injection. (D) Images of the tick midgut. *B. gibsoni* parasites were visualized by use of mouse anti-*B. gibsoni* antibody (green). The two right panels highlight the lumen and epithelium from the merge section of the midgut. (E) Ticks on day 6. The body weights of ticks with silencing *longicin* were significantly lower than those of control ticks at day 6. Suppression of endogenous longicin expression in *longicin* dsRNA-treated ticks was seen up to engorgement. Preoviposition, oviposition, and egg periods of ticks treated with dsRNA were similar to those of PBS control ticks. Smaller engorged ticks silenced with *longicin* subsequently transmitted larger numbers of *Babesia* sp. parasites than did PBS control ticks. One scale, 1 mm. (F) Prevalence and intensity of *B. gibsoni* infection. The numbers of invaded parasites were evaluated by P18 genes on the *B. gibsoni* genome DNA by use of a real-time quantitative PCR. (G) Representative image of the migrating *Babesia* parasite in the tick ovary. (H) Intensity of *B. gibsoni* infection. Data represent the means ± SEM for three experiments with five ticks. Quantitative results demonstrated that repression of *longicin* enhanced the *B. gibsoni* transmission in the vector tick. The error bars indicate SEM for three independent experiments with three ticks.

merozoites (data not shown), indicating that this difference was the result of subsequent interactions of peptides with parasites.

In vivo babesiacidal activity of longicin. For babesiosis or *Babesia*-associated pathology to occur, the invasive merozoites must recognize, bind, and enter into the circulating erythrocytes. Subsequent replication of the babesial blood-stage parasites causes rupture of the animal erythrocytes (40). The efficacy of the hemoparasiticidal activity of longicin was tested in vivo against a murine *Babesia* parasite, *B. microti*. When infected mice were inoculated with a single dose of 3.0 mg/kg longicin at 5% parasitemia, the parasitemia increased significantly more slowly, and a 72% inhibition was noted at the peak

parasitemia. Infected mice inoculated with 1.25 mg longicin/kg of body weight at 15% parasitemia showed a 60% inhibition of parasitemia (Fig. 3A and B). Next, a challenge infection using longicin-treated mice was initiated. Treatment of mice with 3.0 mg/kg maximally inhibited parasitemia by 40% and cleared it at day 12, whereas 1.25 mg/kg allowed a low level of parasitemia throughout the observation period (Fig. 3C). Mice treated with 3.0 mg/kg followed by a second treatment at the same dose did not show any synergistic effects (Fig. 3D). All mice treated with longicin and P4 were as active and healthy as untreated mice, with normal parameters for liver and kidney function as indicated by blood examination (data not shown).

The role of longicin in *H. longicornis*. We next hypothesized that endogenous longicin may directly affect *Babesia* parasite survival. We took an RNAi approach to knock down *longicin* mRNA by dsRNA in adult *H. longicornis*. We then assessed whether the transmission of *Babesia* sp. parasites was affected by the endogenous longicin. The dsRNA-treated ticks were attached to a dog that was preinfected with dog *Babesia* sp. parasite *B. gibsoni*. Interestingly, the knockdown of *longicin* resulted in a significant reduction in the ability of the ticks to feed and engorge. Longicin depression clearly impaired tick blood feeding at day 3, although the underlying mechanism responsible for the developmental effect is unclear (Fig. 4A). Inhibition of *longicin* mRNA and endogenous longicin was clearly seen for the *longicin* dsRNA-treated ticks (Fig. 4B and C). Immunofluorescent studies indicate increasing ratios of the parasites in the midgut of the *longicin* knockdown ticks (Fig. 4D). A significant difference in body weight at engorgement between the knockdown (mean ± standard deviation, 89.5 ± 32.1 mg; $n = 12$) and control (312.5 ± 48.3 mg; $n = 15$) groups was observed (Fig. 4E). In addition, the knockdown of *longicin* showed a significantly increased number of *B. gibsoni* parasites in the midgut and the ovary (Fig. 4F and G). Longicin deletion in ticks resulted in a twofold increase in the transmission ability of *Babesia* sp. parasites into the eggs (Fig. 4H). Although *longicin* dsRNA-treated ticks were attached to non-*Babesia*-infected dogs, no significant differences were observed in the tick feeding and engorgement abilities on the infected and the noninfected dogs.

DISCUSSION

Babesia sp. parasites must complete a complex developmental cycle in the tick for transmission to occur. Their development depends on a balance between the ability of the tick to establish a defense response against the parasite and the ability of the parasite to escape the tick's immune response. Thus, it may be that *H. longicornis* has some defense molecules against invading *Babesia* sp. parasites. An excessive number of parasites may destroy the midgut epithelium, resulting in the hemolymph flowing into the lumen and causing the death of the tick (20, 45). Longicin expressed by *H. longicornis* may have acquired the parasiticidal action of controlling the number of *Babesia* parasites in addition to its antibacterial and antifungal actions. On the other hand, for babesiosis or *Babesia*-associated pathology to occur, the invasive merozoites must recognize, bind, and enter circulating erythrocytes. Subsequent replication of the babesial blood-stage parasites causes rupture of animal erythrocytes (40).

Current information indicates that longicin is not involved in erythrocyte rupture during the blood phase of *Babesia* but rather has a specific role in the invasion of the host cell by extracellular merozoites (7). Antimicrobial peptides recognize pathogen-associated molecular patterns in microbes and may form ion-permeable transmembrane pores, resulting in the rupture of target microbes (31). Some antimicrobial peptides derived from non-parasite-bearing vectors are known to possess antiparasite activity (8). However, these are very toxic for mammalian cells, since they cause hemolysis. The present results suggest that the bactericidal, fungicidal, and babesiacidal active sites are located in the C-terminal amino acid sequence, which consists of a β-sheet, in contrast to insect defensins, whose recognition site is located in an α-helix (38). Ticks are phylogenetically closer to spiders and scorpions (Arachnida, Chelicerata) than to insects (24, 29). Cationic defensin peptides from both spiders and scorpions act as ion channel blockers, and the ion channel recognition site is located in the β-sheet of their C termini (39). Longicin may have evolved from a common ancestral peptide resembling spider and scorpion toxins. Longicin is the first molecule isolated from a parasite-bearing vector that exerts a hemoparasiticidal effect without any demonstrable toxicity to mammalian host cells.

Our data show that longicin can prevent or retard proliferation of merozoites. Moreover, longicin appears to be stable in vivo and therefore able to act against *Babesia* parasites when exported to them. This may be due to its lack of degradation by murine proteases (39). Surprisingly, present therapeutic test results indicate that the efficacy of longicin is superior to that reported for antibabesial drugs commonly used in clinical practice (48). Our data confirm the efficacy of longicin in inhibiting the growth of *Babesia* sp. parasites both in vitro and in vivo and strongly suggest that it may be useful in designing new chemotherapeutic agents.

After the acquisition of a blood meal by adult *H. longicornis*, the ingested *Babesia* sp. parasites invade the midgut epithelium, move to the ovary, and finally enter into the eggs (32). The present results suggest that longicin-mediated killing of *Babesia* may control the number of parasites as they exit and/or invade the midgut epithelium. Prior studies have shown that the tick transmits only a limited number of *Babesia* sp. parasites during blood feeding, suggesting the existence of a partially successful natural defense mechanism against the parasites (20). Thus, longicin expressed by vector ticks may have acquired parasiticidal action in reducing the number of *Babesia* sp. parasites in addition to its antibacterial and antifungal actions.

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REFERENCES

1. **Abraham, E. G., and M. Jacobs-Lorena.** 2004. Mosquito midgut barriers to malaria parasite development. Insect Biochem. Mol. Biol. **34**:667–671.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
3. **Bairoch, A.** 1992. The SWISS-PROT protein sequence data bank. Nucleic Acids Res. **20**:2013–2018.
4. **Beier, J. C.** 1998. Malaria parasite development in mosquitoes. Annu. Rev. Entomol. **43**:519–543.
5. **Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, B. A. Rapp, and D. L. Wheeler.** 2002. GenBank. Nucleic Acids Res. **30**:17–20.
6. **Boldbaatar, D., C. S. Sikasunge, B. Battsetseg, X. Xuan, and K. Fujisaki.** 2006. Molecular cloning and functional characterization of an aspartic protease from the hard tick *Haemaphysalis longicornis*. Insect Biochem. Mol. Biol. **36**:25–36.
7. **Boman, H. G.** 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. **5**:61–92.
8. **Boulanger, N., R. J. Munks, J. V. Hamilton, F. Vovelle, R. Brun, M. J. Lehane, and P. Bulet.** 2002. Epithelial innate immunity. A novel antimicrobial peptide with antiparasitic activity in the blood-sucking insect *Stomoxys calcitrans*. J. Biol. Chem. **277**:49921–49926.
9. **Ceraul, S. M., S. M. Dreher-Lesnick, J. J. Gillespie, M. S. Rahman, and A. F. Azad.** 2007. New tick defensin isoform and antimicrobial gene expression in response to *Rickettsia montanensis* challenge. Infect. Immun. **75**:1973–1983.
10. **Chandrashekar, R., N. Tsuji, T. Morales, V. Ozols, and K. Mehta.** 1998. An ERp60-like protein from the filarial parasite *Dirofilaria immitis* has both transglutaminase and protein disulfide isomerase activity. Proc. Natl. Acad. Sci. USA **95**:531–536.
11. **Dimopoulos, G., H. M. Muller, E. A. Levashina, and F. C. Kafatos.** 2001. Innate immune defense against malaria infection in the mosquito. Curr. Opin. Immunol. **13**:79–88.
12. **Dimopoulos, G., G. K. Christophides, S. Meister, J. Schultz, K. P. White, C. Barillas-Mury, and F. C. Kafatos.** 2002. Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection. Proc. Natl. Acad. Sci. USA **99**:8814–8819.
13. **Ehret-Sabatier, L., D. M. Loew, M. Goyffon, P. Fehlbaum, J. A. Hoffmann, A. van Dorsselaer, and P. Bulet.** 1996. Characterization of novel cysteine-rich antimicrobial peptides from scorpion blood. J. Biol. Chem. **271**:29537–29544.
14. **Fogaca, A. C., P. I. da Silva, Jr., M. T. Miranda, A. G. Bianchi, A. Miranda, P. E. Ribolla, and S. Daffre.** 1999. Antimicrobial activity of a bovine hemoglobin fragment in the tick *Boophilus microplus*. J. Biol. Chem. **274**:25330–25334.
15. **Fogaca, A. C., D. M. Lorenzini, L. M. Kaku, E. Esteves, P. Bulet, and S. Daffre.** 2004. Cysteine-rich antimicrobial peptides of the cattle tick *Boophilus microplus*: isolation, structural characterization and tissue expression profile. Dev. Comp. Immunol. **28**:191–200.
16. **Fujisaki, K., S. Kawazu, and T. Kamio.** 1994. The taxonomy of the bovine *Theileria* spp. Parasitol. Today **10**:31–33.
17. **Fukumoto, S., X. Xuan, Y. Nishikawa, N. Inoue, I. Igarashi, H. Nagasawa, K. Fujisaki, and T. Mikami.** 2001. Identification and expression of a 50-kilodalton surface antigen of *Babesia gibsoni* and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. **39**:2603–2609.
18. **Fukumoto, S., H. Suzuki, I. Igarashi, and X. Xuan.** 2005. Fatal experimental transplacental *Babesia gibsoni* infections in dogs. Int. J. Parasitol. **35**:1031–1035.
19. **Ghosh, D., E. Porter, B. Shen, S. K. Lee, D. Wilk, J. Drazba, S. P. Yadav, J. W. Crabb, T. Ganz, and C. L. Bevins.** 2002. Paneth cell trypsin is the processing enzyme for human defensin-5. Nat. Immunol. **3**:583–590.
20. **Gray, J. S.** 1982. The effects of the piroplasm *Babesia bigemina* on the survival and reproduction of the blue, tick, *Boophilus decoloratus*. J. Invertebr. Pathol. **39**:413–415.
21. **Gura, T.** 2001. Innate immunity. Ancient system gets new respect. Science **291**:2068–2071.
22. **Hirata, H., X. Xuan, N. Yokoyama, Y. Nishikawa, K. Fujisaki, N. Suzuki, and I. Igarashi.** 2003. Identification of a specific antigenic region of the P82 protein of *Babesia equi* and its potential use in serodiagnosis. J. Clin. Microbiol. **41**:547–551.
23. **Hoffmann, J. A.** 1997. Immune responsiveness in vector insects. Proc. Natl. Acad. Sci. USA **94**:11152–11153.
24. **Hoffmann, J. A., F. C. Kafatos, C. A. Janeway, and R. A. Ezekowitz.** 1999. Phylogenetic perspectives in innate immunity. Science **284**:1313–1318.
25. **Homer, M. J., I. Aguilar-Delfin, S. R. Telford III, P. J. Krause, and D. H. Persing.** 2000. Babesiosis. Clin. Microbiol. Rev. **13**:451–469.
26. **Huang, X., N. Tsuji, T. Miyoshi, M. Motobu, M. K. Islam, M. A. Alim, and K. Fujisaki.** 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.
27. **Hynes, W. L., S. M. Ceraul, S. M. Todd, K. C. Seguin, and D. E. Sonenshine.** 2005. A defensin-like gene expressed in the black-legged tick, Ixodes scapularis. Med. Vet. Entomol. **19**:339–344.
28. **Klompen, H.** 2005. Ticks, the Ixodida, p. 45–55. *In* W. C. Marquardt (ed.), Biology of disease vectors, 2nd edition. Elsevier Academic Press, Burlington, MA.
29. **Klompen, J. S., W. C. Black IV, J. E. Keirans, and J. H. Oliver, Jr.** 1996. Evolution of ticks. Annu. Rev. Entomol. **41**:141–161.
30. **Levy, O.** 2000. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. Blood **96**:2664–2672.
31. **Medzhitov, R.** 2001. Toll-like receptors and innate immunity. Nat. Rev. Immunol. **1**:135–145.
32. **Mehlhorn, H., and E. Shein.** 1984. The piroplasms: life cycle and sexual stages. Adv. Parasitol. **23**:37–103.
33. **Miyoshi, T., N. Tsuji, M. K. Islam, T. Kamio, and K. Fujisaki.** 2004. Gene silencing of a cubilin-related serine proteinase from the hard tick *Haemaphysalis longicornis* by RNA interference. J. Vet. Med. Sci. **66**:1471–1473.
34. **Nakajima, Y., A. van der Goes van Naters-Yasui, D. Taylor, and M. Yamakawa.** 2002. Antibacterial peptide defensin is involved in midgut immunity of the soft tick, Ornithodoros moubata. Insect Mol. Biol. **11**:611–618.
35. **Narasimhan, S., R. R., Montgomery, K. DePonte, C. Tschudi, N. Marcantonio, J. F. Anderson, J. R. Sauer, M. Cappello, F. S. Kantor, and E. Fikrig.** 2004. Disruption of *Ixodes scapularis* anticoagulation by using RNA interference. Proc. Natl. Acad. Sci. USA **101**:1141–1146.
36. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10**:1–6.
37. **Nishisaka, M., N. Yokoyama, X. Xuan, N. Inoue, N. Nagasawa, K. Fujisaki, T. Mikami, and I. Igarashi.** 2001. Characterisation of the gene encoding a protective antigen from *Babesia microti* identified it as eta subunit of chaperonin containing T-complex protein 1. Int. J. Parasitol. **31**:1673–1679.
38. **Osaki, T., M. Omotezako, R. Nagayama, M. Hirata, S. Iwanaga, J. Kasahara, J. Hattori, I. Ito, H. Sugiyama, and S. Kawabata.** 1999. Horseshoe crab hemocyte-derived antimicrobial polypeptides, tachystatins, with sequence similarity to spider neurotoxins. J. Biol. Chem. **274**:26172–26178.
39. **Otvos, L., Jr., K. Bokonyi, I. Varga, B. L. Otvos, R. Hoffmann, H. C. Ertl, J. D. Wade, A. M. McManus, D. J. Craik, and P. Bulet.** 2000. Insect peptides with improved protease-resistance protect mice against bacterial infection. Protein Sci. **9**:742–749.
40. **Ristic, M.** 1988. Babesiosis of domestic animals and man. CRC Press, Boca Raton, FL.
41. **Rossignol, P. A., J. M. Ribeiro, M. Jungery, M. J. Turell, A. Spielman, and C. L. Bailey.** 1985. Enhanced mosquito blood-finding success on parasitemic hosts: evidence for vector-parasite mutualism. Proc. Natl. Acad. Sci. USA **82**:7725–7727.
42. **Rudenko, N., M. Golovchenko, M. J. Edwards, and L. Grubhoffer.** 2005. Differential expression of *Ixodes ricinus* tick genes induced by blood feeding or *Borrelia burgdorferi* infection. J. Med. Entomol. **42**:36–41.
43. **Selsted, M. E., and A. J. Ouellette.** 2005. Mammalian defensins in the antimicrobial immune response. Nat. Immunol. **6**:551–557.
44. **Sonenshine, D. E., S. M. Ceraul, W. E. Hynes, K. R. Macaluso, and A. F. Azad.** 2002. Expression of defensin-like peptides in tick hemolymph and midgut in response to challenge with *Borrelia burgdorferi*, *Escherichia coli* and *Bacillus subtilis*. Exp. Appl. Acarol. **28**:127–134.
45. **Sonenshine, D. E.** 1991. Biology of ticks, vol. 1. Oxford University Press, New York, NY.
46. **Tang, Y. Q., J. Yuan, G. Osapay, K. Osapay, D. Tran, C. J. Miller, A. J. Ouellette, and M. E. Selsted.** 1999. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. Science **286**:498–502.
47. **Telford, S. R., and A. Spielman.** 1998. Babesiosis of humans, p. 349–359. *In* L. Collier, A. Balows, and M. Sussman (ed.), Topley & Wilson's microbiology and microbial infection, 9th ed., vol. 5. Arnold, London, England.
48. **Vial, H. J., and A. Gorenflot.** 2006. Chemotherapy against babesiosis. Vet. Parasitol. **138**:147–160.
49. **Vizioli, J., P. Bulet, J. A. Hoffmann, F. C. Kafatos, H. M. Müller, and G. Dimopoulos.** 2001. Gambicin: a novel immune responsive antimicrobial peptide from the malaria vector *Anopheles gambiae*. Proc. Natl. Acad. Sci. USA **98**:12630–12635.
50. **Yang, D., A. Biragyn, L. W. Kwak, and J. J. Oppenheim.** 2002. Mammalian defensins in immunity: more than just microbicidal. Trends Immunol. **23**: 291–296.
51. **You, M., X. Xuan, N. Tsuji, T. Kamio, D. Taylor, N. Suzuki, and K. Fujisaki.** 2003. Identification and molecular characterization of a chitinase from the hard tick *Haemaphysalis longicornis*. J. Biol. Chem. **278**:8556–8663.

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Parasitology in Japan

Disease burden and epidemiology of soil-transmitted helminthiases and schistosomiasis in Asia: the Japanese perspective

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The disease burden due to soil-transmitted helminthiases (STH) and schistosomiasis is not well documented in Asia. Both STH and schistosomiasis are chronic diseases but case detection is not easy because of the absence of clinical symptoms. STH and schistosomiasis are, however, endemic in Asia and their burden is significant. At the preparatory meeting for the Hashimoto Initiative in Japan in 1997, STH and schistosomiasis were categorized as Group 2 diseases. Parasitic infections in this category were well understood at the time but sophisticated control strategies were lacking. Japan has promoted comprehensive collaborative projects to reduce the burden of STH and schistosomiasis throughout Asia, creating an international network to collect epidemiological information and to implement and improve disease control, thus extending the school-based control method that had proved so successful in Japan.

Evaluation of disease burden

Helminth infections are important health problems in many parts of Asia but an exact evaluation of disease burden due to soil-transmitted helminthiases (STH) and schistosomiasis is not available because they are so-called 'neglected diseases'. There is no registration system for STH and schistosomiasis in Japan or other Asian countries. The current status of STH and schistosomiasis varies across the region. A recent increase in trade and human migration within Asia has highlighted the need to evaluate the epidemiological status of STH and schistosomiasis.

Japanese researchers have been proactive at building partnerships with Asian parasitologists to find applicable and effective strategies for parasite control. In the Hashimoto Initiative for global parasite control (HI) of 1997, STH and schistosomiasis were designated as Group 2 diseases [1]. The HI working group categorized parasitic diseases into three groups: Group 1 diseases require investment from the basic research stage to develop new treatments. Group 2 (which includes the filariases, in addition to STH and schistosomiasis) is a group of diseases for which control drugs already exist, and the priority is to establish a mechanism to deliver them to endemic areas. Group 3 falls between Group 1 and Group 2. This means that applied or operational research is needed, rather than basic research, for implementing disease control for STH and schistosomiasis. To discuss the Japanese perspective on STH and schistosomiasis in Asia, it is important to understand the current status of these parasitic infections in Japan and in East or Southeast Asian countries.

STH and schistosomiasis in Japan: then and now

Heavy disease burden due to STH and schistosomiasis in the first half of the 20th century was a strong driving force for parasitology research in Japan. In 1949, the incidence of STH peaked at 73% of the population [2] but the picture regarding schistosomiasis remains unclear. In 1950, only 1.6% of residents in the Kofu basin, which is in the central Yamanashi part of Japan, tested positive for *Schistosoma japonicum* in stool examinations using the direct smear method [3]. This relatively low incidence was probably a gross underestimation because a positive rate of *S. japonicum* infection of 44.2% was reported in the same area when some, but not all, health centers tested stools of schoolchildren using the centrifugal concentration (AMS III) method. Furthermore, single testing results in underestimates because repeated testing of fecal samples from residents of the Kofu basin raised the positive rate to more than twice that detected by the merthiolate-iodine-formaldehyde concentration (MIFC) method [4].

The most common STH in Japan in the first half of the 20th century was *Ascaris lumbricoides* infection, followed by *Necator americanus* infection. Night soil was widely used as a fertilizer for cultivation, resulting in contaminated vegetables, which were the main source of

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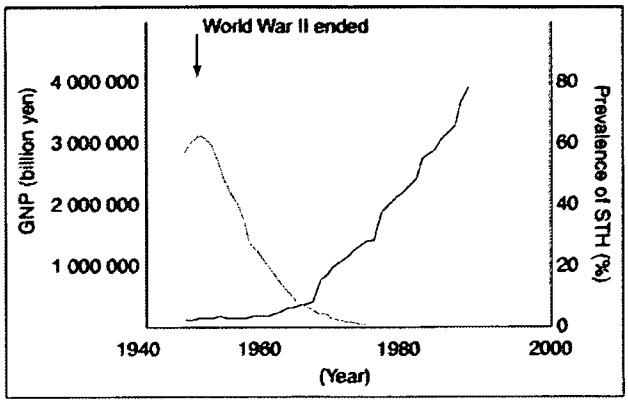

Figure 1. Control of parasitic diseases and economic development in Japan. The GNP (blue line) of Japan started to rise in the mid-1960s, whereas the prevalence of STH (green line) decreased rapidly before the economic growth in Japan. Reproduced, with permission, from M. Shimada, Nagasaki University.

STH. With the rapid improvement in quality of life in Japan (including the cessation of night soil use), the prevalence of STH fell rapidly and reached a negligible level in the late 1970s (Figure 1). Despite rapid economic growth in Japan in the 1960s, economic development was not the main factor behind successful STH control. Japan created a unique and effective scheme for parasite control after World War II, which was led by the private sector, the Japan Association of Parasite Control (JAPC) and supported by local governments [5]. JAPC implemented a school-based approach, in which teachers had the main role in health education and deworming, that was backed by the Japanese Government via proclamation of the School Health Law in 1958. The Japanese Organization for International Cooperation in Family Planning (JOICFP: http://www.joicfp.or.jp/) was also important and was established based on the unique idea of integrating STH control with family planning and nutritional improvement [6]. The parasite control activities of JAPC and the integrated programs contributed substantially to health promotion, not only in schools but also in communities because parasite control has proven to be a good starting point for encouraging community participation. Members of academia also had important roles in parasite control activities, not only by providing scientific guidance but also by evaluating the efficacy of the control interventions.

Currently, a small number of sporadic cases of STH infection still occurs in Japan. Several factors contribute to this: (i) there is a movement to use non-chemical fertilizer for vegetation, such as night soil, the use of which places more people at risk of *A. lumbricoides* infection; (ii) a specific group at risk comprises those who have lived for prolonged periods in other endemic countries, with >10% of people returning from Africa bringing back STH [7]; (iii) recently, food-borne STH from fresh food imported from endemic countries has also been identified [8]; and (iv) strongyloidiasis is still endemic in Okinawa and other southern Japanese tropical islands. A recent survey reported that the incidence of *Strongyloides stercolaris* infection is 5–10% in Okinawa, although infections were observed mainly in older age groups [9]. A strong association between strongyloidiasis and adult T-cell leukemia virus has been reported [10,11], although the biological mechanisms of the association remain to be elucidated. In addition, there is a small number of cases of opportunistic infection with *S. stercolaris* in immunocompromized people in Okinawa [12].

Schistosomiasis japonica was endemic in Japan, with two Japanese pathologists, Katsurada and Fujinami, discovering the causative parasite, *S. japonicum*, in 1904 [13]. Nine years later, Miyairi discovered *Oncomelania nosophora*, which is the intermediate host snail for *S. japonicum* [14]. These discoveries enabled the implementation of schistosomiasis control in the early 20th century [15]. Schistosomiasis is prevalent in several foci where intermediate snail host colonies exist. Although the intermediate host snails in Japan are of a single species, *S. japonicum* in each endemic focus in Japan has adapted only to *O. nosophora* of the same geographical origin [16,17]. This indicates that imported strains of *S. japonicum* are not readily introduced into Japan.

Since 1977, no newly infected cases of schistosomiasis have been reported in Japan and, in 1996, the local government in Yamanashi (Japan) declared that transmission had ceased. Since then, only imported cases have been reported, most of which were schistosomiasis from Africa. The disease control strategy for schistosomiais in Japan comprised three main approaches: (i) control of the snail intermediate host; (ii) treatment of all infected people; and (iii) concreting over the wetland habitat of the snail host [18]. Because schistosomiasis japonica is zoonotic, health checks of human residents and sampling of wild mice were the methods used for case detection. At a health check, intradermal skin tests were used to screen for schistosomal infection [19]. Although the cause is unclear, the hepatitis C virus appeared earlier in schistosomiasis-endemic areas than in schistosomiasis-free areas [20]. A similar association was reported in Egypt [21], where schistosomiasis is also endemic. *S. japonicum* infection might be carcinogenic [22], with epidemiological studies showing significant association between rectal and hepatic cancers and *S. japonicum* infection in Japan [23,24]. Cercarial dermatitis has also been reported, which is caused by schistosomes of birds (e.g. *Gigantobilharzia sturniae*). Rice farmers, in particular, are at risk because there are snails in paddy fields, and birds that feed on the snails perpetuate the life cycle of the parasite.

STH in Southeast Asia and China

STH and schistosomiasis are the most common helminth infections worldwide, especially in poor communities in Southeast Asia. STH are widely distributed throughout the region (Figure 2). It is estimated that, in 2003, 33.9 million people in Vietnam and 74.7 million people in the Greater Mekong Subregion (GMS) countries were infected with *A. lumbricoides*, and 17.6 million people in Vietnam and 32.9 million people in the GMS were infected with *Trichuris trichiura* [25,26].

There is only a small amount of recent published data about the disease burden and epidemiology of STH, and data are not available from many countries. Brooker *et al.*

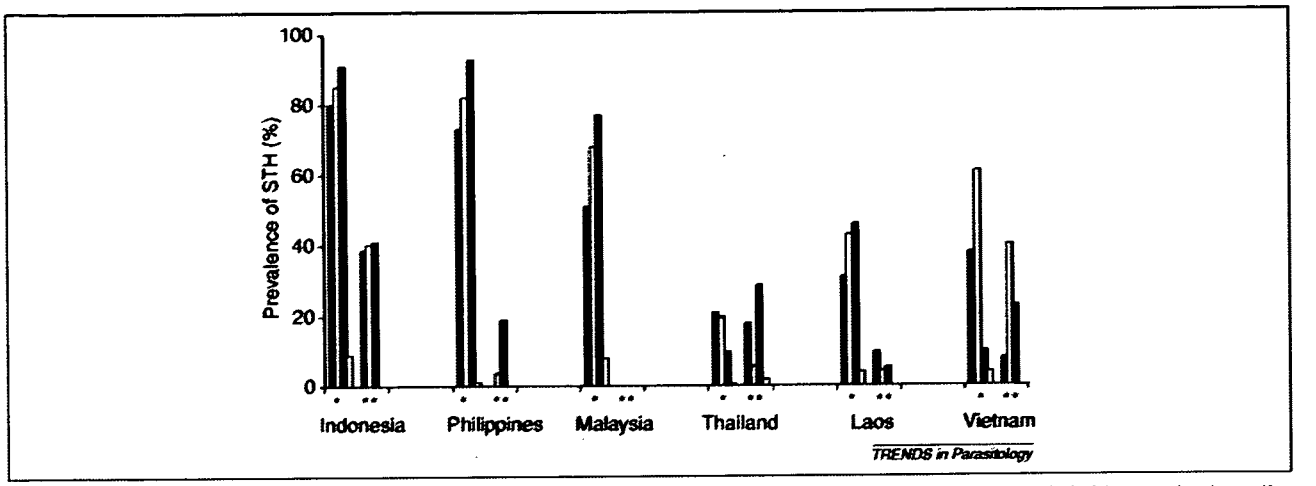

Figure 2. Prevalence of STH in Southeast Asia. The prevalence of STH is high in the south-central part (*) compared with the northern part (**) of the countries shown. Key: *Ascaris lumbricoides*, orange bars; hookworm, green bars; *Strongyloides stercoralis*, unfilled bars; *Trichuris trichiura*, blue bars.

[26] used a geographical information system (GIS) to collate and map STH distribution in Southeast Asia and found a distinct geographical variation throughout the region. In Vietnam, for instance, the prevalence of STH infection declined from the north to the south of the country [25], whereas higher prevalence occurred in the south of Thailand than in other parts of the country [27]. A report from Bali showed that wet highland had significantly higher STH prevalence than did wet lowland, dry highland or dry lowland [28]. In Malaysia, high levels of STH infection were reported in Orang Asli, an aboriginal tribe resettlement village [29]. This variation in STH occurs because transmission is strongly related to environmental and host behavioral factors (Table 1).

A national survey was carried out in China in 2003 to analyze the epidemiological status of helminthic infections [30]. Results showed that the infection rate of STH was 19.6%, of which ascariasis accounted for 12.7% (86 million people), followed by hookworm infections with 6.12% (39 million people) and whipworm infections with 4.63% (29 million people). One-third of infected individuals carried two or more parasites, with some infected by six species of parasite at the same time. The highest infection rate, with >10 000 hookworm and/or whipworm eggs per gram and >50 000 *A. lumbricoides* eggs per gram, was noted in Hainan Province (54.7%), with other southern areas such as Guizhou, Sichuan, Guangxi and Hunan showing an infection rate of at least 30%. STH in northern areas were less frequent, with the lowest infection rate reported in Xinjiang Province (0.72%). STH are most common in primary-school children and illiterate people in China, indicating that the STH infection rate decreases with higher education level. The helminthic infection rate in China seems to be declining, with heavy infections of *A. lumbricoides* and hookworm found in fewer than 2% of infected people in 2003.

Table 1. Factors influencing the transmission of STH and schistosomiasis

STH	Schistosomiasis
Environmental	
Tropical climate	Tropical climate
High humidity	Water (e.g. river)
Unhygienic sanitation	Unhygienic sanitation
Land surface temperature	Snail intermediate host
Night soil fertilizer	Reservoir hosts
Behavioral	
Toilet usage	Toilet usage
Personal cleanliness	Water contact
Occupation (e.g. farmer)	Occupation (e.g. farmer, fisherman)
Wearing shoes	

Schistosomiasis in Southeast Asia and China

Schistosoma blood flukes in Southeast Asia belong to the *S. japonicum* complex, which comprises three different species: *S. japonicum*, *Schistosoma mekongi* and *Schistosoma malaysiensis* [31]. They differ in morphology, geographical distribution, snail intermediate host and enzyme polymorphisms. Approximately 60 000 Laotians and 80 000 Cambodians are estimated to be at risk from schistosomiasis mekongi [32], and ~6.7 million Filipinos are at risk from schistosomiasis japonicum [33]. The best-known species is *S. japonicum*, which is found in China, the Philippines [34] and certain areas of Sulawesi, Indonesia [35]. In China, endemic foci occurred in seven provinces, and 843 000 infected individuals were reported in 2003. Two distinct types of schistosomiasis are endemic in China: marshland type and hill type. Approximately 11 million people are at risk from the marshland type and 5 million people are at risk from the hill type. Marshland schistosomiasis occurs in the mid- or lower reaches of the Yangtze and is under the direct influence of the environmental conditions of both the river and the surrounding lakes. Hill-type schistosomiasis occurs in western China. Although the endemic foci are not big, disease control is difficult because the foci are located in remote areas.

S. mekongi occurs in the Khong District of Laos and along the Mekong in Cambodia [32]. Up to 150 000 inhabitants were at risk of *S. mekongi* infection in the 1980s. However, the recent situation in the endemic area is much improved because of a mass treatment program led by the Cambodian Government, the World Health Organization (WHO: http://www.who.int) and Medicins sans Frontieres (http://www.msf.org). The program started in 1995, with a Japanese non-governmental organization (NGO), the

Sasakawa Memorial Health Foundation (http://www.sasakawa-igaku.or.jp), joining in 1997 [36].

S. malayensis infects various indigenous tribes in the upper Rejang river basin, Sarawak, Malaysia [37]. Among animal schistosome species, *Schistosoma spindale* is a common cause of cercarial dermatitis in humans in Indonesia, Malaysia, Thailand and Vietnam. This dermatitis is strongly associated with farmers and fishermen working in rice paddies [38].

More than ten species of mammal, including water buffalo, wild pigs, deer, horses, dogs, cats and rodents, are reservoir hosts of *S. japonicum*. Water buffalo and cattle are the most important hosts of this parasite in China, whereas dogs, but not water buffalo, are important hosts in the Philippines [32]. In the case of *S. mekongi*, 12.2% of pigs in Laos and 0.3–3.6% of dogs in Cambodia are hosts [39–41]. *Oncomelania hupensis hupensis* and *Oncomelania hupensis quadrasi* are the snail hosts for *S. japonicum* in China and the Philippines, respectively; however, apparent strain differences are noted within *O. h. hupensis* [42]. *Neotrichura aperta* is the only species of snail known to be a host of *S. mekongi* [43], and *S. malayensis* uses *Robertsiella kaporensis* as its intermediate host [37]. The restricted distribution of these snails limits the endemic areas of schistosomiasis. A study of rats in proximity to snail colonies showed that 95.5% of rats caught within a snail colony were positive for schistosomiasis, 56.5% of rats caught within 100 m of a snail colony were positive and no rat caught $\geq$1 km from a snail colony was positive for schistosomiasis. Existing control programs in endemic areas aimed at improving sanitation and reducing both the number and the size of snail habitats have led to decreased infection rates among rats and snails [44]. Common locations relating to snail breeding sites that increase the presence of the disease are irrigation networks and agricultural land [45] (Table 1).

Results of studies on the epidemiology and immunology of schistosomiasis in the Philippines indicate that the individuals who are most vulnerable to rapid reinfection are 5–14-year-old children. In China, however, high incidence is observed even in adults [46]. A drop in incidence at age 15–19 years and decreased intensity of infection at this age and in older Filippino people indicate the development of immunity [47]. Schistosomes, *T. trichiura* and hookworms cause anemia, and co-infections of these species increase the likelihood of anemia, particularly in 5–14-year-old children. Carcinogenesis associated with *S. japonicum* has also been found in China, and specific effects on mutagenicity have been suggested [48].

Schistosomiasis control in China has been implemented since 1955, when endemic situations were serious in areas along the Yangtze, including Shanghai, Wuhan and other big cities. Although the endemic situation has improved, a renewed effort to eliminate schistosomiasis was mounted as a collaborative project with the World Bank (http://www.worldbank.org) in 1992. Over eight years, a nationwide mass chemotherapy program was implemented and one endemic province, Zejiang, declared the disease to be eradicated in 1996. During the program, >200 research projects, both applied and operational, were promoted and new therapeutics, diagnostics, epidemiological techniques and cost-effective operational approaches were investigated [49]. China is considered to be in the final stage of disease eradication; however, there are several obstacles to overcome before reaching this goal [50]. Despite the intensive control program, a warning was recently issued by the Chinese Center for Disease Control and Prevention (http://www.chinacdc.net.cn) that there is a reemergence of schistosomiasis japonica in China. A nationwide survey was carried out in 2005, the results of which will be made public in the near future. Construction of the Three Gorges Dam will be completed in 2006 and the distribution of endemic foci is anticipated to change because of the changing water levels of the Yangtze. Because selective chemotherapy is undertaken during low disease prevalence in China, it is important to develop a sensitive, but cost-effective, case-detection system. Intensive surveys that use new tools and techniques are needed to create a new strategy for schistosomiasis control in China.

The viewpoint in Japan

Historically, the transmission of STH has been related to social infrastructure, including water supply, toilet facilities and sanitation, lifestyle, cultivation techniques and food distribution. Therefore, STH are considered to be a socioeconomic matter. However, in the case of Japan, the economy did not have an important role in the control of STH. Instead, the gross national product increased in Japan just after the successful control of infectious diseases such as STH (**Figure 1**). This means that improved public health conditions preceded economic growth. Attitude changes, based on improved knowledge and experiences, resulted in successful parasite control. Control is not expensive, yet the presence of STH and schistosomiasis is an inhibitory factor for socioeconomic development, and this is the most obvious consequence of disease burden due to helminth infection.

Japanese scientists have sought to build a close relationship with researchers in other Asian countries in both basic and applied research into parasitic diseases. Bilateral cooperative overseas aid orchestrated by the Japan International Cooperation Agency (JICA: http://www.jica.go.jp) has helped to strengthen the training aspects needed for parasite control, which are based on the lessons learned during previous success in Japan. One of the successful programs promoted by JICA is the HI [51]. In 2000, JICA established the Asian Center of International Parasite Control (ACIPAC: http://www.tm.mahidol.ac.th/en/seameo/thailand.htm) at Mahidol University, Bangkok, as the first center within the HI. Training courses for the school-based control of malaria and STH for program managers were organized by ACIPAC for health personnel and educators from central to provincial levels. Approximately 111 personnel, mostly from GMS countries, were trained between 2001 and 2005. Small-scale pilot projects (SSPPs) on school-based STH control, supported by JICA, have been conducted in Cambodia, Laos, Myanmar and Vietnam.

The contribution made by Japanese NGOs to parasite control should also be emphasized. The JAPC supported the Asian Parasite Control Organization (APCO), which was established in 1974. Between 1977 and 1999, an APCO

training course that used school-based STH control to gain entry into the community was conducted at Mahidol University and its partner institutions (the Faculty of Public Health and the Ministry of Public Health). More than 530 health personnel in Asia have been trained and evaluation of the training, conducted after the 20th course, showed that >50% of ex-participants continue to work in the field of parasite control (J. Waikagul, unpublished). Another bilateral cooperation scheme is the US–Japan Cooperative Medical Science Program, which was established in 1964 to focus research on diseases that are prevalent in Asia, with parasitology research being one of the main subjects. Through these cooperation schemes, the exchange of scientific information among Asian parasitologists is increasing and, by combining various bilateral cooperation programs, the Federation of Asian Parasitologists was officially established in 2001. More recently, the Japanese Government launched a new strategy for the research and control of infectious diseases by collecting biological and epidemiological information in Asia and Africa. In addition, three centers were established in Thailand, Vietnam and China as cooperative projects with Osaka University (http://ww.osaka-u.ac.jp), Nagasaki University (http://www.nagasaki-u.ac.jp) and the University of Tokyo (http://www.u-tokyo.ac.jp), respectively.

Future perspectives

Collaborative projects between Japan and other Asian countries have been ongoing in the field of basic research into parasitology and disease control, and will increase in number in the future. In particular, the development of vaccines and new therapeutics is an urgent research subject for *S. japonicum*. Several groups from Japan are, with Chinese colleagues, undertaking research projects on schistosomiasis vaccine development. Paramyosin and calpain of *S. japonicum* were tested as vaccine candidates [52–54], and partial but significant vaccine effects were observed in a field trial using domestic pigs [55,56]. Qinhaosu derivatives have been intensively investigated as new therapeutic and/or prophylactic drugs for Asian schistosomiasis [57,58]. Artemether was also shown to have prophylactic effects on various schistosome species [59]. Artesunate was also effective, not only against *S. japonicum* but also against *S. mansoni* infection; however, the optimal protocols for artesunate treatment were different between *S. japonicum* and *S. mansoni* infections [60]. Side effects were not observed and complete cure rate was confirmed.

The monitoring of intermediate host snails by remote sensing was investigated as a novel epidemiological tool for schistosomiasis. In the Philippines, a research group from Japan proposed a monitoring system that uses digital maps of Landsat images to which epidemiological information is added [61]. A similar system was developed to monitor the reemergence of schistosomiasis japonica in a former endemic focus of the disease in Japan [62].

Cooperative projects are being intensively promoted for STH and schistosomiasis control. Children of primary-school age are most affected by STH and schistosomiasis. Deworming is a preventive control measure but, to keep reinfection rates as low as possible, preventive education must be implemented widely and continuously throughout the region. A regional training program of effective education for trainers is necessary but the program remains in a low profile at present. ACIPAC activities are continuing as a collaborative project between Japan and Thailand, and SSPPs in the surrounding countries are encouraged to develop into country-level projects. In fact, as a result of this activity and the support of other international organizations, a National Intestinal Parasite Control Program has been started in Cambodia and Laos [63].

Although the prevalence of STH has decreased compared with the prevalence in the 1980s, these diseases remain a major public health problem in Southeast Asia. Extensive training programs are still needed in the region to support national programs for parasite control. ACIPAC, together with collaboration from Japan, is ready to be a partner of other international and local organizations and agencies to provide training based on the successful Japanese model of school-based control programs for parasites and other infectious diseases.

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References

1 Working Group on Global Parasite Control (1998) The global parasite control for the 21st century. Government of Japan
2 Yokogawa, M. (1993) 40 year history of parasite control, preventive medicine. In *Parasitic Infection Special Issue*, Kanagawa Association of Preventive Medicine
3 Yokogawa, M. (1970) Schistosomiasis japonica in Japan. In *Recent Advances in Research on Filariasis and Schistosomiasis in Japan* (Sasa, M., ed.), pp. 231–235, University of Tokyo Press
4 Iijima, T. *et al.* (1962) Studies on diagnosis of schistosomiasis. I. Statistical studies on recovering schistosome eggs in human feces with repeated MIFC technique. *Jpn J. Parasitol.* 11, 483–487
5 Kunii, C. (1992) *It All Started From Worms*, The Hoken Kaikan Foundation (http://www.joicfp.or.jp/eng/publications/worms.shtml)
6 Kobayashi, A. *et al.* (2006) Historical aspects for the control of soil-transmitted helminthiasis. *Parasitol. Int.* 55, S289–S291
7 Hamada, A. *et al.* (2003) Changes in the prevalence of intestinal parasites among Japanese expatriates in developing countries. *Kansensyogakuzassi* 77, 138–145
8 Ohta, N. *et al.* Parasite eggs contaminated in imported pickles in Japan. *Clinical Parasitol* (in press)
9 Zaha, O. *et al.* (2000) Strongyloidiasis: progress in diagnosis and treatment. *Intern. Med.* 39, 695–700
10 Sato, Y. and Shiroma, Y. (1989) Concurrent infections with *Strongyloides* and T-cell leukemia virus and their possible effect on immune responses of host. *Clin. Immunol. Immunopathol.* 52, 212–224
11 Pulummele, Y. and Edouard, A. (1996) *Strongyloides stercoralis* in T-cell leukemia/lymphoma in adults and acquired immunodeficiency syndrome. *Rev. Med. Interne* 17, 125–129
12 Ohnishi, K. *et al.* (2004) Strongyloidiasis in a patient with acquired immunodeficiency syndrome. *J. Infect. Chemother.* 10, 178–180
13 Fujinami, A. (1904) Further discussion of the Katayama disease and its causative parasite. *Kyoto Igakkai Zasshi* 1, 201–213
14 Sasa, M. (1972) A historical review of the early Japanese contributions to the knowledge of schistosomiasis japonica. In *Researches in Filariasis and Schistosomiasis* (Yokogawa, M., ed.), pp. 235–261, US–Japan Cooperative Medical Science Program
15 Tanaka, H. and Tsuji, M. (1997) From discovery to eradication of schistosomiasis in Japan: 1847-1996. *Int. J. Parasitol.* 27, 1465–1480

16 Iwanaga, Y. *et al.* (1976) Observation on the susceptibility of *Oncomelania* spp. to *Schistosoma japonicum*, Yamanashi strain. 2. The susceptibility of *Oncomelania* spp. to the different geographical strains of *S. japonicum*. *Jpn J. Parasitol.* 25, 69–79
17 Ohmae, H. *et al.* (2003) Biological characteristics and control of intermediate snail host of *Schistosoma japonicum*. *Parasitol. Int.* 52, 409–417
18 Hunter, G.E., III and Yokogawa, M. (1984) Control of schistosomiasis japonica in Japan. A review – 1950–1998. *Jpn J. Parasitol.* 33, 341–351
19 Minai, M. *et al.* (2003) Histological view of schistosomiasis japonica in Japan: implementation and evaluation of disease-control strategies in Yamanshi Prefecture. *Parasitol. Int.* 52, 321–326
20 Tanaka, Y. *et al.* (2005) Molecular evolutionary analyses implicate injection treatment for schistosomiasis in the initial hepatitis C epidemics in Japan. *J. Hepatol.* 42, 47–53
21 Heral, T. *et al.* (1998) The relationship between hepatitis C virus and schistosomiasis: histologic evaluation of liver biopsy specimen. *Hum. Pathol.* 29, 743–749
22 International Agency for Research on Cancer, (1994) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Schistosomiasis, Liver Flukes and* Helicobacter pylori (Vol. 61), IARC (**http://monographs.iarc.fr/ENG/Monographs/vol61/volume61.pdf**)
23 Inaba, Y. (1984) A cohort study on the causes of death in an endemic area of schistosomiasis japonica in Japan. *Ann. Acad. Med. Singapore* 13, 142–148
24 Takemura, Y. *et al.* (1998) Epidemiologic study of the relationship between schistosomiasis due to *Schistosoma japonicum* and liver cancer/cirrhosis. *Am. J. Trop. Med. Hyg.* 59, 551–556
25 Van der Hoek, W. *et al.* (2003) Current status of soil-transmitted helminths in Vietnam. *Southeast Asian J. Trop. Med. Public Health* 34 (Suppl. 1), 1–11
26 Brooker, S. *et al.* (2003) Mapping soil-transmitted helminths in Southeast Asia and implications for parasite control. *Southeast Asian J. Trop. Med. Public Health* 34, 24–36
27 Jongsuksantigul, P. *et al.* (2003) Evaluation of helminthiasis control program in Thailand at the end of the 8th health development plan, 2001. *J. Trop. Med. Parasitol.* 26, 38–46
28 Widjana, D.P. and Sutisna, P. (2000) Prevalence of soil-transmitted helminth infections in the rural population of Bali, Indonesia. *Southeast Asian J. Trop. Med. Public Health* 31, 454–459
29 Zulkifli, A. *et al.* (1999) The prevalence and intensity of soil-transmitted helminthiasis among pre-school children in Orang Asli resettlement villages in Kelantan. *Med. J. Malaysia* 54, 453–458
30 Ministry of Health, China (2005) Report on the National Survey of current situation of major human parasitic diseases in China.
31 Agatsuma, T. (2003) Origin and evolution of *Schistosoma japonicum*. *Parasitol. Int.* 52, 335–340
32 Urbani, C. *et al.* (2002) Epidemiology and control of mekongi schistosomiasis. *Acta Trop.* 82, 157–168
33 Leonardo, L.R. *et al.* (2002) Difficulties and strategies in the control of schistosomiasis in the Philippines. *Acta Trop.* 82, 295–299
34 Blas, B.L. *et al.* (2004) The schistosomiasis problem in the Philippines: a review. *Parasitol. Int.* 53, 127–134
35 Izhar, A. *et al.* (2002) Recent situation of schistosomiasis in Indonesia. *Acta Trop.* 82, 283–288
36 Ohmae, H. *et al.* (2004) Schistosomiasis mekongi: from discovery to control. *Parasitol. Int.* 53, 135–142
37 Sagin, D.D. *et al.* (2001) Schistosomiasis malayensis-like infection among the Penan and other interior tribes (Orang Ulu) in upper Rejang River Basin, Sarawak Malaysia. *Southeast Asian J Trop. Med. Public Health* 32, 27–32
38 Nithiuthai, S. *et al.* (2004) Waterborne zoonotic helminthiases. *Vet. Parasitol.* 126, 167–193
39 Iijima, T. *et al.* (1971) Studies on schistosomiasis in the Mekong Basin. I. Morphological observation of the schistosomes and detection of them in reservoir hosts. *Jpn J. Parasitol.* 20, 24–33
40 Strandgaard, H. *et al.* (2001) The pig as a host for *Schistosoma mekongi* in Laos. *J. Parasitol.* 87, 708–709
41 Matsumoto, J. *et al.* (2002) The first reported cases of canine schistosomiasis mekongi in Cambodia. *Southeast Asian J. Trop. Med. Public Health* 33, 458–461
42 Wilke, T. *et al.* (2001) *Oncomelania hupensis* (*Gastropoda*: rissooidea) in eastern China: molecular physiology, population structure, and ecology. *Acta Trop.* 77, 215–227
43 Attwood, S.W. *et al.* (1997) Infectivity of a Cambodian isolate of *Schistosoma mekongi* to *Neotricula aperta* from northeast Thailand. *J. Helminthol.* 71, 183–187
44 Fedoko, J.M. (1999) *Schistosoma japonicum* in the black rat, *Rattus rattus mindanensis*, from Leyte, Philippines in relation to *Oncomelania* snail colonies with reference to other endoparasites. *Southeast Asian J. Trop. Med. Public Health* 30, 343–349
45 Leonardo, L.R. *et al.* (2005) A study of the environmental determinants of malaria and schistosomiasis in the Philippines using remote sensing and geographic information systems. *Parasitologia* 47, 105–114
46 Ministry of Health, China (1993) Epidemiological situation of schistosomiasis in China. Results from a nationwide sampling survey in 1989
47 Acosta, L.P. (2002) Immune correlate study on human *Schistosoma japonicum* in a well-defined population in Leyte, Philippines: I. Assessment of 'resistance' versus 'susceptibility' to *S. japonicum*. *Acta Trop.* 84, 127–136
48 Zhang, R.L. *et al.* (1998) Mutations in *p53* gene in Chinese patients with rectal cancer associated with schistosomiasis japonica. *Cancer Lett.* 135, 215–221
49 Yuan, H.C. *et al.* (2000) The 1992-1999 World Bank schistosomiasis Research Initiative in China: outcome and perspective. *Parasitol. Int.* 49, 195–207
50 Kojima, S. (2005) Schistosomes: Asia. In *Topley and Wilson's Microbiology and Microbial Infections* (10th edn) (Cox, F.E.G. *et al.*, eds), pp. 628–636, Hodder Arnold
51 Kojima, S. and Takeuchi, T. (2006) Global parasite control initiative of Japan (Hashimoto Initiative). *Parasitol. Int.* 55, S293–S296
52 Nara, T. *et al.* (1994) Demonstration of the target molecule of a protective IgE antibody in secretary glands of *Schistosoma japonicum* larvae. *Int. Immunol.* 6, 963–971
53 Kalinna, B.H. and McManus, D.P. (1997) A vaccine against the Asian schistosome, *Schistosoma japonicum*: an update on paramyosin as a target of protective immunity. *Int. J. Parasitol.* 27, 1213–1219
54 Zhang, R.L. *et al.* (2001) Vaccination with calpain induces a Th1-biased protective immune response against *Schistosoma japonicum*. *Infect. Immun.* 69, 386–391
55 Chen, H.G. *et al.* (2000) Vaccination of domestic pig with recombinant paramyosin against *Schistosoma japonicum* in China. *Vaccine* 18, 2142–2146
56 Ohta, N. *et al.* (2004) Research on calpain of *Schistosoma japonicum* as a vaccine candidate. *Parasitol. Int.* 53, 175–181
57 Xiao, S. *et al.* (1995) Experimental studies on early treatment of schistosomal infection with artemether. *Southeast Asian J. Trop. Med. Public Health* 23, 306–318
58 Li, S. *et al.* (1996) Studies on prophylactic effect of artesunate on schistosomiasis japonica. *Chin. Med. J. (Engl.)* 109, 848–853
59 Xiao, S. *et al.* (2002) Recent investigations on artemether, a novel agent for the prevention of schistosomiasis japonica, mansoni and hematobia. *Acta Trop.* 82, 175–181
60 Lu, S.H. *et al.* (2005) Evaluation of the anthelmintic effects of artesunate against experimental *Schistosoma mansoni* infection in mice using different treatment protocols. *Parasitol. Int.* 55, 63–68
61 Nihei, N. *et al.* (1998) Soil factors influencing the distribution of *Oncomelanis quadrasi*, the intermediate host of *Schistosoma japonicum*, on Bohol Island, Philippines. *Ann. Trop. Med. Parasitol.* 12, 669–710
62 Nihei, N. *et al.* (2004) Fixed-point observation of *Oncomelania nosophora* in Kofu basin – establishment monitoring system of schistosomiasis japonica in Japan. *Parasitol. Int.* 53, 197–205
63 Kojima, S. *et al.* (2006) Asian Centre of International Parasite Control (ACIPAC): five years achievements. I. Introduction. *Southeast Asian J. Trop. Med. Public Health* 36 (Suppl. 3), 1–12

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Review

Toxocariasis in Japan

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Abstract

Toxocariasis has long been considered a parasitic disease affecting pet owners and children who often play in sandboxes at public parks. Recent cases of this animal-borne infection, however, indicate that its clinical manifestations and etiologies are changing. In this article, we will describe the critical characteristic features of toxocariasis alongside the contributions of Japanese researchers to a better understanding of the disease.

Keywords: *Toxocara canis*; *Toxocara cati*; Toxocariasis; Visceral larva migrans

Contents

1. Introduction

Among animal-borne diseases, toxocariasis is one of the most popular parasitic infections in the world, caused by the larval stage of *Toxocara* spp. Humans are infected mainly by the tiny developmental stage of the parasite, which belong to the family Ascaridoidea, through their pet dogs and cats. Other natural hosts include wild Canidae for *Toxocara canis* and wild felines for *Toxocara cati*. Symptoms depend on organs affected and the magnitude of infection. It is usually a non-fatal disease, but the larvae migrate through the eyes and can cause severe vision disability or even blindness.

In 1950, Dr. Wilder, an American ophthalmologist, histopathologically identified a nematode of unknown etiology in the retinas of 26 out of 46 enucleated eyes with retinoblastoma [1].

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Two years later, Beaver et al. [2] recognized the same parasite in the liver of three young children. Shortly afterwards, the parasite was correctly identified as an infectious stage larva of *T. canis* [3–5]. Since then, many clinicians and biologists have been accumulating knowledge of *Toxocara* and toxocariasis.

In this review article, we describe the lesser-known contributions of Japanese researchers to the understanding of *Toxocara* and toxocariasis. This articles builds on the work of Kondo [6], focusing on the topics that he did not cover in his review and on new findings since his publication.

2. Toxocariasis in humans

2.1. Clinical cases

Toxocariasis is clinically classified into four types: visceral, ocular, neurologic, and covert [7,8]. In 1963, the first report on toxocariasis in Japan was presented orally at the 32nd Annual Meeting of the Japanese Society of Parasitology by Fushimi et al. [9]. A 14 year-old boy was admitted to a university hospital because of fever, hepatomegaly and persistent eosinophilia. The patient died from severe anemia six months later. Though no autopsy or serological examinations were performed, the patient was strongly suspected to have suffered from visceral toxocariasis. In the early 1960s, immunological tests for parasitic infections, especially for helminthiasis, had only just begun, and antigen for the diagnosis of toxocariasis was not yet known.

Just as in other parasitic infections, direct demonstration is the only way to make definite diagnosis of toxocariasis. However, it is difficult to find the larva in either tissue biopsies or autopsies due to its very small size. So far in Japan, one morphologically and two pathologically confirmed cases have been reported [10–12]. Two additional reports, both of ocular toxocariasis, were doubtful because of the lack of characteristic features of the parasite; the authors nevertheless reproduced the microscopic findings of the purported larva in their papers [13,14]. One of these two cases showed increased antibody production in vitreous fluid against *Toxocara* antigen prepared from larval excretory–secretory product (LES), suggesting that the case might be attributable to ocular toxocariasis.

Serology is an alternative method for the diagnosis of toxocariasis. A method has been established for *in vitro* cultivation of the larvae, with LES prepared from the culture medium serving as an antigen. Detection of specific antibodies against LES provides evidence of *Toxocara* infection in individual patients and useful tool for understanding the epidemiological characteristics of this disease. The first serological survey in Japan was reported by Matsumura and Endo [15] using sera of 83 clinically healthy children. In their sample, 3.6% tested were positive for LES. In another study, Matsumura and Endo [16] demonstrated that 20 of 530 adults possessed the IgG antibody to LES. The positive individuals were thought to have a latent or past infection. In a large-scale seroepidemiolgical survey, Kondo et al. [17] collected 3277 sera from 14 prefectures in Japan and tested for LES antibodies. Antibodies were confirmed in 52 individuals (1.6%), but geographical patterns were notable: the highest prevalence rate was observed in Miyagi Prefecture (6.1%), and the lowest was in Ibaragi Prefecture (0.5%). The researchers concluded that the overall seroprevalence rate was in good agreement with those reported from other countries [17–19].

Based on improvements in the field of serology, diagnosis of toxocariasis is usually made by detection of the specific antibody to LES, along with clinical manifestations such as eosinophilia, eosinophilic pneumonia, or ophthalmoscopic findings.

2.2. Characteristic features of toxocariasis

2.2.1. Toxocariasis as a food-borne infectious disease

Using serological methods, there were nearly 200 reports of toxocariasis in the database of Japana Centra Revuo Medicina, and almost 300 cases have been diagnosed in Japan in the past two decades. Among these cases, some significant reports have provided a new perspective on the pathogenic mechanisms of toxocariasis.

Since Beaver et al. [2] introduced the concept of visceral larva migrans, characterized by chronic eosinophilia with granulomatous lesions in the liver, toxocariasis was regarded as a disease in children who were infected by soil contaminated with embryonated eggs [20]. In 1983, Sakai et al. [21] reported a case toxocariasis after ingestion of raw chicken liver. The 57-year-old man was admitted to a hospital due to cough, fever and weight loss. Complete blood count revealed a marked increase in eosinophils in peripheral blood with leukocytosis, and serum antibody against *T. canis* was strongly positive. Before onset, he and his friends had eaten raw chicken livers derived from his poultry and boar farm. Soon after the meal, they experienced abdominal pain, vomiting and diarrhea, but the symptoms improved within two days after ingestion. One month later, his chief complaints emerged. Two similar cases were subsequently reported by the same group [22].

These cases clearly indicates that the disease should be considered a food-borne parasitic infection. Four additional papers describing six patients were published in Japan in the 1980s [22–25]. These patients, all male and between 22 and 51 years of age, had a history of eating raw meat or liver of fowl and/or cattle before onset of symptoms. The possibility that raw liver of domestic animals can transmit the pathogens of human visceral larva migrans was substantiated by Lee et al. [26] of Yonsei University College of Medicine in Korea. They found that a dietary habit of raw liver was much more frequently seen in males than in females, especially in the 31–40 age group. Experimental studies revealed that chicken, cattle and swine were able to act as paratenic hosts for *T. canis* [27–29]. Most of the adult cases reported in recent years in Japan are categorized as this type of infection [30].

2.2.2. Respiratory illness and toxocariasis

In animal models in rodents, hatched larvae migrate into the lungs through the liver after ingestion, resulting in liver dysfunction and pneumonia [31–33]. In humans, similar manifestations are well documented in the literature [30,34–36]. Pulmonary lesions appear on computed tomography as multifocal subpleural nodules with halos or ground-glass

opacities and ill-defined margins. Additionally, transient pulmonary infiltrates are a characteristic finding. Morimatsu et al. [30] recently reported a familial case of visceral toxocariasis after consumption of raw chicken livers. In this case, the patients, a father (71 years old) and his son (45 years old), ate raw chicken livers three weeks before onset and then developed mild fever, general fatigue, headache and respiratory disorder. The specific antibody to LES was identified both in their serum samples and in bronchoalveolar lavage fluid (BALF). *T. canis* larvae were recovered from chicken liver from the same source as that ingested by the patients. These cases showed that BALF is a reliable specimen to demonstrate LES antibodies when the patient shows respiratory illness.

2.2.3. Urticaria-like skin lesions and toxocariasis

Parasitic infection is often said to be associated with chronic urticaria [37]. This is still a controversial issue, but acute urticaria is certainly associated with infection with larva from the marine fish parasite, *Anisakis simplex* [38]. Japanese have long tradition of eating raw fish, sashimi and sushi, and anisakidosis is a common parasitic infection in Japan. It is well documented that urticaria is closely related to the infestation of *Anisakis* larva [38,39]. As with anisakidosis, an allergic reaction could be elicited by the invasion of *Toxocara* larvae and result in skin rash that looks like hives. These skin manifestations might occur as a result of immunological response to larval metabolites [40,41].

In 1999, the first confirmed case of toxocariasis with larva in subcutaneous tissue was reported [11]. A 26-year-old female with fever, headache, and dry cough was admitted to a university hospital. Her peripheral blood smear showed an eosinophilia (61%) and her chest radiograph revealed multiple nodules. A diagnosis of visceral toxocariasis was made after detection of LES antibodies. During her hospitalization, several brown itchy nodules, which were thought to be prurigo, developed on her legs. Histological examination showed *Toxocara* larva in the center of an eosinophilic and lymphocytic abscess. The patient admitted frequently eating raw beef liver almost one year before her hospitalization for its purported health benefits. We can learn from this case that larvae migrating into subcutaneous tissue directly elicit pruriginous skin lesions.

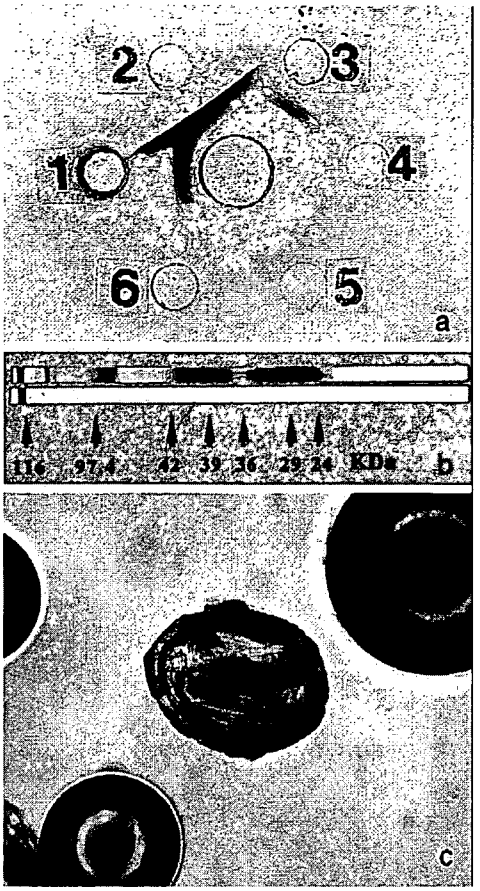

Fig. 1. The results of double gel diffusion (a) and western blot (b) tests of a patient of visceral toxocariasis. Strong precipitin bands were obviously observed between larval excretory–secretory products (LES) of *Toxocara canis* and patient's serum by means of double gel diffusion test. Antigens used in this test were adult worm extract (AEX) of *T. canis* (1), LES of *T. canis* (2), AEX of *Dirofilaria immitis* (3), AEX of *Ascaris suum* (4), LES of *Anisakis simplex* (5) and AEX of *Ascaris lumbricoides* (6). Western blot test shows a whole range of LES molecules were reacted with the patient's serum (upper strip) but not with a normal control serum (bottom strip). An embryonated egg recovered from the soil in the park where the patient often played (c). A fully developed and live *Toxocara* larva was found in the egg.

2.2.4. Toxocariasis is a disease that affects adults rather than children

Many reviews from western countries indicated that children under 12 years old, who often play outside, are the most affected age group for toxocariasis [42,43]. They are accidentally infected with *T. canis*/*T. cati* eggs, which expelled in feces puppies and fully develop in the surrounding environment within two to four weeks. Therefore, contaminated soil is the most important etiological source for toxocariasis [44,45]. Hori et al. [46] reported a case of visceral toxocariasis in a 1.5-year-old girl with fever, hepatomegaly, and eosinophilia (73%). The patient had a history of pica, particularly eating soil from a nearby park where she frequently played with her brother. Serological examination strongly suggested that she was suffering from *Toxocara* infection (Fig. 1a, b). They also found many embryonated eggs from the soil in the park that contained a live larva closely resembling *T. canis* eggs (Fig. 1c). Fortunately, her brother showed a negative result in serological tests.

In a review article of Barriga [47], the average age of visceral toxocariasis was 9.5 years, and only 18% of patients were adults. However, in recent investigations, adults rather than young children were more frequently affected by this parasite. This tendency is particularly true for ocular toxocariasis. Yoshida et al. [48] described that, among 38 Japanese cases of ocular toxocariasis, 34 (89%) were older than 20 years of age, and suggested that clinical features observed in these patients were somewhat different from those of previously reported cases [49]. Therefore, ocular toxocariasis is no longer merely a disease of young children, but affects any age group having a risk factor such as consumption of raw meat or close contact with contaminated soil.

As of the end of 2006, 584 clinically suspected cases of toxocariasis (112 of visceral type and 472 of ocular type) have been referred to our laboratory for detection of the anti-*Toxocara* antibody. We omitted 109 cases from this study due to a lack of description of the patient's age and sex. In visceral toxocariasis, the male-to-female ratio in the remaining sample was 2.04 (male: 53, female 26). The average age was 39.2± 21.7 (range, 0–83 years old) in male and 31.3±23.9 8range, 0.5–82 years old) in female. On the other hand, the male-to-female ratio in ocular toxocariasis group was 1.16 (male: 213, female: 183). The average age was 39.3±18.5 among males (range, 2–83 years old) and 37.6±18.2 among females (range, 2–74 years old). There were no significant differences in age distribution between males and females (Fig. 2). A similar result was obtained by Fujino et al. in 1998 [50].

2.2.5. Myelitis and toxocariasis

According to the case-control study by Magnaval et al. [51], migration of *T. canis* larvae in the human brain does not frequently induce recognizable neurological signs, but is possibly responsible for repeated low-dose infections. These light parasitic burdens usually do not appear to elicit a special clinical symptom, but in some cases, sever neurological disorders such as encephalitis, myelitis and meningitis are manifested [52]. In Japan, Ota et al. [53] reported a case of eosinophilic meningo-encephalo-myelitis due to *Toxocara* infection. The patient, a 21-year-old woman, showed frontal headache, low-grade fever and convulsion. She had a long history of close contact with her pet dog. Immunological tests were strongly positive for LES antigen in both her serum and cerebrospinal fluid. Based on clinical evidence and characteristic features in similar patients, Kira and his colleagues proposed a new disease entity: "atopic myelitis" or "parasitic myelitis." They assumed that allergic reaction to LES might be involved in this neurologic disorder [54]. Interestingly, most of the patients lived in Kyushu District, in the south of Japan, suggesting that myelitis due to *Toxocara* infection might be a regional clustering disease.

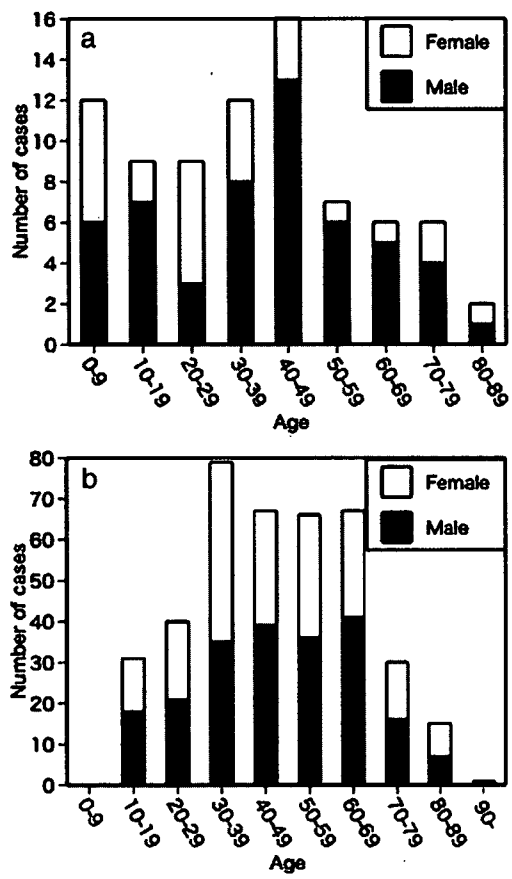

Fig. 2. Age distribution of suspected cases of visceral (n=79)(a) and ocular toxocariasis (n=396)(b) referred to our laboratory from August 1994 to December 2006.

2.3. T. cati

Because morphological differences between *T. canis* and *T. cati* in the adult stage are apparent [55], *T. cati* is easy to identify when patients expel adult worms. It has been suggested that *T. cati* could develop in children through the ingestion of the immature worm of *T. cati* [56]. More than 26 cases were reported so far [56,57], but there was only one case was reported from Japan. A 5-year-old male boy was admitted to a hospital due to a complaint of vomiting 3 worm-like foreign bodies. These worms were morphologically identified as two female and one male immature worms [58].

On the contrary, there are few reports of human intestinal infection with adult worms of *T. canis* [59], and many of these are believed to be erroneous observations [60]. Serological discrimination between toxocariasis canis and toxocariasis cati, however, is not so apparent, because of complete cross-reactivity between the two LESs, although *T. cati*-specific LES has been identified [61]. Therefore, distinguishing between *T. canis* and *T. cati* is even more difficult if somatic antigens are used in the serological diagnosis [62–64]. For the precise serodiagnosis of toxocariasis, a great deal of additional research effort is needed to obtain *T. cati*-specific LES antigens.

3. Advances in serological diagnosis

3.1. Antigens

As mentioned above, the most reliable and suitable antigen for the diagnosis of toxocariasis is LES from *T. canis*. Once the larvae are cultivated *in vitro*, they are viable for up to two years. During this period, no morphological changes have been observed, but chemosusceptibility to some compounds were found to have changed [65], suggesting that the physiological natures of the larva do change over this time period. The nature of LES was extensively studied by Maizels and colleagues [61,66–68]. Around the same time, Sugane and Oshima demonstrated that LES had an ability to induce not only IgG and IgM antibodies, but also IgE antibody in mice. Allergenic activity was lost when LES was treated with guanidine hydrochloride and 2-mercaptoethanol. LES also showed a cross-reaction with serum from *Ascaris suum*-infected mice