In vitro *growth inhibition assay*

B. bovis Texas strain and *B. bigemina* Argentina strain were grown in a micro-aerophilous stationary-phase culture system using bovine erythrocytes (RBCs), and sera as described previously (Bork *et al.* 2004*a*). Serum-free GIT medium also was used to study the effect of serum-free medium on the drug inhibition of *B. bovis* (Bork *et al.* 2005*b*). The *in vitro* growth inhibition assay for (-) Epigallocatechin-3-gallate followed a method previously described (Bork *et al.* 2003*a*, 2004*a*) with some modifications. Parasite-infected RBCs were diluted with uninfected RBC to obtain 1% parasitaemia. Twenty μl of RBC with 1% parasitaemia were dispensed to a 96-well microtitre plate together with 200 μl of the culture medium containing the indicated concentration of (-) Epigallocatechin-3-gallate (5, 10, 25, 50, 100 μM), diminazene aceturate (0·5, 1, and 2 μM), and tetracycline hydrochloride (5, 50, and 100 μM) (Matsuu *et al.* 2008) and then incubated at 37 °C in a humified multi-gas water-jacketed incubator. For the control, cultures without the drug and another containing only PBS or distilled water at a similar concentration to the highest drug concentration were prepared. The experiments were carried out in triplicate for each drug concentration in 3 separate trials for a period of 4 days. The culture medium was replaced daily with 200 μl of fresh medium containing the appropriate concentration of the drug. Parasitaemia was monitored daily by counting the parasitized RBC to approximately 1000 in Giemsia-stained thin blood smears. Values of a 50% inhibitory concentration (IC_{50}) of the tested drugs against all parasites were calculated by interpolation after curve fitting, based on parasitaemia observed at day 3 after drug treatment.

AQ2

Viability test

After the fourth day of the treatment, 6 μl of each of the control and drug-treated (at the various concentrations) RBC was mixed with 14 μl of parasite-free RBC and suspended in 200 μl of fresh growth medium without drug supplementation. The plates were incubated for the next 10 days. The culture medium was replaced daily, and parasite recrudescence was determined by light microscopy to evaluate the parasite viability (Bork *et al.* 2004*a*).

In vivo *inhibition assay*

The Munich strain of *B. microti* was maintained by passage in the blood of BALB/c mice as previously described (Yokoyama *et al.* 2003). The *in vivo* growth inhibitory assay for EGCG was carried out for *B. microti* twice per parasite according to the method previously described (Bork *et al.* 2004*a*; Li *et al.* 2007) with some modifications. Eighteen 8-week-old female BALB/c mice (CLEA Japan, Tokyo, Japan) were divided into 3 groups. Each group consisted of 6 mice that were inoculated intraperitoneally with 1×10^6 *B. microti*-infected RBC. EGCG was administered at a dosage of 5 and 10 mg/kg after dissolving in 0·3 ml of phosphate-buffered saline (PBS) for the first and second groups. PBS alone was administered at 0·3 ml to the third group as a placebo control. EGCG-treated groups underwent daily intraperitoneal injections from days 3 to 12 post-infection when the parasitaemia was approximately 1%. The levels of parasitaemia in all mice were monitored daily until day 26 post-infection by examination of stained thin blood smears prepared from venous tail blood. All animal experiments were conducted in accordance with the Standard Relating to the Care and Management of Experimental Animals promulgated by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

AQ3

Statistical analysis

JMP statistical software version 5.1(SAS Institute Inc., USA) was used to compare the means in the *in vitro* and *in vivo* experiments by independent Student's *t*-test and considered to be significantly different when $P<0{\cdot}05$.

RESULTS

In vitro *growth inhibition assay*

The growth of *B. bovis* (Fig. 1A) and *B. bigemina* (Fig. 1B) from an initial parasitaemia of 1% was significantly (Student's *t*-test, $P<0{\cdot}05$) inhibited at 10 and 25 μM respectively and significantly suppressed in the presence of 50 μM EGCG. Complete clearance of *B. bovis* and *B. bigemina* (Fig. 1A and B) was observed as early as the second day of drug treatment. The concentrations of 50 μM tetracycline hydrochloride and 1 μM diminazene aceturate significantly (Student's *t*-test, $P<0{\cdot}05$) inhibited the growth of *B. bovis* and *B. bigemina*. However, higher concentrations of 100 μM tetracycline hydrochloride and 2 μM diminazene aceturate could not completely clear the parasites on the fourth day of culture (Fig. 1A and B). The 50% inhibitory concentration (IC_{50}) values of EGCG on the third day of culture were 18 μM (*B. bovis*) and 25 μM (*B. bigemina*) (Table 1). Subsequent cultivation of the parasites without the drug for a 10-day period showed no regrowth of the parasites at 25 μM for *B. bovis* and *B. bigemina* (Fig. 1A and B). Parasites exposed to lower drug concentrations started to grow again, as shown by light microscopy. The IC_{50} values of tetracycline hydrochloride were 48 μM (*B. bovis*) and 43 μM (*B. bigemina*), while those of diminazene aceturate were 0·67 μM (*B. bovis*) and 0·5 μM (*B. bigemina*) (Table 1). Parasites could not re-grow at the concentrations of

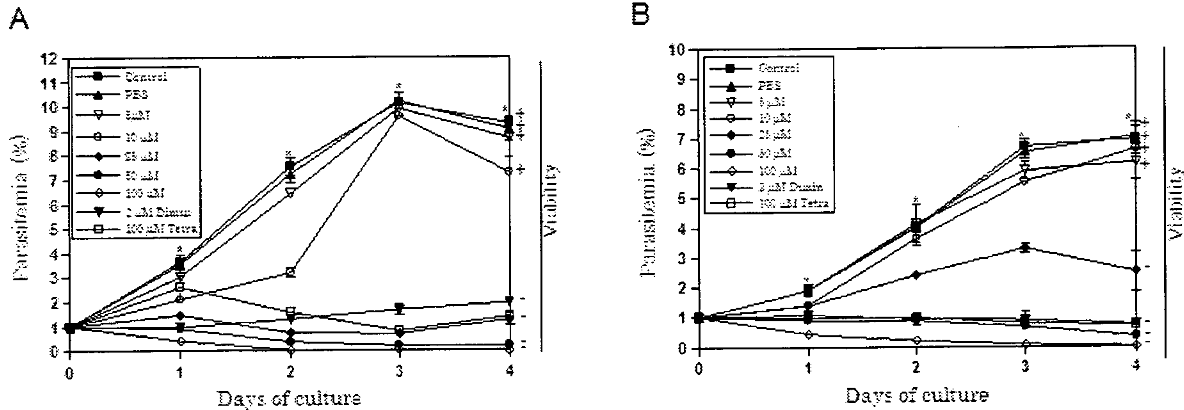


Fig. 1. Inhibitory effect of EGCG on the *in vitro* growth of *Babesia bovis* (A) and *B. bigemina* (B). Diminazene aceturate (Dimin) and tetracycline hydrochloride (Tetra) were used as positive controls. Each value represents the mean ± standard deviation of 3 separate experiments carried out in triplicate. Asterisks, indicate significant difference (Student's *t*-test; * $P<0{\cdot}05$) between the 10 μM and 25 μM EGCG-treated and the control cultures of *B. bovis* and *B. bigemina*, respectively. AQ9

Table 1. IC_{50} values of EGCG, tetracycline hydrochloride and diminazene aceturate for growth inhibition of *Babesia bovis* and *B. bigemina*

	IC_{50} (μM)[a]		
Parasite	EGCG	Tetracycline hydrochloride	Diminazene aceturate
B. bovis	18	48	0·67
B. bigemina	25	44	0·5

[a] IC_{50} values expressed as drug concentration are in micromoles of the growth medium and were determined on day 3 of *in vitro* culture using a curve fitting technique. IC_{50} values obtained from 3 separate experiments.

100 μM tetracycline hydrochloride and 1 μM diminazene aceturate in the subsequent viability test (Fig. 1A and B). There was no difference in the growth inhibition of EGCG for *B. bovis* with GIT medium (data not shown), therefore, serum has no effect on the activity of EGCG. EGCG affected the morphology of the parasites in treated cultures as indicated by light microscopy. Some parasites appeared degenerate, or abnormally multidividing forms were observed (Fig. 2B and D). In addition to these reported changes, treated *B. bigemina* parasites lost the normal appearance of the acute angle and had AQ4 an obtuse angle between their pairs (Fig. 2D). Based on light microscopic observations of the changes in the host cell shape, size, and colour, and the appearance of perforations, EGCG was non-toxic to erythrocytes.

In vivo *effect of EGCG on the growth of* B. microti

In *B. microti* infected mice, the levels of parasitaemia in the treated groups increased significantly (Student's *t*-test, $P<0{\cdot}05$) more slowly than the control, achieving peak parasitaemia of 10·5 and 9·1% in the presence of 5 and 10 mg/kg on day 11 p.i., respectively. In contrast, in the control group, the peak parasitaemia was 60·8% (PBS) on day 11 p.i. (Fig. 3). The 5 mg/kg treated mice completely cleared the parasites on the day 14 p.i. achieving a parasitaemia of zero, while the parasites completely cleared on day 16 p.i. in 10 mg/kg treated mice. There were no signs of toxicity observed in treated mice.

DISCUSSION

In the present study, the inhibitory effect of EGCG on the *in vitro* growth of bovine *Babesia* was evident. The presence of a higher concentration of EGCG in the culture completely suppressed the growth of all parasites tested in this study. As the presence of the solvents alone did not affect the growth of the parasites, the growth inhibition observed in this study was due to the effects of EGCG. The IC_{50} values of EGCG were lower than those of tetracycline hydrochloride, while they were higher than those of diminazene aceturate. The IC_{50}s values of EGCG for *Babesia* parasites were lower than other drugs used in previous studies: ketoconazole (Bork *et al.* 2003*c*), gossypol (Bork *et al.* 2004*b*), heparin (Bork *et al.* 2004*a*), EGTA (Okubo *et al.* 2006), and chloroquine diphosphate (Matsuu *et al.* 2008). They were a similar range to the other drugs tested as babesicidal drugs: triclosan (Bork *et al.* 2003*a*), clotrimazole (Bork *et al.* 2003*b*, *c*), tetracyclines (Nott *et al.* 1990; Matsuu *et al.* 2008), staurosporine (Bork *et al.* 2006), purvalanol A (Nakamura *et al.* 2007), and ALLN (Okubo *et al.* 2007). The IC_{50} values of EGCG were higher than other babesicidal drugs: quinuronium sulfate (Brockelman and Tan-ariya, 1991), imidocarb dipropionate (Rodriguez and Trees, 1996;

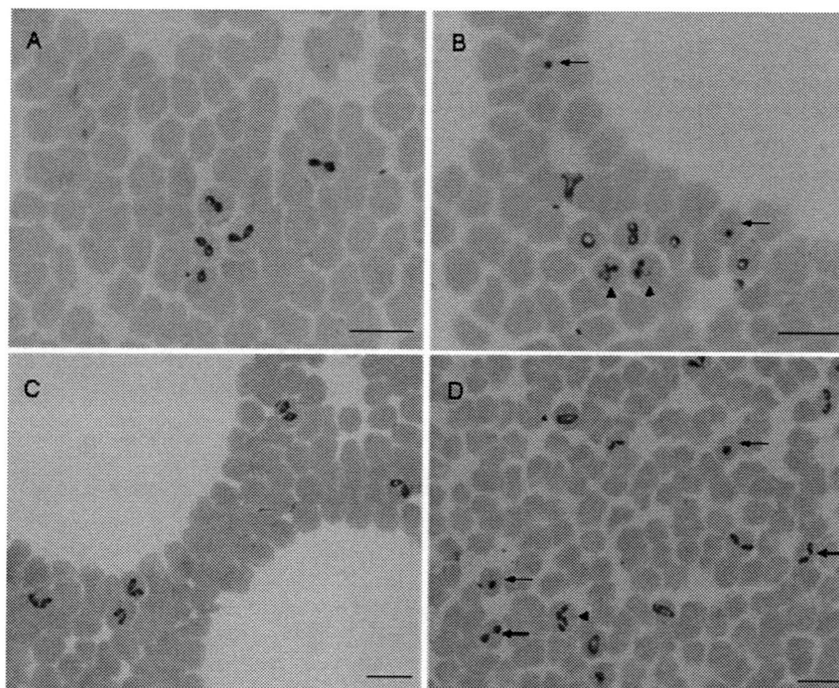
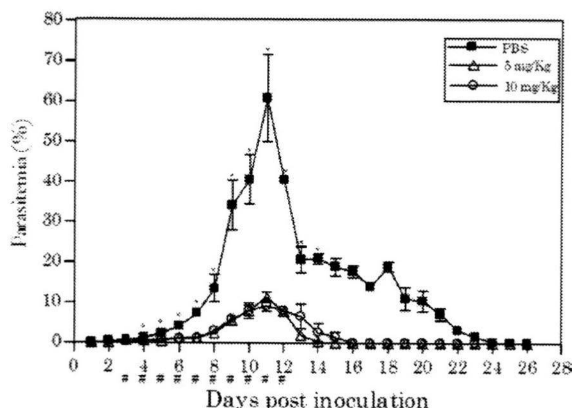


Fig. 2. EGCG-treated *Babesia* parasites in an *in vitro* culture. *B. bovis*: control (A), treated with 10 μM EGCG (B). *B. bigemina*: control (C), treated with 10 μM EGCG (D). Drug-treated cultures showed a higher number of degenerated parasites (thin arrows) and abnormally multidividing forms (arrowheads) and parasites with an obtuse angle (thick arrows). Scale bars = 10 μm.



AQ9 Fig. 3. Inhibitory effects of EGCG (5 and 10 mg/kg) on the *in vivo* growth of *Babesia microti* for observations of 6 mice per experimental group. Each value represents the mean ± S.D for 2 experiments. Asterisks, indicate significant difference (Student's *t*-test; * $P < 0.05$) between drug-treated and PBS groups.

Brasseur *et al.* 1998), Clindamycin phosphate (Brasseur *et al.* 1998), and atovaquone (Pudney and Gray, 1997; Matsuu *et al.* 2008). The IC_{50}s values of EGCG were very high comparable to those reported for *T. cruzi* (Paveto *et al.* 2004) indicating a higher sensitivity of *T. cruzi* to EGCG than *Babesia* parasites. The IC_{50}s values of EGCG for *Babesia* are very low when compared with that reported for the normal mammalian cells of H9 (174·8 μM) and THP-1 (440·3 μM) types (Yamagouchi *et al.* 2002); AQ8 furthermore, the concentration of 20 μM had no effect on the normal mammalian cells (Shammas *et al.* 2006). Thus, EGCG is non-toxic to the mammalian cells.

EGCG had a strong inhibitory effect on the growth of the cultured parasites; thus, we were encouraged to investigate its *in vivo* effect on *B. microti* infection in mice. The inhibitory effect of EGCG on the growth of *B. microti* was evident. The difference in growth inhibition between the control and the drug-treated groups was significant on days 4–14 p.i. Interestingly, there was no significant difference in the inhibition of *B. microti* between the two doses used and this was consistent with the report by Wang *et al.* (2006) in which doses up to 10 mg/kg did not inhibit Concanavalin A-induced alanine aminotransferase (ALT) release from hepatocytes more efficiently than the dose of 5 mg/kg. *B. microti*-infected mice that were treated with 5 and 10 mg/kg did not show signs of toxicity and were alive during and after the end of the experiment. In the current study, EGCG effectively suppressed *B. microti* in mice treated for 10 days at 5 and 10 mg/kg with 83·3 and 84% inhibition, respectively, on day 11 p.i., while mice treated with 2·5 mg heparin for 10 days had 86% inhibition on day 8 p.i. (Bork *et al.* 2004*a*). In the hamster model, atovaquone at doses of 300, 150, and 80 mg/kg/day for 8 days was effective in the recovery of all animals compared with 50% of those

receiving 10 mg/kg/day (Hughes and Oz, 1995), while recrudescences occurred at the dosage of 100 mg/kg/day (Wittner *et al.* 1996). Clindamycin plus quinine was used at 150 mg/kg for 7 days, and the inhibition was 70% on day 7 post-treatment (Marley *et al.* 1997). Azithromycin, either alone (150 and 300 mg/kg) or in combination (150 mg/kg) with quinine (250 mg/kg), was effective in reducing levels of parasitaemia during the period of drug administration, but parasitaemia rapidly returned to pre-treatment levels when the drug was stopped (Wiess AQ5 *et al.* 1993). In the gerbil model, atovaquone at 50 mg/kg for 10 days resulted in rapid reduction of AQ6 parasitaemia but did not eliminate the infection (Gray and Pudney, 1999). The direct comparison of EGCG effectiveness with other drugs used for *in vivo* treatment of *Babesia* infection requires further study. Our results encourage the potential use of EGCG as a chemotherapeutic tool against animal and human babesiosis.

EGCG was used as anti-oxidative (Fraga *et al.* 1987), anti-inflammatory (Lin and Lin, 1997), anti-proliferative (Ramirez-Mares *et al.* 2004; Shammas *et al.* 2006), anti-cancer (Chen and Zhang, 2007), anti-bacterial (Mabe *et al.* 1999), anti-viral (Fassina *et al.* 2002; Yamaguchi *et al.* 2002; Williamson *et al.* 2006) and anti-trypanocidal (Paveto *et al.* 2004). The mechanism of inhibition of EGCG for *Babesia* parasites is not known while there are several reported mechanisms of inhibition in the previous research on other microorganisms. EGCG inhibits arginine kinase of *T. cruzi* (Paveto *et al.* 2004), but this enzyme was not found in the genome data-base of either *B. bovis* or *B. bigemina* (http://www.sanger.ac.uk) indicating a different mechanism of action for *Babesia* parasites. EGCG has been shown to be an inhibitor of dihydrofolate reductase (DHFR) of *Stenotrophomonas maltophilia* (Navarro-Martínez *et al.* 2005); therefore, EGCG may inhibit the homologous enzyme of *Babesia* parasites, but this requires further investigation. EGCG binds to the haemagglutinin (HA) spike proteins of influenza virus which results in blocking the viral attachment to the receptors of target cells (Nakayama *et al.* 1993); furthermore, EGCG inhibited the binding of HIV1-gp120 to CD4$^+$T cells in a dose-dependent manner (Williamson *et al.* 2006). However, EGCG only affected the morphology of the intracellular parasites, and the percentages of the extracellular parasites in treated cultures were not different from those of the control. Therefore, EGCG had no effect on parasite invasion and further studies are required to identify the potential mechanism of action.

In summary, EGCG inhibited the *in vitro* growth of 2 bovine *Babesia* species and the *in vivo* growth of *B. microti*. EGCG may have potential for practical use in *in vivo* therapy of babesiosis; however, further studies will be required to confirm its mechanism of inhibition to *Babesia* parasites.

ACKNOWLEDGEMENTS

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Grants from the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), the 21st Century COE Program (A-1), Ministry of Education, Culture, Sports, Science, and Technology, Japan, and Ministry of Higher Education, Egypt.

REFERENCES

Bork, S., Das, S., Okubo, K., Yokoyama, N. and Igarashi, I. (2006). Effects of protein kinase inhibitors on the *in vitro* growth of *Babesia bovis*. *Parasitology* **132**, 775–779.

Bork, S., Okamura, M., Boonchit, S., Hirata, H., Yokoyama, N. and Igarashi, I. (2004*b*). Identification of *Babesia bovis* L-lactate dehydrogenase as a potential chemotherapeutic target against bovine babesiosis. *Molecular and Biochemical Parasitology* **136**, 165–172.

Bork, S., Okamura, M., Matsu, T., Kumar, S., Yokoyama, N. and Igarashi, I. (2005*b*). Host serum modifies the drug susceptibility of *Babesia bovis in vitro*. *The Journal of Parasitology* **130**, 489–492.

Bork, S., Yokoyama, N. and Igarashi, I. (2005*a*). Recent advances in the chemotherapy of babesiosis by Asian scientist: Toxoplasmosis and Babesiosis in Asia. *Asian Parasitology* **4**, 233–242.

Bork, S., Yokoyama, N., Ikehara, Y., Kumar, S., Sugimoto, C. and Igarashi, I. (2004*a*). Growth-Inhibitory effect of heparin on *Babesia* parasites. *Antimicrobial Agents and Chemotherapy* **48**, 236–241.

Bork, S., Yokoyama, N., Matsuo, T., Claveria, F. G., Fujisaki, K. and Igarashi, I. (2003*a*). Growth inhibitory effect of triclosan on equine and bovine *Babesia* parasites. *The American Journal of Tropical Medicine and Hygiene* **68**, 334–340.

Bork, S., Yokoyama, N., Matsuo, T., Claveria, F. G., Fujisaki, K. and Igarashi, I. (2003*b*). Clotrimazole, ketoconazole, and clodinafop-propargyl as potent growth inhibitors of equine *Babesia* parasites during *in vitro* cultures. *The Journal of Parasitology* **89**, 604–606.

Bork, S., Yokoyama, N., Matsuo, T., Claveria, F. G., Fujisaki, K. and Igarashi, I. (2003*c*). Clotrimazole, ketoconazole, and clodinafop-propargyl inhibit the *in vitro* growth of *Babesia bigemina* and *Babesia bovis* (Phylum Apicomplexa). *Parasitology* **127**, 311–315.

Brasseur, P., Lecoublet, S., Kapel, N., Vennec, L. and Ballet, J. J. (1998). *In vitro* evaluation of drug susceptibilities of *Babesia divergens* isolates. *Antimicrobial Agents and Chemotherapy* **42**, 818–820.

Brockelman, C. R. and Tan-ariya, P. (1991). Development of an *in vitro* microtest to assess drug susceptibility of *Babesia bovis* and *Babesia bigemina*. *The Journal of Parasitology* **6**, 994–997.

Chen, L. and Zhang, H. Y. (2007). Cancer preventive mechanisms of the green tea polyphenol (-)-epigallocatechin- 3-gallate. *Molecules* **12**, 946–957.

Cox, F. E. and Young, A. S. (1969). Acquired immunity to *Babesia microti* and *Babesia rodhaini* in mice. *Parasitology* **59**, 257–268.

Fassina, G., Buffa, A., Benelli, R., Varnier, O. E., Noonan, D. M. and Albini, A. (2002). Polyphenolic

antioxidant (-)-epigallocatechin-3-gallate from green tea as a candidate anti-HIV agent. *AIDS* **16**, 939–941.

Fraga, C. G., Martino, V. S., Ferraro, G. E., Coussio, J. D. and Boveris, A. (1987). Flavonoids as antioxidants evaluated by *in vitro* and *in situ* liver chemiluminescence. *Biochemical Pharmacology* **36**, 717–720.

Graham, H. N. (1992). Green tea composition, consumption, and polyphenol chemistry. *Preventive Medicine* **21**, 334–350.

Gray, J. S. and Pudney, M. (1999). Activity of atovaquone against *Babesia microti* in the Mongolian gerbil, *Meriones unguiculatus*. *The Journal of Parasitology* **85**, 723–728.

Homer, M. J., Aguilar-Delfin, I., Telford, S. R., Krause, P. J. and Persing, D. H. (2000). Babesiosis. *Clinical Microbiology Reviews* **13**, 451–469.

Hughes, W. T. and Oz, H. S. (1995). Successful prevention and treatment of babesiosis with atovaquone. *The Journal of Infectious Diseases* **172**, 1042–1046.

Kjemtrup, A. M. and Conrad, P. A. (2000). Human babesiosis: An emerging tick-borne disease. *International Journal for Parasitology* **30**, 1323–1337.

Kuttler, K. (1988). World-wide impact of babesiosis. In *Babesiosis of Domestic Animals and Man* (ed. Ristic, M.), pp. 1–22. CRC Press Inc., Florida, USA.

Li, W., Ashok, M., Li, J., Yang, H., Sama, A. E. and Wang, H. (2007). A major ingredient of green tea rescues mice from lethal sepsis partly by inhibiting HMGB1. *PLoS ONE* **2**, e1153.

Lin, Y. L. and Lin, J. K. (1997). (−)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-κ B. *Molecular Pharmacology* **52**, 465–472.

Mabe, K., Yamada, M., Oguni, I. and Takahashi, T. (1999). *In vitro* and *in vivo* activities of tea catechins against *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy* **43**, 1788–1791.

Marley, S. E., Eberhard, M. L., Steurer, F. J., Ellis, W. L., McGreevy, P. B. and Ruebush IInd., T. K. (1997). Evaluation of selected antiprotozoal drugs in the *Babesia microti* hamster model. *Antimicrobial Agents and Chemotherapy* **41**, 91–94.

Matsuu, A., Yamasaki, M., Xuan, X., Ikadai, H. and Hikasa, Y. (2008). *In vitro* evaluation of the growth inhibitory activities of 15 drugs against *B. gibsoni* (Amory strain). *Veterinary Parasitology* **157**, 1–8.

Nakamura, K., Yokoyama, N. and Igarashi, I. (2007). Cyclin-dependent kinase inhibitors block erythrocyte invasion and intraerythrocytic development of *Babesia bovis in vitro*. *Parasitology* **135**, 1–7.

Nakayama, M., Suzuki, K., Toda, M., Okubo, S., Hara, Y. and Shimamura, T. (1993). Inhibition of the infectivity of influenza virus by tea polyphenols. *Antiviral Research* **21**, 289–299.

Navarro-Martínez, M. D., Navarro-Perán, E., Cabezas-Herrera, J., Ruiz-Gómez, J., García-Cánovas, F. and Rodríguez-López, J. N. (2005). Antifolate Activity of Epigallocatechin Gallate against *Stenotrophomonas maltophilia*. *Antimicrobial Agents and Chemotherapy* **49**, 2914–2920.

Nott, S. E., O'Sullivan, W. J., Gero, A. M. and Bagnara, A. S. (1990). Routine screening for potential babesicides using cultures of *Babesia bovis*. *International Journal for Parasitology* **20**, 797–802.

Okubo, K., Wilawan, P., Bork, S., Okamura, M., Yokoyama, N. and Igarashi, I. (2006). Calcium-ions are involved in erythrocyte invasion by equine *Babesia* parasites. *Parasitology* **133**, 289–294.

Okubo, K., Yokoyama, N., Govind, Y., Alhassan, A. and Igarashi, I. (2007). *Babesia bovis*: effects of cysteine protease inhibitors on *in vitro* growth. *Experimental Parasitology* **117**, 214–217.

Paveto, C., Güida, M. C., Esteva, M. I., Martino, V., Coussio, J., Flawiá, M. M. and Torres, H. N. (2004). Anti-*Trypanosoma cruzi* activity of green tea (*Camellia sinensis*) catechins. *Antimicrobial Agents and Chemotherapy* **48**, 69–74.

Pudney, M. and Gray, J. S. (1997). Therapeutic efficacy of atovaquone against the bovine intraerythrocytic parasite, *Babesia divergens*. *The Journal of Parasitology* **83**, 307–310.

Ramirez-Mares, M. V., Chandra, S. and de Mejia, E. G. (2004). *In vitro* chemopreventive activity of *Camellia sinensis*, *Ilex paraguariensis* and *Ardisia compressa* tea extracts and selected polyphenols. *Mutation Research* **554**, 53–65.

Rodriguez, R. I. and Trees, A. J. (1996). *In vitro* responsiveness of *B. bovis* to imidocarb dipropionate and the selection of adapted lines. *Veterinary Parasitology* **62**, 357–341. AQ7

Shammas, M. A., Neri, P., Koley, H., Batchu, R. B., Bertheau, R. C., Munshi, V., Prabhala, R., Fulciniti, M., Tai, Y. T., Treon, S. P., Goyal, R. K., Anderson, K. C. and Munshi, N. C. (2006). Specific killing of multiple myeloma cells by (-)-epigallocatechin-3-gallate extracted from green tea: biologic activity and therapeutic implications. *Blood* **108**, 2804–2810.

Vial, H. J. and Gorenflot, A. (2006). Chemotherapy against babesiosis. *Veterinary Parasitology* **138**, 147–160.

Wang, Y., Mei, Y., Feng, D. and Xu, L. (2006). (−)-Epigallocatechin- 3-gallate protects mice from Concanavalin A induced hepatitis through suppressing immune-mediated liver injury. *Clinical and Experimental Immunology* **145**, 485–492.

Weiss, L. M., Wittner, M., Wasserman, S., Oz, H. S., Retsema, J. and Tanowitz, H. B. (1993). Efficacy of azithromycin for treating *Babesia microti* infection in the hamster model. *The Journal of Infectious Diseases* **168**, 1289–1292. AQ5

Williamson, M. P., McCormick, T. G., Nance, C. L. and Shearer, W. T. (2006). Epigallocatechin gallate, the main polyphenol in green tea, binds to the T-cell receptor, CD4: Potential for HIV-1 therapy. *Journal of Allergy and Clinical Immunology* **118**, 1369–1374.

Wittner, M., Lederman, J., Tanowitz, H. B., Rosenbaum, G. S. and Weiss, L. M. (1996). Atovaquone in the treatment of *Babesia microti* infections in hamsters. *The American Journal of Tropical Medicine and Hygiene* **55**, 219–222.

Yamaguchi, K., Honda, M., Ikigai, H., Hara, Y. and Shimamura, T. (2002). Inhibitory effects of AQ8

(-)-epigallocatechin gallate on the life cycle of human immunodeficiency virus type 1 (HIV-1). *Antiviral Research* **53**, 19–34.

Yang, C. S. and Landau, J. M. (2000). Effects of tea consumption on nutrition and health. *Journal of Nutrition* **130**, 2409–2412.

Yokoyama, N., Bork, S., Nishisaka, M., Hirata, H., Matsuo, T., Inoue, N., Xuan, X., Suzuki, H., Sugimoto, C. and Igarashi, I. (2003). Roles of the Maltese cross form in the development of parasitemia and protection against *Babesia microti* infection in mice. *Infection and Immunity* **71**, 411–417.

Veterinary Parasitology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Development and evaluation of a nested PCR based on spherical body protein 2 gene for the diagnosis of *Babesia bovis* infection

Mahmoud AbouLaila, Naoaki Yokoyama, Ikuo Igarashi *

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-13, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

ARTICLE INFO

Article history:
Received 7 July 2009
Received in revised form 3 December 2009
Accepted 10 December 2009

Keywords:
Nested PCR
Spherical body protein 2 gene
Babesia bovis

ABSTRACT

We developed and evaluated a nested PCR assay for the diagnosis of *Babesia bovis* infection in cattle based on the spherical body protein 2 gene (SBP2) from *B. bovis*. The specificity and sensitivity of the test were compared with the *B. bovis* RAP-1 gene nPCR. The SBP2 primers have specificities of 100% for *B. bovis* DNA. The sensitivity of the SBP2 nPCR to *B. bovis* from the *in vitro* cultured parasites was higher than that of the *B. bovis* RAP-1 gene nPCR, and a parasitemia as low as 10^{-8}% was detected. The sensitivity of the SBP2 nPCR to *B. bovis*-diluted genomic DNA was also higher than that of *B. bovis* RAP-1 gene nPCR, and as little as 1 fg per test detected. For field applications, the sensitivity to a total of 145 field samples from Ghana, Mongolia, and Brazil was evaluated. The nPCR assay of spherical body protein-2 gene detected 87.6% (127/145), while *B. bovis* RAP-1 gene nPCR detected 37.2% (51/145) of the total samples examined. This nPCR assay provides a good diagnostic tool for the laboratory diagnostic assessment of *B. bovis* infection in cattle worldwide.

© 2009 Published by Elsevier B.V.

1. Introduction

Bovine babesiosis is an economically important tick-borne disease of cattle in tropical and subtropical regions of the world (McCosker, 1981). The disease is mainly caused by two bovine intraerythrocytic protozoan parasites, *Babesia bovis* and *Babesia bigemina*. Although the clinical signs induced by these parasites are similar, as characterized by fever, anemia, and icterus in the infected cattle (de Vos and Potgieter, 2004), generally, the disease caused by *B. bovis* is more severe than that by *B. bigemina* (Ristic, 1981).

Microscopic techniques for blood examination remain the most appropriate for the diagnosis of acute babesiosis, but the low sensitivity of these methods does not permit its use in epidemiological studies, in which it is necessary to identify carrier animals (Almeria et al., 2001). Several serological methods standardized for the diagnosis of babesiosis have been extensively employed in epidemiological field studies. Among the drawbacks of these techniques is the occurrence of cross-reactions between *B. bovis* and *B. bigemina* (Passos et al., 1998) and the lack of discrimination between previous exposure and current infections (Wagner et al., 1992).

The efficiency of the polymerase chain reaction (PCR) method has been verified in the course of epidemiological studies of babesiosis by several investigators because of its high specificity and sensitivity, in particular, through the nested PCR assay (Fahrimal et al., 1992; Figueroa et al., 1993; Smeenk et al., 2000; Almeria et al., 2001; Gayo et al., 2003; Oliveira-Sequeira et al., 2005; Costa-Júnior et al., 2006).

The spherical body is an apical organelle thought to be analogous to dense granules in other apicomplexan organisms (Hines et al., 1995). Spherical body protein 2 (SBP2) (also known as BvVa1) is a 225-kD immunostimulatory protein from the spherical body that is released into the host erythrocyte post-invasion and localizes to the cytoplasmic side of the erythrocyte membrane (Jasmer et al., 1992; Dalrymple, 1993; Dalrymple et al., 1993;

* Corresponding author. Tel.: +81 155 49 5641; fax: +81 155 49 5641.
E-mail address: igarcpmi@obihiro.ac.jp (I. Igarashi).

0304-4017/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.vetpar.2009.12.013

Please cite this article in press as: AbouLaila, M., et al., Development and evaluation of a nested PCR based on spherical body protein 2 gene for the diagnosis of *Babesia bovis* infection. Vet. Parasitol. (2009), doi:10.1016/j.vetpar.2009.12.013

Dowling et al., 1996; Yokoyama et al., 2006). There were multiple copies of the 5′-end of the gene, while the 3′-end appeared to be a single copy (Jasmer et al., 1992). The genome sequence revealed that there are 12 truncated copies of the SBP2 gene, corresponding to the 5′-end of the full-length gene, and one full-length copy. The full-length gene and one truncated gene are on chromosome 4, with all remaining truncated copies on chromosome 3. The truncated genes on chromosome 3 occur in three clusters of two, four, and five genes. The genes occurring in the 2- and 5-gene clusters are interspersed with another set of highly conserved (88–100%) five gene repeats that have no homologues in the public databases (Brayton et al., 2007). Unlike other apical complex proteins (e.g., Rhoptry-Associated Protein 1), which are conserved among *Babesia* species, the gene encoding spherical body protein 2 (SBP2) has not been detected in *B. bigemina* (Ruef et al., 2000).

The aim of this study was to evaluate the diagnostic efficiency of the spherical body protein 2 gene for the diagnosis of *B. bovis* in nested PCR and compare it with that of the *B. bovis* Rhoptry-Associated Protein 1 (RAP-1) nested PCR used in most previous studies.

2. Materials and methods

2.1. Parasites

The Texas strain of *B. bovis* was maintained in purified bovine red blood cells (RBC) with a microaerophilic stationary-phase culture system (Bork et al., 2004). Medium M199 (Sigma-Aldrich, Tokyo, Japan) was supplemented with 40% normal bovine serum to prepare the culture medium for the parasites.

2.2. DNA extraction

To evaluate the sensitivities of the PCR and nested PCR methods of the newly developed primers, *B. bovis* DNA samples were extracted from a 10-fold serial dilution of the culture and then subjected to the PCR and nested PCR methods. DNA was extracted as previously described (Iseki et al., 2007) with some modifications. *B. bovis*-infected RBC were washed three times with cold phosphate-buffered saline (PBS) by centrifuging at 1000 × g for 5 min at 4 °C and resuspended in PBS. The infected RBC were serially diluted 10-fold with normal RBC to adjust the parasite concentrations from 2.7×10^{-2} (parasitemia: 0.0000000001%) to 2.7×10^{6} (1%) infected RBC/200 μl of the total RBC. Another concentration of 7×10^{7} (5.2%) infected RBC/200 μl of total RBC was also prepared (as a positive control). Then, all the dilutions were separately subjected to DNA extraction with a QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan). The purified DNA samples were used as templates for the subsequent PCR method. Genomic DNA was extracted from the cultured parasites as reported above to measure the sensitivity of the primers to genomic DNA (Alhassan et al., 2005). The DNA was measured spectrophotometrically and diluted 10-fold from 200 ng/μl to 2 fg/μl and used for PCR amplification. DNA from field samples was extracted from blood-spotted filter papers (Abe and Konomi, 1998; da Silva et al., 2004). Briefly, the spotted filter papers were cut out with a 2-mm hole puncher (2.0-mm Harris Micro Punch; Whatman, Middlesex, UK). DNA samples were extracted from the cut portion containing the spotted blood using a QIAamp DNA Mini Kit (QIAGEN). As negative controls, distilled water, extracted DNA samples of normal bovine blood, and other cultured parasites (*B. bigemina*, *Theileria parva*, *Trypanosoma congolonse*, and *Neospora caninum*) were prepared as described above.

2.3. PCR and nested PCR amplifications

PCR and nested PCR (nPCR) amplifications were carried out with the developed primers specific to the *B. bovis* spherical body protein 2 (SBP2) gene (gene bank accession numbers of XM_001611726, XM_001611728, and XM_001611730 (Table 1)). The published pairs of species-specific primers for the detection of *B. bovis* (Figueroa et al., 1993) were used as a control for the developed primers. PCR was performed in 25 μl of a mixture containing 0.5 μl of the extracted DNA template, 50 pmol of each primer, 200 μM of each dNTP, and 1.25 U of Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) in a PCR buffer (Applied Biosystems). The reactions were performed under the following conditions: 5 min at 95 °C to activate the Taq Gold DNA polymerase, at 35 cycles (1 min of denaturation at 94 °C, 1 min of annealing at 64 °C, 90 s of extension at 72 °C), and 10 min of final extension at 72 °C in a Gene Amp PCR system 9700 (Applied Biosystems). The amplified PCR products of 0.5 μl were used for the subsequent nPCR with a limited annealing temperature at 58 °C and 1 min at 72 °C. The PCR and nPCR conditions for *B. bovis* RAP-1 followed the method previously described (Figueroa et al., 1993). Negative controls were included in all runs. Cross-contamination was prevented by using plugged tips and performing the PCR in a separate room from that used for DNA extraction. The PCR and nested PCR products were subjected to electrophoresis in 2% agarose gel and then visualized under an ultraviolet (UV) light after staining with ethidium bromide (SIGMA).

2.4. DNA sequencing

Positive DNA products from the specificity test PCR and nPCR and from field samples nPCR were purified after 2% agarose gel electrophoresis and then cloned into a pCR2.1 cloning vector using the original TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). The nucleotide sequences of inserts were determined using a Big Dye Terminator Kit (Applied Biosystems Japan, Ltd.) with an automated DNA sequencer (ABI PRISM 3100 genetic analyzer, Applied Biosystems Japan, Ltd.). The Genetyx 7 package (Software

Table 1
Primers sets of SBP2 developed for *B. bovis* DNA amplification in the present study.

Assay	Primers	Sequences (5′ → 3′)	Amplicon size
PCR	F	ccgaattcctggaagtggatctcatgcaacc	1236
	R	atctcgagtcacgagcactctacggctttgcag	
nPCR	F1	cgaatctaggcatataaggcat	580
	R1	atcccctcctaaggttggctac	

Please cite this article in press as: AbouLaila, M., et al., Development and evaluation of a nested PCR based on spherical body protein 2 gene for the diagnosis of *Babesia bovis* infection. Vet. Parasitol. (2009), doi:10.1016/j.vetpar.2009.12.013

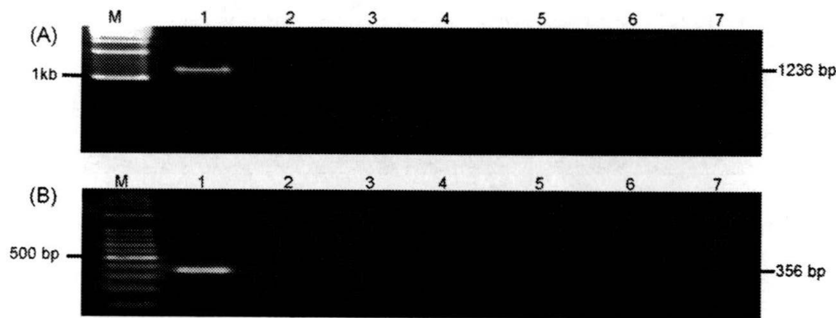


Fig. 1. Specificities of the PCR methods for the detection of *B. bovis*. SBP2 (Panel A) and *B. bovis* RAP-1 (Panel B) with each extracted DNA. In all panels, lane 1, *B. bovis*; lane 2, *B. bigemina*; lane 3, *T. parva*; lane 4, *T. congolonse*; lane 5, *N. caninum*; lane 6, bovine DNA; and lane 7, DDW. Lane M shows a 100-bp ladder size marker, and the band of 500 bp is indicated on the left. The size of the positive bands is indicated on the right.

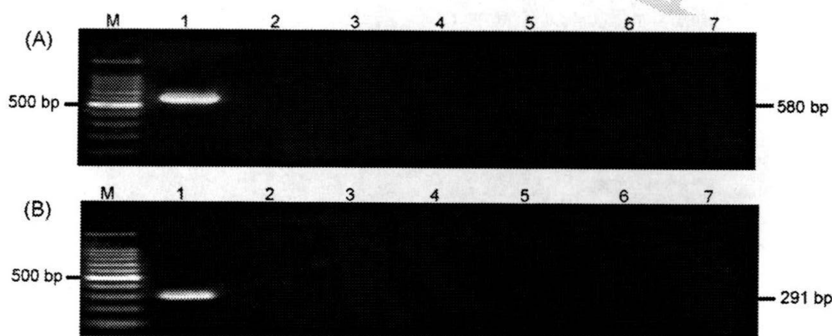


Fig. 2. Specificities of the nested PCR methods. nPCR reactions carried out for the detection of *B. bovis*. SBP2 nPCR (Panel A) and *B. bovis* RAP-1 nPCR (Panel B) with each extracted DNA. In all panels, lane 1, *B. bovis*; lane 2, *B. bigemina*; lane 3, *T. parva*; lane 4, *T. congolonse*; lane 5, *N. caninum*, lane 6, bovine DNA, and lane 7, DDW. Lane M shows a 100-bp ladder size marker, and the band of 500 bp is indicated on the left. The size of the positive bands is indicated on the right.

151 Development Co., Ltd., Tokyo, Japan) was used to align the
152 determined sequences.

153 ### 2.5. Field samples

154 Field blood samples were collected from jugular vein on
155 FTA cards. Appropriate care was exercised during blood
156 collection and application of the samples to the FTA cards.
157 The samples were 40, 24, and 81 from cattle living in
158 Ghana, Mongolia, and Brazil, respectively. The DNA was
159 extracted from FTA cards as described above.

3. Results 160

3.1. Specificities of PCR and nested PCR methods 161

162 The species-specific primers for *B. bovis* specifically
163 produced positive amplicons of 1236 bp for the SBP2 gene
164 (Fig. 1, Panel A, lane 1) and 356 bp for *B. bovis* RAP-1 (Fig. 1,
165 Panel B, lane 1) of only *B. bovis* DNA. The nested primers
166 targeted 580 bp for the SBP2 gene (Fig. 2, Panel A, lane 1)
167 and 291 bp for RAP-1 (Fig. 2, Panel B, lane 1) only from *B.*
168 *bovis* DNA, while no amplification was reported for DNA
169 from the other parasites used as negative controls. In order

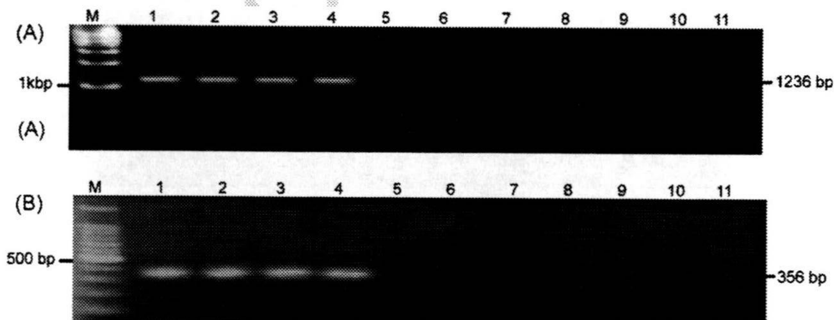


Fig. 3. Sensitivities of the PCR methods. PCR methods were carried out using the extracted DNAs from dilutions of infected RBC in the *in vitro* culture. SBP2 PCR (Panel A) and *B. bovis* RAP-1 PCR (Panel B). In all panels, lane 1, 7×10^7; lanes 2–10, 2.7×10^6, 2.7×10^5, 2.7×10^4, 2.7×10^3, 2.7×10^2, 2.7×10^1, 2.7×10^0, 2.7×10^{-1}, and 2.7×10^{-2} infected RBC; and lane 11, bovine DNA. Lane M shows a 100-bp ladder size marker, and the band of 500 bp is indicated on the left. The size of the positive bands is indicated on the right.

Please cite this article in press as: AbouLaila, M., et al., Development and evaluation of a nested PCR based on spherical body protein 2 gene for the diagnosis of *Babesia bovis* infection. Vet. Parasitol. (2009), doi:10.1016/j.vetpar.2009.12.013

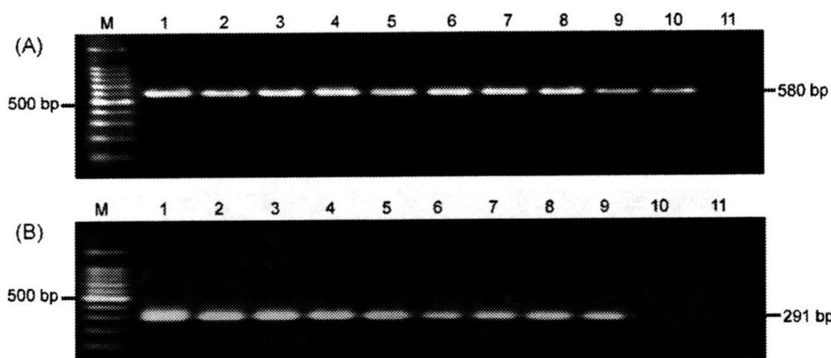


Fig. 4. Sensitivities of the nPCR methods. The nPCR methods were carried out using the extracted DNAs from dilutions of the infected RBC in the *in vitro* culture. SBP2 nPCR (Panel A) and *bovis* RAP-1 nPCR (Panel B). In all panels, lane 1, 7×10^7; lanes 2–10, 2.7×10^6, 2.7×10^5, 2.7×10^4, 2.7×10^3, 2.7×10^2, 2.7×10^1, 2.7×10^0, 2.7×10^{-1}, and 2.7×10^{-2} infected RBC; and lane 11, bovine DNA. Lane M shows a 100-bp ladder size marker, and the band of 500 bp is indicated on the left. The size of the positive bands is indicated on the right.

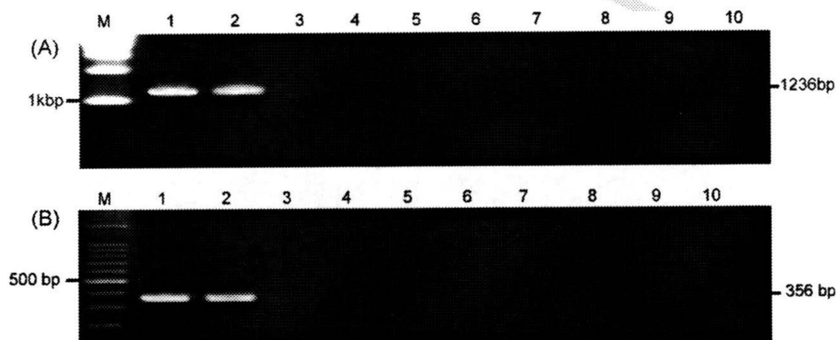


Fig. 5. Sensitivities of the PCR methods. PCR methods were carried out using the 10-fold diluted genomic DNA. SBP2 PCR (Panel A) and *B. bovis* RAP-1 PCR (Panel B). In all panels, lane 1, 100 ng; lane 9, 1 fg; and lane 10, bovine DNA. Lane M shows a 100-bp ladder size marker, and the band of 500 bp is indicated on the left. The size of the positive bands is indicated on the right.

170 to confirm the nucleotide sequences of the PCR and nested
171 PCR products, the amplified DNA products were purified
172 from the positive reactions and cloned into a vector. The
173 determined sequences of all DNA fragments were 100%
174 identical to the reported ones of SBP2 and RAP-1 genes
175 (Gene Bank accession numbers: XM_001611726,
176 XM_001611728, XM_001611730, and AF027149) (data
177 not shown).

3.2. Sensitivities of the PCR and nested PCR methods 178

3.2.1. Sensitivities to DNA extracted from diluted infected RBC 179

In the PCR methods (Fig. 3), positive bands of 1236 bp 180
(Panel A, lane 4) and 356 bp (Panel B, lane 4) were detected 181
from DNA extracted from RBC with 0.01% parasitemia 182
(2.7×10^4 infected RBC) for the SBP2 and RAP-1 genes, 183
respectively. Subsequently, in the nPCR methods (Fig. 4), 184

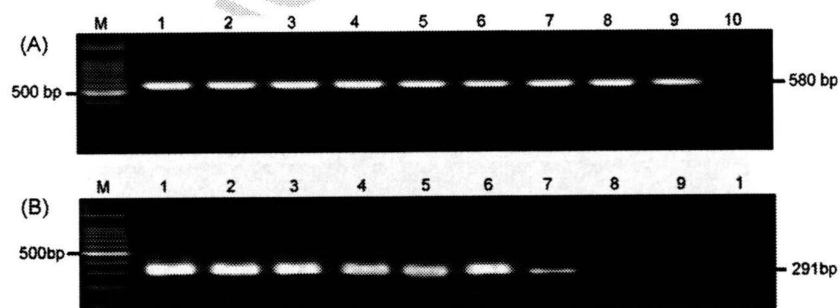


Fig. 6. Sensitivities of the nPCR methods. The nPCR methods were carried out using the 10-fold diluted genomic DNA. SBP2 nPCR (Panel A) and *bovis* RAP-1 nPCR (Panel B). In all panels, lane 1, 100 ng; lane 9, 1 fg; and lane 10, bovine DNA. Lane M shows a 100-bp ladder size marker, and the band of 500 bp is indicated on the left. The size of the positive bands is indicated on the right.

Please cite this article in press as: AbouLaila, M., et al., Development and evaluation of a nested PCR based on spherical body protein 2 gene for the diagnosis of *Babesia bovis* infection. Vet. Parasitol. (2009), doi:10.1016/j.vetpar.2009.12.013

Table 2
Comparison of the positive numbers among PCR and nested PCR methods of SBP2 and *B. bovis* RAP-1 genes for the detection of *B. bovis* from field bovine blood samples collected from Ghana, Mongolia, and Brazil.

Country	Number	SBP2		RAP-1	
		Positive numbers (%)			
		PCR	nPCR	PCR	nPCR
Ghana	40	0 (0)	36 (90)	0 (0)	10 (25)
Mongolia	24	0 (0)	23 (95.8)	0 (0)	13 (54.2)
Brazil	81	3 (3.7)	67 (82.7)	2 (2.5)	31 (38)
Total	145	3 (2)	127 (87.6)	2 (1.4)	54 (37.2)

specific bands of 580 bp (Panel A, lane 10) and 291 bp (Panel B, lane 9) could be observed from the diluted samples of 0.000000001% parasitemia (2.7×10^{-2} infected RBC) for the SBP2 gene and of 0.00000001% parasitemia (2.7×10^{-1} infected RBC) for the RAP-1 gene.

3.2.2. Sensitivities to genomic DNA diluted 10-fold

We evaluated the sensitivities of the PCR and nested PCR methods of the newly developed primers for *B. bovis* genomic DNA. The detection limit for the PCR (Fig. 5) was 10 ng genomic DNA for the SBP2 (Panel A, lane 2) and RAP-1 genes (Panel B, lane 2). The nPCR (Fig. 6) detected as low as 1 fg genomic DNA for the SBP2 gene (Panel A, lane 9) and 100 fg genomic DNA for the RAP-1 gene (Panel B, lane 2).

3.2.3. Detection of B. bovis from field samples by the PCR and nPCR methods

Field blood samples collected from cattle living in Ghana, Mongolia, and Brazil were surveyed using the PCR and nPCR methods in order to demonstrate the field utility of these methods as a diagnostic tool for epidemiological studies. The PCR of the SBP2 gene detected *B. bovis* in 3.7% (3/81) of the samples from Brazil, but *B. bovis* could not be detected in any of the samples from Ghana and Mongolia. The RAP-1 PCR only detected *B. bovis* in 2.5% (2/81) of the samples from Brazil (Table 2). Subsequently, the detection rates of *B. bovis* from these samples by the SBP2 gene nPCR were 90% (36/40) for Ghana; 95.8% (23/24) for Mongolia; and 82.7% (67/81) for Brazil (Table 2). The determined sequences of all positive DNA nPCR fragments were 100% identical to the reported ones of SBP2 and *B. bovis* RAP-1 genes (Gene Bank accession numbers: XM_001611726, XM_001611728, XM_001611730, and AF027149) (data not shown).

4. Discussion

In this report, we described the successful development of a nested PCR method for the detection of *B. bovis*. In the method, sets of the designed primers specifically amplified the target DNAs derived from the respective SBP2 gene. The specificity of 100% reported for the SBP2 gene is consistent with those of the *B. bovis* RAP-1 gene in PCR and nPCR methods. The PCR primers of the SBP2 gene were found to have similar sensitivity to *B. bovis* RAP-1 with diluted infected RBC and provide the detection of only 2.7×10^4 infected RBC (0.01% parasitemia). The sensitivity of nPCR primers of the SBP2 gene is 10 times higher than that of *B. bovis* RAP-1 and permitted the detection of 2.7×10^{-2} infected RBC (10^{-8}% parasitemia). The PCR of the SBP2 gene has similar sensitivity to that of the RAP-1 gene with the diluted genomic DNA and permitted the detection of 10 ng per test. The nPCR primers of the SBP2 gene have higher sensitivity than those of the *B. bovis* RAP-1 gene and detected as low as 1 fg per test of the genomic DNA. The PCR primers of the SBP2 gene have nearly identical sensitivity to the *B. bovis* RAP-1 genes for the field samples collected from Ghana, Mongolia, and Brazil. The nPCR primers of the SBP2 gene had higher detection rates for the field samples (87.6%) than those of *B. bovis* RAP-1 (37.2%). The sequence of SBP2 nested fragments was conserved among geographic isolates, which was consistent with the sequence conservation of the SBP3 gene, the third gene in SBP family, among geographical isolates (Ruef et al., 2000). Unlike other apical complex proteins (e.g., RAP-1), which are conserved among *Babesia* species, genes encoding spherical body proteins (SBP1, SBP2, and SBP3) have not been detected in *B. bigemina* (Ruef et al., 2000); therefore, this gene family may have a high potential in the diagnosis of *B. bovis*, in particular, SBP2.

There are several reported methods describing the detection of *B. bovis*, but only some of them have been tested with random field samples. They include the nPCR (Figueroa et al., 1993; Almeria et al., 2001; Gayo et al., 2003; Oliveira et al., 2005; Costa-Júnior et al., 2006), the RLB (Gubbels et al., 1999; Brígido et al., 2004; Oura et al., 2004), the LAMP assay (Iseki et al., 2007), and, recently, the seminested hot-start PCR (Martins et al., 2008). The sensitivity of this nPCR is higher than the reported sensitivities in previous studies using nPCR of around 10^{-7}% parasitemia (Oliveira-Sequeira et al., 2005), 10^{-6}% parasitemia (Costa-Júnior et al., 2006), and 10^{-4}% parasitemia (Iseki et al., 2007).

The high detection rates for *B. bovis* in the field samples from Brazil were consistent with those detected by Oliveira-Sequeira et al. (2005), indicating a high prevalence of *B. bovis* infection in Brazil. The detection rates of *B. bovis* from Ghana by our newly developed nPCR were consistent with the detection levels using the LAMP test by Iseki et al. (2007), and these high levels indicate a high prevalence of *B. bovis* infection in Ghana. To our knowledge, this is the first detection of bovine babesiosis caused by *B. bovis* from Mongolia, and the high detection rate by nPCR indicates that not only equine babesiosis (Boldbaatar et al., 2005) but also bovine babesiosis caused by *B. bovis* has a high prevalence in Mongolia.

In conclusion, we developed nPCR-based method for the detection of *B. bovis* from cattle with high levels of

Please cite this article in press as: AbouLaila, M., et al., Development and evaluation of a nested PCR based on spherical body protein 2 gene for the diagnosis of *Babesia bovis* infection. Vet. Parasitol. (2009), doi:10.1016/j.vetpar.2009.12.013

specificity and sensitivity. This method is broadly applicable to strains of the parasite from diverse geographic regions.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science, Grants from the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), the 21st Century COE Program (A-1), Ministry of Education, Culture, Sports, Science, and Technology, Japan, and the Ministry of Higher Education, Egypt.

References

Abe, K., Konomi, N., 1998. Hepatitis C virus RNA in dried serum spotted onto filter paper is stable at room temperature. J. Clin. Microbiol. 36, 3070–3072.

Alhassan, A., Pumidonming, W., Okamura, M., Hirata, H., Battsetseg, B., Fujisaki, K., Yokoyama, N., Igarashi, I., 2005. Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. Vet. Parasitol. 129, 43–49.

Almeria, S., Castella, J., Ferrer, D., Ortuno, A., Estrada-Pena, A., Gutierrez, J.F., 2001. Bovine piroplasms in Minorca (Balearic Islands, Spain): a comparison of PCR-based and light microscopy detection. Vet. Parasitol. 99, 249–259.

Boldbaatar, D., Xuan, X., Battsetseg, B., Igarashi, I., Battur, B., Batsukh, Z., Bayambaa, B., Fujisaki, K., 2005. Epidemiological study of equine piroplasmosis in Mongolia. Vet. Parasitol. 127, 29–32.

Bork, S., Yokoyama, N., Ikehara, Y., Kumar, S., Sugimoto, C., Igarashi, I., 2004. Growth-inhibitory effect of heparin on *Babesia* parasites. Antimicrob. Agents Chemother. 48, 236–241.

Brayton, K., Lau, A., Herndon, D., Hannick, L., Kappmeyer, L., Berens, S., et al., 2007. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS Pathogens 3, 1401–1413.

Brígido, C., da Fonseca, I.P., Parreira, R., Fazendeiro, I., do Rosário, V.E., Centeno-Lima, S., 2004. Molecular and phylogenetic characterization of *Theileria* spp. parasites in autochthonous bovines (Mirandesa breed) in Portugal. Vet. Parasitol. 123, 17–23.

Costa-Júnior, L.M., Rabelo, É.M.L., Filho, O.A.M., Ribeiro, M.F.B., 2006. Comparison of different direct diagnostic methods to identify *Babesia bovis* and *Babesia bigemina* in animals vaccinated with live attenuated parasites. Vet. Parasitol. 139, 231–236.

Dalrymple, B.P., 1993. Molecular variation and diversity in candidate vaccine antigens from *Babesia*. Acta Trop. 53, 227–238.

Dalrymple, B.P., Peters, J., Goodger, B.V., Bushell, G., Waltisbuhl, D., et al., 1993. Cloning and characterization of cDNA clones encoding two *Babesia bovis* proteins with homologous amino- and carboxy-terminal domains. Mol. Biochem. Parasitol. 59, 181–189.

da Silva, E.S., Gontijo, C.M., Pacheco Rda, S., Brazil, R.P., 2004. Diagnosis of human visceral leishmaniasis by PCR using blood samples spotted on filter paper. Genet. Mol. Res. 3, 251–257.

de Vos, A.J., Potgieter, F.T., 2004. Bovine babesiosis. In: Coetzer, J.A.W., Thomson, G.R., Tustin, R.C. (Eds.), Infectious Diseases of Livestock. Oxford University Press, Cape Town, South Africa, pp. 278–294.

Dowling, S.C., Perryman, L.E., Jasmer, D.P., 1996. A *Babesia bovis* 225-kilodalton spherical-body protein: localization to the cytoplasmic face of infected erythrocytes after merozoite invasion. Infect. Immun. 64, 2618–2626.

Fahrimal, Y., Goff, W.L., Jasmer, D.P., 1992. Detection of *Babesia bovis* carrier cattle by using polymerase chain reaction amplification of parasite DNA. J. Clin. Microbiol. 30, 1374–1379.

Figueroa, J.V., Chieves, L.P., Johnson, G.S., Buening, G.M., 1993. Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis*, and *Anaplasma marginale* DNA in bovine blood. Vet. Parasitol. 50, 69–81.

Gayo, V., Romito, M., Nel, L.H., Solari, M.A., Viljoen, G.J., 2003. PCR-based detection of the transovarial transmission of Uruguayan *Babesia bovis* and *Babesia bigemina* vaccine strains. Onderstepoort J. Vet. Res. 70, 197–204.

Gubbels, J.M., de Vos, A.P., van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 37, 1782–1789.

Hines, S., Palmer, G., Brown, W., McElwain, T., Suarez, C., Vidotto, O., Rice-Ficht, A., 1995. Genetic and antigenic characterization of *Babesia bovis* merozoite spherical body protein Bb-1. Mol. Biochem. Parasitol. 69, 149–159.

Iseki, H., Alhassan, A., Ohta, N., Thekiose, O.M., Yokoyama, N., Inoue, N., Nambota, A., Yasuda, J., Igarashi, I., 2007. Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites. J. Microbiol. Methods 71, 281–287.

Jasmer, D., Reduker, D., Perryman, L., McGuire, T., 1992. A *Babesia bovis* 225-kilodalton protein located on the cytoplasmic side of the erythrocyte membrane has sequence similarity with a region of glycogen phosphorylase. Mol. Biochem. Parasitol. 52, 263–269.

McCosker, P.J., 1981. The global importance of babesiosis. In: Ristic, M., Kreier, J.P. (Eds.), Babesiosis. Academic Press, New York, NY, pp. 1–24.

Martins, T.M., Pedro, O.C., Caldeira, R.A., do Rosário, V.E., Neves, L., Domingos, A., 2008. Detection of bovine babesiosis in Mozambique by a novel seminested hot-start PCR method. Vet. Parasitol. 153, 225–230.

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

Oliveira-Sequeira, T.C.G., Oliveira, M.C.S., Araújo Jr., J.P., Amarante, A.F., 2005. PCR-based detection of *Babesia bovis* and *Babesia bigemina* in their natural host *Boophilus microplus* and cattle. Int. J. Parasitol. 35, 105–111.

Oura, C.A.L., Bishop, R.P., Wampande, E.M., Lubega, G.W., Tait, A., 2004. Application of a reverse line blot assay to the study of hemoparasites in cattle in Uganda. Int. J. Parasitol. 34, 603–613.

Passos, L.M.F., Bell-Sakyi, L., Brown, C.G.D., 1998. Immunochemical characterization of *in vitro* culture-derived antigens of *Babesia bovis* and *Babesia bigemina*. Vet. Parasitol. 76, 239–249.

Ristic, M., 1981. Babesiosis. In: Ristic, M., MacIntyre, I. (Eds.), Disease of Cattle in the Tropics. Martinus Nijhof Publishers, The Hague, The Netherlands, pp. 443–468.

Ruef, B.J., Dowling, S.C., Conley, P.G., Perryman, L.E., Brown, W.C., Jasmer, D.P., Rice-Ficht, A.C., 2000. A unique *Babesia bovis* spherical body protein is conserved among geographic isolates and localizes to the infected erythrocyte membrane. Mol. Biochem. Parasitol. 105, 1–12.

Smeenk, I., Kelly, P.J., Wray, K., Musuka, G., Trees, A.J., Jongejan, F., 2000. *Babesia bovis* and *B. bigemina* DNA detected in cattle and ticks from Zimbabwe by polymerase chain reaction. J. S. Afr. Vet. Assoc. 71, 21–24.

Wagner, G., Cruz, D., Holman, P., Waghela, S., Perrone, J., Shompole, S., Rurangirwa, F., 1992. Non-immunologic methods of diagnosis of babesiosis. Mem. Inst. Oswaldo Cruz 87, 193–199.

Yokoyama, N., Okamura, M., Igarashi, I., 2006. Erythrocyte invasion by *Babesia* parasites: current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. Vet. Parasitol. 138, 22–32.

Please cite this article in press as: AbouLaila, M., et al., Development and evaluation of a nested PCR based on spherical body protein 2 gene for the diagnosis of *Babesia bovis* infection. Vet. Parasitol. (2009), doi:10.1016/j.vetpar.2009.12.013

■ MINI-REVIEW

Korean J Parasitol. Vol. 47, No. 1: 1-5, March 2009 DOI: 10.3347/kjp.2009.47.1.1

Modeling the Dynamics and Control of Transmission of *Schistosoma japonicum* and *S. mekongi* in Southeast Asia

Hirofumi Ishikawa[1,*] and Hiroshi Ohmae[2]

[1]Deptartment of Human Ecology, Graduate School of Environmental Science, Okayama University, Okayama 700-8530, Japan;
[2]Department of Parasitology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

Abstract: A mathematical model for transmission of schistosomes is useful to predict effects of various control measures on suppression of these parasites. This review focuses on epidemiological and environmental factors in *Schistosoma japonicum* and *Schistosoma mekongi* infections and recent advances in mathematical models of *Schistosoma* transmission.

Key words: *Schistosoma japonicum, Schistosoma mekongi*, mathematical model, control strategies

INTRODUCTION

Schistosomiasis is an important disease problem in several Asian countries. Schistosomiasis japonica is prevalent in China and the Philippines, where millions of people are affected. Schistosomiasis mekongi is prevalent in the Mekong River basin (MRB) from the Khong district in southern Laos to Kratie province in northern Cambodia. The total population at risk for schistosomiasis mekongi is estimated to be 60,000 in Laos and 80,000 in Cambodia [1]. The first human case of *Schistosoma mekongi* infection was reported as *Schistosoma japonicum* infection in 1957 [2]. Afterwards, *S. mekongi* was identified as a new species of *Schistosoma* [3]. *S. japonicum* and *S. mekongi* have a complicated mode of transmission. As part of the life cycle occurs in the environment outside of the host, it is difficult to measure the transmission rate on the basis of field observations [4]. Therefore, a mathematical model for *Schistosoma* transmission could be useful for estimating its prevalence, and model simulations can be instrumental in managing various control strategies. There have been many studies involving the mathematical modeling of transmission for *S. japonicum* since 1965 [4-12], while there have been only 2 studies on mathematical modeling of the transmission of *S. mekongi* [13,14]. This review focuses on the epidemiological and environmental factors in *S. japonicum* and *S. mekongi* infections and the recent advances in mathematical models of *Schistosoma* transmission.

S. japonicum and *S. mekongi* carry out their transmission cycle in definitive and intermediate hosts. Humans are the major definitive hosts, and many domestic and wild mammals were found to be reservoirs [15-19], whereas the intermediate hosts are only snails. Cercariae, which are released from infected snails, penetrate into the definitive host via the skin and develop into mature adults in the host; female adults produce eggs throughout their life-span. A mathematical model that quantitatively describes *Schistosoma* transmission needs to include the following components [4,14].

1. Dynamics of the human population and behavior of humans related to water contact
2. Dynamics of the intermediate host population
3. Fluctuation of water level in the Mekong River (only for *S. mekongi*)
4. Contribution of animal reservoirs
5. Effect of control measures

DYNAMICS OF THE INTERMEDIATE HOSTS: SNAILS FOR *S. JAPONICUM*

Oncomelania spp. snails were recognized as intermediate hosts of *S. japonicum*; *O. quadrasi* and *O. hupensis* were identified in the Philippines [20,21] and in China [16], respectively. As snail density varies according to time and circumstances, it is difficult to make accurate estimates of snail density. In Bohol, the Philippines, a water area fluctuates seasonally according to rainfalls [4]. In regard to the longevity of snails, the mortality rate among infected snails is higher than that among uninfected snails [20,22].

• Received 9 October 2008, revised 26 January 2009, accepted 27 January 2009.
* Corresponding author (ishikawa@ems.okayama-u.ac.jp)

DYNAMICS OF THE INTERMEDIATE HOSTS: SNAILS FOR *S. MEKONGI* AND FLUCTUATION OF THE MEKONG RIVER FLOW

Neotricula aperta is recognized as the intermediate host of *S. mekongi*, which is composed of 3 races (α, β, and γ). Races α and γ range in the MRB from Khong to Kratie, whereas race β lives in the Mun River, a tributary of the Mekong River [23]. Race γ shows the highest susceptibility to miracidia among the 3 races [24].

In Cambodia, the rainy season begins in March, and heavy rainfall lasts from June to October; the rainfall drops dramatically in November, and thereafter the dry season lasts from December to February. The heavy rainfall and the arrival of the dry season results in rising and dropping water levels in the Mekong River (World Weather Information Service; Mekong River Commission) (Fig. 1). The biology of *N. aperta* is still largely unknown because of the impracticality of field observations during the high water period of the Mekong River. The "Post-Spate Survival" hypothesis that *N. aperta* survive and copulate during the high water period of the Mekong River, but that laying eggs could be delayed until next January, and that thereafter the eggs hatch from February has been used to represent the population dynamics of *N. aperta* [14,25] (Fig. 2).

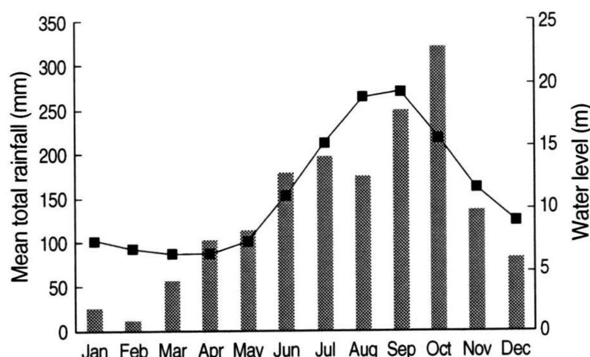


Fig. 1. Monthly average rainfall levels (bars) for 5 years during 1997-2001 in Phnom Penh (World Weather Information Service) and monthly average water levels of the Mekong River (line) for 14 years during 1989-2002 in Kratie province, Cambodia (Mekong River Commission) [14].

DEFINITIVE HOSTS: ANIMAL RESERVOIRS

When animal reservoirs play a part in the reproduction of *Schistosoma*, it is necessary to give careful consideration to them because of the difficulty in eliminating *Schistosoma* by means of chemotherapy for humans only [26]. In Lyte, the Philippines, many domestic animals such as dogs, pigs, and cows were found to be reservoirs for *S. japonicum* [15], while in Bohol, the Philippines, only rats were infected by *S. japonicum* with a low prevalence rate from the results of field surveys [21]. In China, more than 40 species of domestic and wild mammalians have been identified as reservoirs for *S. japonicum* [16]. In the MRB, dogs and pigs have been recognized as reservoirs for *S. mekongi* [18, 19]. In Laos, the prevalence in dogs was estimated as 11% [18] and 29.2% [17].

MATHEMATICAL MODELS OF *SCHISTOSOMA* TRANSMISSION

Macdonald [5] first proposed a mathematical model for *Schistosoma* transmission, and thereafter a number of mathematical models for *Schistosoma* transmission have been published, mainly from the theoretical point of view [5-8]. Anderson and May [22] studied the prevalence of snail infection based on empirical evidence. A stochastic model for schistosomiasis developed

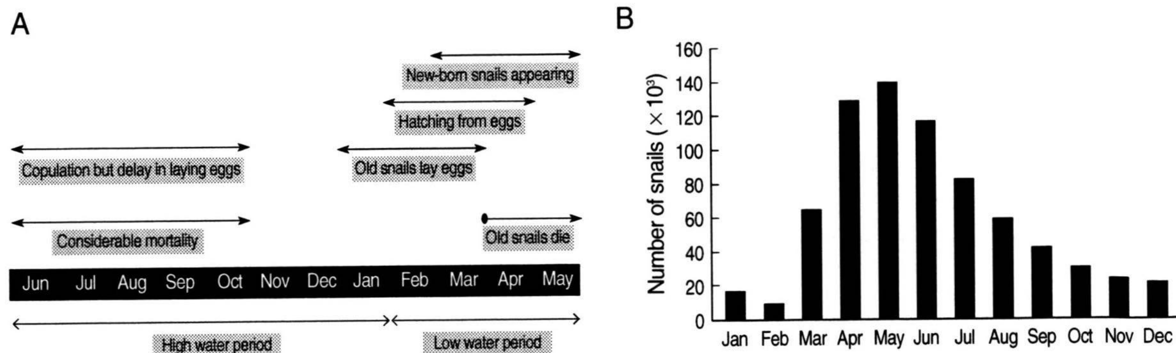


Fig. 2. Population dynamics of *Neotricula aperta* on the basis of the Post-Spate Survival hypothesis. (A) The life cycle of *N. aperta*, (B) The monthly variation of the total snail population [14].

from a model for onchocerciasis aimed to evaluate control strategies [9]. A series of studies on modeling of *S. japonicum* transmission and control in China have been performed [10-12]. Ishikawa et al. [4] developed a model of *S. japonicum* transmission that took account of seasonal variations in snail density, animal reservoirs, rats, and high and low cercarial shedding stages in snails to predict the effects of control measures against *S. japonicum* in Bohol, the Philippines (Fig. 3). A couple of studies proposed a mathematical model for the transmission of *S. mekongi* in Cambodia with age-structure, which was aimed at estimating the coverage rate and range of ages in targeted mass treatment (TT) to interrupt *Schistosoma* transmission [13,14]. The fluctuation of water level in the Mekong River, dynamics of the intermediate snail host population, and the contribution of an animal reservoir, dogs, were incorporated into this model [14].

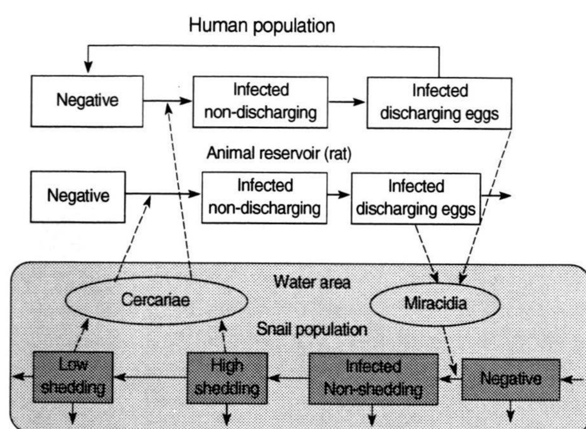


Fig. 3. The basic scheme of the transmission model for *S. japonicum* showing the transfers among epidemiological classes [4].

CONTROL MEASURES AND THEIR EVALUATION THROUGH SIMULATIONS

A collaborative project of the Schistosomiasis Control Service of the Philippine Department of Health and the Sasakawa Memorial Health Foundation of Japan has been continuing since 1981 in Bohol [21]. The major approach to the control of *S. japonicum* consists of 2 methods: the detection of infected individuals and chemotherapeutic treatment, snail control by environmental changes such as land reclamation and cement lining of ditches, and using molluscicides. The comparative simulations showed that the prevalence in inhabitants and the density of infected snails could be restored swiftly after the completion of 4 courses of yearly selected mass treatments without snail control, that the prevalence in inhabitants could be reduced gradually by snail control measures alone, and that the prevalence in inhabitants and the density of infected snails would be eliminated by human control together with snail control [4] (Fig. 4).

In the MRB, snail control measures such as using molluscicides are ineffective because of the Mekong River flow rate. In Cambodia, a universal treatment campaign (UT) was initiated by the Cambodian government, WHO, and Medecine Sans Frontieres in 1995. The Sasakawa Memorial Health Foundation has rendered support for control of schistosomiasis mekongi since 1997. In Laos, although the average prevalence of schistosomiasis mekongi decreased to less than 1% after mass treatment with praziquantel during a 10-year control program [27], a resurgence of schistosomiasis in the Khong district was confirmed by epidemiological surveys by WHO in 2003 [28]. Com-

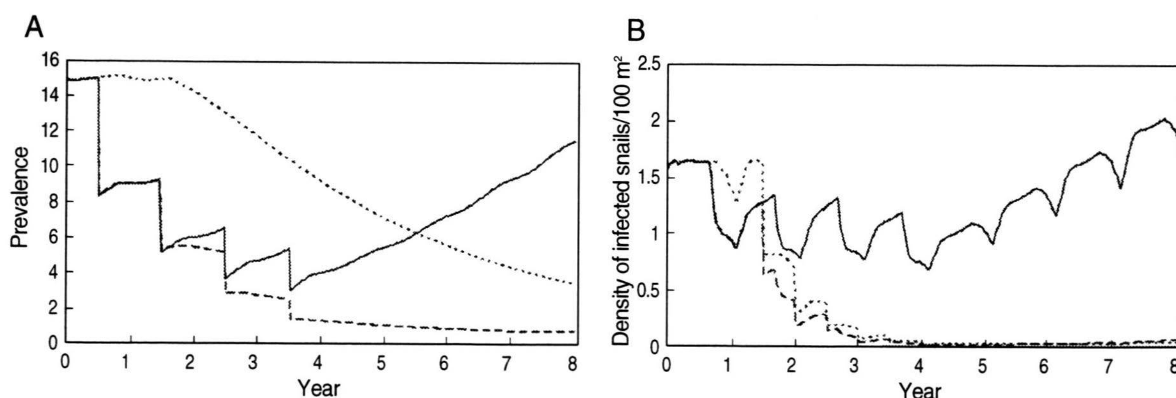


Fig. 4. Variations in the infection of *S. japonicum* in Bohol (Sto. Thomas) for the human-control case with selective mass treatment at 1-year interval with a coverage rate of 50% (solid line), the snail-control case with the use of molluscicides at half-year intervals under the assumption that its effective rate would be 50% (dotted line), and both the human and snail-control case (dashed line), respectively. (A) Variations in prevalence (%) in the human population, (B) Variations in infected snail densities per 1 *a* (100 m²) [4].

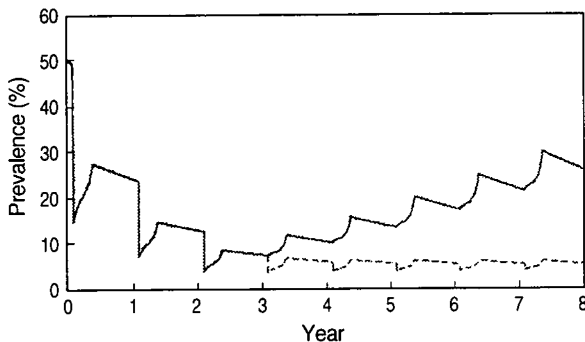


Fig. 5. Variations in the prevalence in humans with 2 control measures. 1: Yearly universal treatment (UT) with a 70% coverage rate for the initial 3 years (solid line), 2: After 3 years of annual UT targeted mass treatment (TT) with a 85% coverage rate (dashed line) [14].

parative simulations for the situation of an interruption in mass treatments after 3 courses of yearly UT showed that the prevalence in inhabitants increased swiftly, while the effect of yearly TT for children of 5-19 years old, who show higher prevalence and intensity of infection, after 3 courses of yearly UT would keep the prevalence in inhabitants low throughout an 8-year simulation [14] (Fig. 5).

PROSPECTS

Recent advances in mathematical modeling of the transmission of *S. japonicum* and *S. mekongi* are summarized here. There has been steady progress in the mathematical modeling of *Schistosoma* transmission taking into consideration of the ecology of snails and the behavior of inhabitants. The model simulations suggested that, among various possible control measures, a selective mass treatment program coordinated with snail control would be effective for the elimination of *S. japonicum*. The model simulations also predicted that the suppression of schistosomiasis mekongi could be possible in Cambodia by maintaining control strategies for humans such as biyearly UT or yearly TT with high coverage.

ACKNOWLEDGEMENTS

We appreciate the helpful comments of Profs. H. Matsuda, Y. Chigusa, and M. Kirinoki of Dokkyo Medical University. We are indebted to Dr. T. Matsumoto of the National Institute for Rural Engineering for providing Mekong River water level data.

REFERENCES

1. Urbani C, Sinoun M, Socheat D, Pholsena K, Strandgaard H, Odermatt P, Hatz C. Epidemiology and control of mekongi schistosomiasis. Acta Trop 2002; 82: 157-168.
2. Vic-Dupont BE, Soubrane J, Halle B, Richier C. Bilharzoise á *Schistosoma japonicum* á forme hépatosplénique révélée par une grande hématémèse. *Bull Mém Soc Méd Hôpit Paris* 1957; 73: 933-994 (in French).
3. Voge M, Bruckner D, Bruce JI. *Schistosoma mekongi* sp. n. from man and animals, compared with four geographic strains of *Schistosoma japonicum*. J Parasitol 1978; 64: 577-584.
4. Ishikawa H, Ohmae H, Pangilinan R, Redulla A, Matsuda H. Modeling the dynamics and control of *Schistosoma japonicum* transmission on Bohol island, the Philippines. Parasitol Int 2006; 55: 23-29.
5. Macdonald G. The dynamics of helminth infections with special reference to schistosomes. Trans R Soc Trop Med Hyg 1965; 59: 489-506.
6. Woolhouse MEJ. On the application of mathematical models of schistosome transmission dynamics. I. Natural transmission. Acta Trop 1991; 49: 241-270.
7. Woolhouse MEJ. On the application of mathematical models of schistosome transmission dynamics. II. Control. Acta Trop 1992; 50: 189-204.
8. Allen EJ, Victory JrHD. Modelling and simulation of a schistosomiasis infection with biological control. Acta Trop 2003; 87: 251-267.
9. de Vlas SJ, Van Oortmarssen GJ, Gryseels B, Polderman AM, Plaisier AP, Habbema JDE. SCHISTOSIM: a microsimulation model for the epidemiology and control of schistosomiasis. Am J Trop Med Hyg 1996; 55: 170-175.
10. Williams GM, Sleigh, AC, Li Y, Feng Z, Davis GM, Chen H, Ross AGP, Bergquist R, McManus DP. Mathematical modelling of schistosomiasis japonica: comparison of control strategies in the People's Republic of China. Acta Trop 2002; 82: 253-262.
11. Liang S, Maszle D, Spear RC. A quantitative framework for a multigroup model of schistosomiasis japonicum transmission dynamics and control in Sichuan, China. Acta Trop 2002; 82: 263-277.
12. Liang S, Spear RC, Seto E, Hubbard A, Qiu D. A multi-group model of *Schistosoma japonicum* transmission dynamics and control: model calibration and control prediction. Trop Med Int Health 2005; 10: 263-278.
13. Hisakane N, Ishikawa H, Kirinoki M, Sinuon M, Socheat D, Matsuda H. Mathematical modeling for transmission of *Schistosoma mekongi*: Kratie province in Cambodia. In Nagao I, Takahashi Y eds, Parasitic Zoonoses in Asian-Pacific Regions. Nagoya, Japan. Sankeisha. 2006, p 81-89.
14. Hisakane N, Kirinoki M, Chigusa Y, Sinuon M, Socheat D, Matsuda H, Ishikawa H. The evaluation of control measures against *Schistosoma mekongi* in Cambodia by a mathematical model. Parasitol Int 2008; 57: 379-385.
15. Pesigan TP, Farooq M, Hairston NG, Jauregui JJ, Garcia EG, Santos

AT, Santos BC, Besa AA. Studies on *Schistosoma japonicum* infection in the Philippines. 1. General considerations. Bull WHO 1958; 18: 345-455.

16. Minggang C, Zheng F. Schistosomiasis control in China. Parasitol Int 1999; 48: 11-19.
17. Iijima T, Lo CT, Ito Y. Studies on schistosomiasis in the Mekong Basin, I. Morphological observations of the schistosomes and detection of their reservoir hosts. Jpn J Parasitol 1971; 20: 24-33.
18. Sornmani S, Kitikoon V, Thirachantra S, Harinasuta C. Epidemiology of Mekong schistosomiasis. The Mekong schistosome. Malacol Rev 1980; suppl 2: 9-18.
19. Strandgaard H, Johansen MV, Pholsena K, Teixayavong K, Christensen NO. The pig as a host for *Schistosoma mekongi* in Laos. J Parasitol 2001; 87: 708-709.
20. Pesigan TP, Hairston NG, Jauregui JJ, Garcia EG, Santos AT, Santos BC, Besa AA. Studies on *Schistosoma japonicum* infection in the Philippines. 2. The molluscan host. Bull WHO 1958; 18: 481-578.
21. Yasuraoka K, Blas BL, Matsuda H, Irie Y, Nihei N, Ohmae H, Yokoi H, Hambre R, Pangilinan R, Autentico C, Tanaka H. Approaches to the elimination of schistosomiasis on Bohol Island, Philippines. Jpn J Parasitol 1996; 45: 391-399.
22. Anderson RM, May RM. Prevalence of schistosome infections within molluscan populations: observed patterns and theoretical predictions. Parasitology 1979; 79: 63-94.
23. Davis GM, Rao NVS, Hoagland KE. In search of *Tricula* (Gastropoda: Pomatiopsidae): *Tricula* defined, and a new genus described. Proc Acad Natl Sci Philadelphia 1986; 138: 426-442.
24. Ohmae H, Sinuon M, Kirinoki M, Matsumoto J, Chigusa Y, Socheat D, Matsuda H. Schistosomiasis mekongi: from discovery to control. Parasitol Int 2004; 53: 135-142.
25. Attwood SW. Schistosomiasis in the Mekong region: epidemiology and phylogeography. Adv Parasitol 2001; 50: 87-152.
26. Guo J, Li Y, Gray D, Ning A, Hu G, Chen H, Davis G, Sleigh AC, Feng Z, McManus DP, Williams GM. A drug-based intervention study on the importance of buffaloes for human *Schistosoma japonicum* infection around Poyang Lake, People's Republic of China. Am J Trop Med Hyg 2006; 74: 336-341.
27. Khamkeo T, Pholsena K. Control of schistosomiasis due to *Schistosoma mekongi* in Khong District, 1989-1999. In Crompton DWT, Montresor A, Nesheim MC, Savioli L eds, Controlling Disease Due to Helminth Infections. Geneva, Switzerland. World Health Organization. 2003, p 170-181.
28. Vongsouvan S. Presentation hand out: updated status of schistosomiasis mekongi in the Lao PDR. Meeting on Regional Network for Research, Surveillance and Control for Asian Schistosomiasis, Vientiane, Lao PDR. 2003.

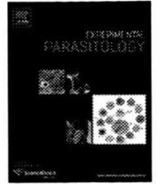
Author's personal copy

Experimental Parasitology 124 (2010) 325–329

Contents lists available at ScienceDirect

Experimental Parasitology

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



Identification and differentiation of human schistosomes by polymerase chain reaction

Naoko Kato-Hayashi [a,*], Masashi Kirinoki [a], Yukio Iwamura [b], Tamotsu Kanazawa [c], Viroj Kitikoon [d], Hajime Matsuda [e], Yuichi Chigusa [a]

[a] Laboratory of Tropical Medicine and Parasitology, Dokkyo Medical University, Tochigi, Japan
[b] Center for Arts and Humanities, Ibaraki Prefectural University of Health Sciences, Ibaraki, Japan
[c] Department of Parasitology and Tropical Public Health, University of Occupational and Environmental Health, Fukuoka, Japan
[d] Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
[e] Institute of International Education and Research, Dokkyo Medical University, Tochigi, Japan

ARTICLE INFO

Article history:
Received 5 March 2009
Received in revised form 6 November 2009
Accepted 16 November 2009
Available online 3 December 2009

Keywords:
Schistosoma mansoni
S. haematobium
S. japonicum
S. mekongi
PCR
Multiplex
Mitochondial DNA
cox1

ABSTRACT

Recent increasing number of travelers, immigrants and foreign workers from schistosomiasis endemic area has thus resulted in the importation of schistosomiasis to non-endemic countries. To avoid ova-induced pathogenicity, sensitive and specific diagnostic means at an early stage of infection are therefore crucial. In this study, we developed polymerase chain reaction (PCR) primers specific for human schistosome species. The PCR products were obtained in a species-specific manner (479 bp, *Schistosoma mansoni*; 365 bp, *S. haematobium*; 614 bp, *S. japonicum*; 303 bp, *S. mekongi*) and were detectable from 0.01 pg of total worm DNA (*S. haematobium, S. japonicum, S. mekongi*). The primer sets were also available for multiplex use. Although some difficulties were experienced in amplifying the parasite DNA from the infected animals, schistosome DNA could be detected from one day post infection. The PCR method described herein will therefore be beneficial to detect human schistosomiasis, after some improvements in this method.

© 2009 Elsevier Inc. All rights reserved.

1. Introduction

Schistosomiasis affects about 200 million people worldwide, and more than 650 million live in endemic areas (WHO, 2005). Moreover, the threat is now spreading globally in non-endemic areas, such as Western countries (Houston et al., 2004; Bierman et al., 2005; Bottieau et al., 2006) and Japan. Recently, increases in the number of travelers, migrants and foreign workers have resulted in the importation of schistosomiasis. Because the main pathogenicity of this disease is severe hypersensitivity (e.g., acute Katayama syndrome and chronic granulomatous diseases) caused by the parasite eggs trapped in the host tissues, the early detection and treatment of this disease are therefore crucial to avoid any subsequent fatalities. The current frequently used diagnostic methods are the detection of parasite eggs and parasite-specific antibodies. The former is distinguishable between species based on the egg morphology and it shows direct evidence of an actual schistosome infection. However, this diagnostic modality is not feasible during the prepatent period when parasite eggs are not detectable. Furthermore, stool examinations such as the Kato–Katz method are insensitive in individuals with only light infections (Doenhoff et al., 2004) and the egg excretion reduced/suppressed with the time course of infection (Cheever et al., 1994) and/or by the host immune status (Hermeto et al., 1994; Montes et al., 2004). The latter has a high sensitivity and it is applicable for mass examinations by means of ELISA. However, it has a rather low specificity due to cross reactions and the existence of antibodies per se shows an unclear state of infection. Namely, according to Hayashi et al. (2000), anti *Schistosoma japonicum* antibody has been detected by ELISA even 33 years after treatment. Moreover, the clue antigens for antibody production tend to be associated with egg deposition (Hillyer and Bruce, 1980; Doenhoff et al., 2004). In this context, there is a need to establish new diagnostic measures that are independent of the pathogenic eggs.

Recently, several investigators have launched new molecular based approaches for detecting schistosomiasis: schistosome DNA in human clinical samples such as feces (Pontes et al., 2002, 2003; Gobert et al., 2005), sera (Pontes et al., 2002) and urine (Sandoval et al., 2006a). The existence of parasite DNA in the host is direct evidence of an actual infection. The data from experimental animals have suggested that potentially effective diagnostic

* Corresponding author. Fax: +81 282 86 6431.
E-mail address: nkato@dokkyomed.ac.jp (N. Kato-Hayashi).

0014-4894/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2009.11.008

Author's personal copy

tools can be developed for use during the prepatent period (Sandoval et al., 2006b; Suzuki et al., 2006; Xia et al., 2009). Furthermore, the detection of parasite DNA may also be a helpful guideline for selecting the optimal treatment for schistosomiasis.

In this study, we developed new PCR primers for the identification and differentiation of major human schistosomes: *S. mansoni*, *S. haematobium*, *S. japonicum* and *S. mekongi*, using the currently available genetic information and assessed their potentiality/availability.

2. Materials and methods

2.1. Parasite materials and the extraction of parasite DNA

Adult worms of *S. mansoni* (Puerto Rican strain), *S. haematobium* (Kenyan strain), *S. japonicum* (Japanese Yamanashi strain) and *S. mekongi* (Laotian strain) used for template DNA were obtained from either experimentally infected mice or Mongolian gerbils. The infected animals were maintained at the Center for Tropical Medicine and Parasitology, Dokkyo Medical University. The handling and care of all experimental animals in this study strictly complied with the Guidelines for Animal Experiments of Dokkyo Medical University according to the Japanese law. Originally, *S. mekongi* was provided from Mahidol University, Thailand in 2000 and *S. haematobium* was provided from the University of Occupational and Environmental Health, Japan in 2004, and, thereafter, they were maintained in our laboratory. Adult worms of each species were picked up from either the mesenteric or portal veins of all experimental animals, washed with PBS several times and then were stored at −80 °C until use.

The total DNA of adult worms was extracted using commercially available DNA extraction Kits (e.g., Easy-DNA™ Kit, Invitrogen™, USA). The adult worms were lyophilized and minced with scissors, and then were processed according to the manufacturer's instructions.

2.2. Primer design, PCR and sequencing

The complete sequences of the mitochondrial gene of *Schistosoma* spp. were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html): *S. mansoni* (GenBank Accession No. AF216698, Le et al., 2000), *S. haematobium* (GenBank Accession No. DQ157222, Littlewood et al., 2006), *S. japonicum* (GenBank Accession No. AF215860, Le et al., 2000) and *S. mekongi* (GenBank Accession No. AF217449, Le et al., 2000). The sequences of the cytochrome c oxidase subunit 1 (cox1) gene of *Schistosoma spp.* were compared using CLUSTALW (http://align.genome.jp/). Candidate primers were checked not to form primer dimer and then *in silico* PCR was performed by FastPCR (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm). The primer pairs were specific for *S. mansoni* (SmF/CR), *S. haematobium* (ShF/CR), *S. japonicum* (SjF/CR), *S. mekongi* (SmF/CR) and common *Schistosoma* spp. (CF/CR) are shown in Table 1. Basically, PCR was carried out in a final volume of 20 μl with 2 μl of 10× PCR Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.4 U of Platinum® *Taq* DNA polymerase (Invitrogen™, USA), 0.5 μM of each primer (Tsukuba Oligo Service Co., Ltd., Japan) and 1 μl of template DNA. The reactions were performed initially at 94 °C for 2 min, then 35 cycles, each consisting of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s and a final cycle of 72 °C for 7 min.

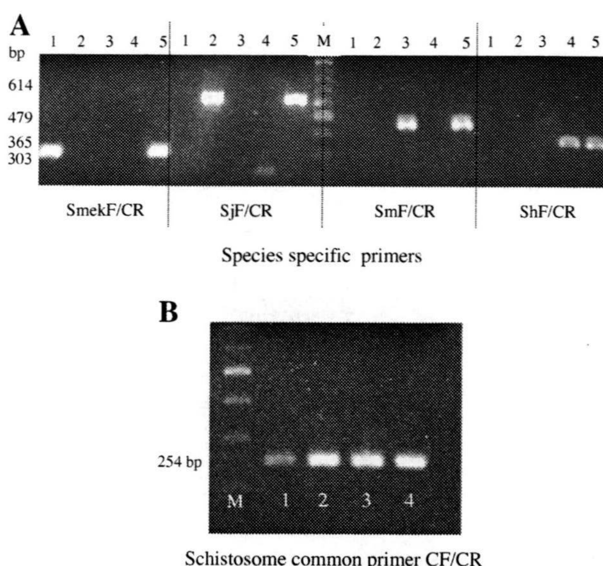


Fig. 1. Specificity tests for the schistosome species-specific primers (A) and common primer (B) using different DNA. Lane M, 100 bp molecular marker; Lane 1, *Schistosoma. mekongi*; Lane 2, *S. japonicum*; Lane 3, *S. mansoni*; Lane 4, *S. haematobium*; Lane 5, mixed four species.

The PCR products were electrophoresed in 2% agarose (Certified™ Low Range Ultra Agarose, BIO-RAD Laboratories, USA) in tris–acetate–EDTA gels with 0.3 μg/ml ethidium bromide, and then were visualized in an UV transilluminator.

The PCR products were purified using the Qiaquick® PCR Purification Kit (Qiagen, USA), and then the samples were prepared to undergo DNA sequencing by BigDye® Terminator FS Cycle Sequencing Kit (Applied Biosystems, USA). DNA sequencing was performed using the ABI Prism 3100 genetic analyzer, and the data were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 1
Primer sets for cox1 gene amplification of *Schistosoma* spp.

Parasite	Primers		PCR Product length (bp)
	Name	Sequence 5′ → 3′	
S. mansoni	SmF	TCCTTTATCAATTTGAGAGG	479
	CR	CCAACCATAAACATATGATG	
S. haematobium	ShF	AGTCGTGTCGATTTTAAGAC	365
	CR	CCAACCATAAACATATGATG	
S. japonicum	SjF	CCGTTTTTTTTGAGTATGAG	614
	CR	CCAACCATAAACATATGATG	
S. mekongi	SmekF	GTTAATATCATTGCCTGTAC	303
	CR	CCAACCATAAACATATGATG	
Schistosoma spp. Four species common	CF	GATCGTAAATTTGGW*ACTGC	254 (*S mansoni*: 253)
	CR	CCAACCATAAACATATGATG	

* W: mixture of A and T.

Author's personal copy

Table 2
Detection limits of monoplex PCR.

Template DNA	*S. mansoni*		*S. haematobium*		*S. japonicum*		*S. mekongi*	
Primers	SmF/CR	CF/CR	ShF/CR	CF/CR	SjF/CR	CF/CR	SmekF/CR	CF/CR
Detection limit (pg)	0.1	0.01	0.01	0.01	0.01	0.01	0.01	0.01

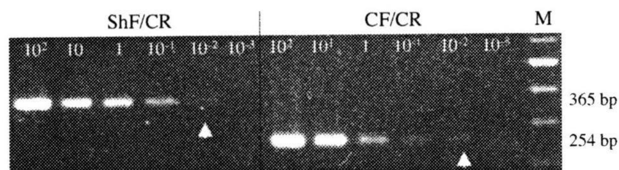


Fig. 2. Representative results of the sensitivity tests for monoplex PCR. The genomic DNA extracted from adult worms of *Schistosoma. haematobium* was serially diluted (10^2 to 10^{-3} pg) and applied *S. haematobium* specific PCR (ShF/CR) or schistosome common PCR (CF/CR). The arrow shows the detection limit of the assay.

2.3. Detection of parasite DNA from biological samples of schistosome infected animals

The biological samples (sera and/or urine) were obtained from the animals experimentally infected with *Schistosoma* spp. These animals were intended to be used either for the maintenance of the parasites or for the other experiments. These samples were collected and stored at −80 °C or in ethanol until use. The DNA was extracted using some commercially available kits (e.g., NucleoSpin® Tissue, Macherey-Nagel, Germany; InstaGene™ Whole Blood Kit, Bio-Rad Laboratories, USA, etc.). The PCR reactions were carried out in almost the same manner as described above, with some modification: 2.5 mM of $MgCl_2$, 0.5–1.0 U of *Taq* DNA polymerase, 0.5–2.0 μM of each primer and the number of reactions were increased up to 50 cycles.

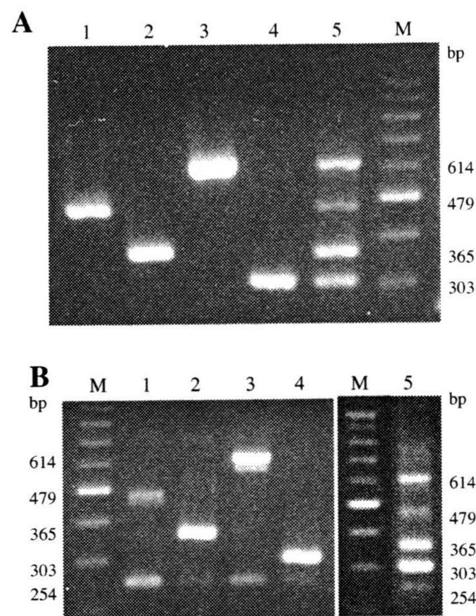


Fig. 3. Specificity tests for multiplex PCR using four sets of schistosome specific primers (A) and multiplex PCR using five sets of schistosome specific primers and common primer (B). Lane M, 100 bp molecular marker; Lane 1, *Schistosoma. mansoni*; Lane 2, *S. haematobium*; Lane 3, *S. japonicum*; Lane 4, *S. mekongi*; Lane 5, mixed four species.

3. Results and discussion

The PCR products were successfully amplified in a species-specific manner. The product sizes obtained were 479 bp (*S. mansoni*), 365 bp (*S. haematobium*), 614 bp (*S. japonicum*), 303 bp (*S. mekongi*) and 253/254 bp (schistosome common) (Fig. 1A and B). The sequenced data were analyzed by BLAST and then were confirmed to be identical to each species. The specificity of the primers was checked by PCR combined with primer sets and template DNA from each schistosome species. In the monoplex PCR, no cross amplification was observed to occur between the species (Fig 1A). To evaluate the sensitivity of the species-specific PCR, the total worm DNA ($OD_{260/280}$ > 1.8) from each species was serially diluted and applied for PCR. The minimum detection limit of the monoplex PCR (individual species) was 0.01 pg on *S. haematobium*, *S. japonicum* and *S. mekongi* (Table 2/Fig. 2). The low detection limits obtained with a low concentration of the primer (0.5 μM) and Taq polymerase (0.4 U/reaction) demonstrated the high sensitivity of the PCR system.

For multiplex PCR, each species-specific reaction mix was mixed in equal ratios. The conditions were equal to those for the monoplex PCR. Similarly, no cross amplification was observed in multiplex use (Fig. 3).

Table 3 shows the representative data of the PCR positive results from biological samples of the schistosome infected animals. Although some difficulties were encountered in amplifying the parasite DNA from the infected animals (data not shown), schistosome DNA could be detected from 1 DPI (days-post-infection). Because a lower sensitivity may be attributed to the small amount of parasite DNA in comparison to a large amount of host DNA, the methods available for concentrating and/or purifying parasite DNA are thus required to improve sensitivity.

In the area of infectious diseases, the detection of pathogen derived nucleic acid by PCR or LAMP (loop-mediated isothermal amplification) have thus been introduced as a diagnostic tool (Yam et al., 2003; Thai et al., 2004). Because the main pathology derived by schistosome is triggered by parasite eggs, we therefore need a more sensitive diagnostic means which can be effectively used during the early stage of infection. The detection of schistosome DNA has been reported in the snail host (Jannotti-Passos et al., 1997; Driscoll et al., 2005; Abbasi et al., 2007), in water (Hamburger et al., 1998) and in clinical specimens (Pontes et al., 2002, 2003; Gobert et al., 2005; Sandoval et al., 2006a; ten Hove et al., 2008; Obeng et al., 2008; Allam et al., 2009). It is encouraging that worm DNA was detected in the urine and sera of experimentally infected mice at one and two weeks-post-infection, respectively (Sandoval et al., 2006b; Suzuki et al., 2006). In addition, our cumulative data also showed parasite DNA to be detectable from 1 DPI (Table 3). It is therefore suggested that cell-free circulating DNA of schistosome exists in both the host serum and urine. Circulating schistosome DNA in the host closely reflects the existence live worms and/or eggs. Cell-free circulating nucleic acids in the plasma, serum and urine have been of interest as a clinical diagnostic tool for cancer, for prenatal diagnoses, transplantation and traumatology (Chan et al., 2003). The detection of circulating parasite DNA is therefore expected to become a useful diagnostic tool, not only for identifying the early stage of infection, but also for selecting the optimal treatment regimen in old cases, namely