demonstration of antibodies by serological methods. Serological tests to detect the specific antibodies from the serum samples, such as the enzyme-linked immunosorbent assay (ELISA) and the indirect immunofluorescence assay (IFAT), have proved very useful in the clinical diagnosis and seroepidemiology of babesiosis (Weiland and Reiter, 1988; Reiter and Weiland, 1989; Wright, 1990; Bose et al., 1995; Ferrer et al., 1998; Molloy et al., 1998; Boonchit et al., 2002, 2004). However, these serological tests also have several limitations for the diagnosis of babesiosis. For example, these tests require laboratory materials, equipment, and trained personnel and additionally, are labor-intensive and time-consuming. Hence, a convenient, rapid, and sensitive diagnostic test, such as an immunochromatographic test (ICT) that does not require any instrument (Shiff et al., 1993; Chandler et al., 2000; Richardson et al., 2002), would be extremely valuable for use in clinical and field applications for the diagnosis of babesiosis.

The ICT is a nitrocellulose membrane-based immunoassay, which has been developed for a variety of applications over the past decade (Sumathy et al., 1992; Shiff et al., 1993; De Saeger and Van Peteghem, 1996). Assays using this format are rapid, taking approximately 15 min to run, and also convenient, only requiring small volume (<200 μl) of the tested serum on the test strip. Recently, the efficacy of recombinant C-terminal-truncated antigens of *B. bovis* and *B. bigemina* rhoptry-associated protein 1 (RAP-1) (both of rRAP-1/CTs) has been reported on ELISA for the diagnosis of bovine babesiosis (Boonchit et al., 2004, 2006). This study is the first to report that convenient and rapid ICTs were developed for the diagnosis of *B. bovis* and *B. bigemina* infections, in which the two ICTs (BoICT and BiICT, respectively) were based on the RAP-1/CTs for the constructions.

2. Materials and methods

2.1. Preparation of recombinant antigens

B. bigemina rRAP-1/CT, which consists of a C-terminal portion (aa 390–480, GeneBank accession number M60878) of the RAP-1, was prepared as described previously (Boonchit et al., 2006), while *B. bovis* rRAP-1/CT (Boonchit et al., 2004) was prepared with some modifications. Briefly, a gene encoding the *B. bovis* RAP-1/CT (aa 388–490, GeneBank accession number AF030062) was cloned into an expression vector, pGEX 4T-3 (Amersham Pharmacia Biotech, Little Chalfont, UK), and expressed as a RAP-1/CT with glutathione S-transferase (GST) in *E. coli*-competent cells (strain DH5α). *B. bovis* rRAP-1/CT was purified with glutathione sepharose 4B beads to bind GST-fused protein and thrombin protease to cleave rRAP-1/CT from GST–glutathione sepharose 4B complex according to the manufacturer's instructions (Amersham Phamacia Biotech). In the case of GST-fused *B. bigemina* rRAP-1/CT, elution buffer (50 mM Tris–HCL, pH 7.5, 100 mM NaCl, and 2 mM EDTA) was used for purification of GST-fused protein instead of thrombin protease. The purified proteins were confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described previously (Boonchit et al., 2004, 2006). Each of the purified rRAP-1/CTs was used to produce the specific immune serum in rabbit and prepare the conjugated and immobilized antigens as described below.

2.2. Production of rabbit anti-rRAP-1/CT IgG antibody

Two rabbits (CLEA Japan, Tokyo, Japan) were immunized with 500 μl of non-GST-fused *B. bovis* (1 mg/ml) or GST-fused *B. bigemina* (2 mg/ml) rRAP-1/CT mixed with an equal volume of TiterMax (CytRx corporation, Norcross, GA) by multiple intradermal injections into backs. On days 21 and 42 post-initial immunization, the rabbits were infected with the same amount of the antigen. The sera of the immunized rabbit were collected 7 days after the last booster and tested for the specifie immunoglobulin G (IgG) antibody titers by standard ELISA and IFAT (Boonchit et al., 2004, 2006). After the titer of each anti-rRAP/CT IgG antibody was confirmed to reach more than 1/8192, the total IgG was purified from the serum with an Econo-Pac Protein A Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacture's instructions.

2.3. Determination of the minimum amount of the antigens and optimal pH for their conjugation with gold colloid

Fifty microliters of the recombinant antigens were diluted to the final concentrations of 1000, 500, 200, 100, 50, 25, and 10 μg/ml with a 5 mM phosphoric acid buffer that had been separately adjusted at pH 5.0, 5.5, 6.0, 6.3, 6.5, 6.8, and 7.0. Gold colloid (50 nm; British BioCell International, SDX, UK) was also adjusted at the same pH as above with 20 mM phosphoric acid buffer. Five hundred microliters of the pH-adjusted gold colloid was mixed with 50 μl of the diluted and pH-adjusted antigens. After incubation at room temperature

(RT) for 5 min, 50 μl of 10% NaCl was added to the mixture and then agitated at RT for 1 min. The minimum amount of antigens required to stabilize the gold colloid was determined according to the color of the tube, in which the color of the stabilized mixture never changes from red to blue upon the addition of 10% NaCl (Slot and Geuze, 1985).

2.4. Conjugation of rRAP-1/CT with gold colloid

The antigens prepared based on the optical amount and pH were gently mixed with gold colloids (1:10, v/v) and then incubated at RT for 10 min. Subsequently, polyethylene glycol 20,000 (PEG; Sigma Chemical Co., St. Quentin, France) and bovine serum albumin (BSA; Sigma–Aldrich Inc., St. Louis, MO) were added to stabilize and block the conjugated particles, at the final concentrations of 0.05 and 1%, respectively, and then mixed gently. After the mixtures were centrifuged at 10,000 × *g* for 30 min, the precipitates were suspended with 0.05% PEG and 0.5% BSA in phosphate-buffered saline (PBS). After the subsequent centrifugation at 10,000 × *g* for 30 min, 520 nm absorbance of the antigen-conjugated colloid solutions was measured and adjusted at more than 5.0 with 0.05% PEG and 0.5% BSA in PBS (Huang et al., 2004a,b). The conjugates were diluted in 10 mM Tris–HCl (pH 8.2) with 5% sucrose, soaked into a borosilicate fiberglass paper (Schleicher and Schuell, Keene, NH), and then dried in a vacuum overnight.

2.5. Preparation of ICT strips

Non-conjugated and dialyzed *B. bovis* (75 μg/ml) and *B. bigemina* (100 μg/ml) rRAP-1/CTs were immobilized onto a nitrocellulose (NC) membrane (Schleicher and Schuell) using a BioDot's BioJet 3050 quanti-dispenser (BioDot Inc., Irvine, CA) to assess as a capture reagent for the test line detection. The purified rabbit anti-rRAP-1/CT IgG antibody (1.2 μg/ml) was sprayed onto the NC membrane at a 5-mm distance from the test line to serve as a procedural control. In the case of *B. bigemina*, the GST (100 μg/ml) line was newly inserted onto the NC membrane because only *B. bigemina* rRAP-1/CT remained fused with GST (Huang et al., 2004a). To assemble the strips, materials including the NC membrane, sample application, and absorbent pads were attached as slightly overlapping strips to a plastic card (Schleicher and Schuell) and then cut into 2 mm-wide strips using a BioDot's cutter (BioDot Inc.). The strips were stably stored with dehumidification in foil pouches at ambient temperature until use.

2.6. IFAT and ELISA

Reference standard IFAT and ELISA were performed as described previously (Boonchit et al., 2004, 2006). The results of the ICTs were compared with those of these tests and percentages of overall concordance were calculated (percent concordance (%) = (number of concordances between ICT and the standard reference × 100)/the number of tested sample).

2.7. Testing of ICT strips

Forty microliters of diluted serum with PBS (1:1) was applied on the sample pad. The result was judged within 10 min at RT. A sample was considered (i) positive if two colored lines (purple or pink) appeared on the NC membrane, one at the test line and one at the control line, (ii) negative if the colored line appeared only at the control line, and (iii) invalid if there was no colored line at the control line or the colored line appeared at the GST line (Fig. 1).

Sequential bovine sera from experimentally infected cattle with *B. bovis* ($n = 13$; 0–93 days post-infection) or *B. bigemina* ($n = 13$; 0–274 days post-infection) were kindly provided by National Institute of Animal Health (Tsukuba, Ibaraki, Japan). Non-infected control sera ($n = 10$) were obtained from healthy cattles bred at Obihiro University of Agriculture and Veterinary Medicine in Japan where *B. bovis* and *B. bigemina* infections have not been reported and used as negative control. Bovine sera used for specificity test were as follows: 5 *Neospora caninum*- and 5 *Cryptosporidium parvum*-positive bovine sera diagnosed by ELISA (Chahan et al., 2003; Takashima et al., 2003). Infected and non-infected sera were used for the evaluation of the two constructed ICTs, designated as BoICT and BiICT. Subsequently, 100 and 86 bovine sera, which had been collected from cattle living in Yanbian, China and Mato Grosso do Sul, Brazil (Chahan et al., 2003), were evaluated for field utilities of two ICTs.

3. Results

3.1. Productions of recombinant antigens

The recombinant proteins were successfully expressed in *E. coli* as recombinant fusion proteins with GST, and sufficient amounts of the soluble GST-fused (*B. bigemina* rRAP-1/CT-GST) and non-fused proteins (*B. bovis* rRAP-1/CT) were obtained as shown in Fig. 2. For the constructions of BoICT and BiICT, *B.*

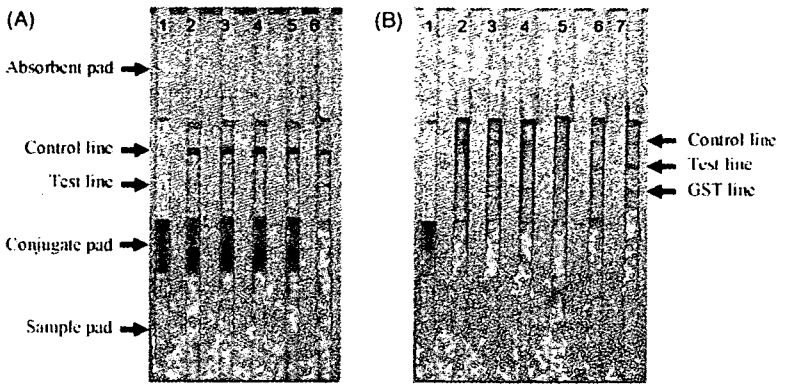

Fig. 1. Efficacies of BoICT (A) and BiICT (B) for the detections of the specific antibodies against *B. bovis* and *B. bigemina*. After the test serum was applied for the indicated ICT, clear development of color on the test line within 10 min was identified as positive, while no color change of the test line indicated a negative reaction. Lanes A1 and B1 show the strips before the application of serum. *Note:* No color development is observed on the control line. (A) BoICT for *B. bovis* infection. Negative reactions with non-infected cattle (lane 2), *N. caninum* (lane 3), *C. parvum* (lane 4), and *B. bigemina* (lane 5). Positive reactions with *B. bovis* (lane 6). *Note:* clear reactions are observed on the test lines in the positive reactions. (B) BiICT for *B. bigemina* infection. Negative reactions with non-infected cattle (lane 2), *N. caninum* (lane 3), *C. parvum* (lane 4), and *B. bovis* (lane 5). Positive reactions with *B. bigemina* (lane 6) and rabbit anti-GST serum (lane 7). *Note:* Clear reactions are observed on the test and GST lines in the GST-positive reaction (lane 7).

bovis rRAP-1/CT (lane 2) and *B. bigemina* rRAP-1/CT-GST (lane 1) were used.

3.2. Development of serodiagnostic ICTs for B. bovis and B. bigemina infections

In order to achieve a strong conjugation of a gold colloid with the target antigen, the minimum amounts of applied rRAP-1/CTs were first determined in preliminary titrations. The optical amounts and pH conditions were 100 μg/ml in pH 6.0 and 200 μg/ml in pH 5.0 for *B. bovis* and *B. bigemina* rRAP-1/CTs, respectively (data not shown). Next, the efficacy of two ICTs was evaluated using 13 positive sera from experimentally infected cattle with *B. bovis* or *B. bigemina* and 10 negative sera from non-infected cattle. The BoICT (Fig. 3A) and ELISA (Fig. 3B) detected antibodies to *B. bovis* in sera collected from 14 to 93 days post-infection, while BiICT (Fig. 3C) and ELISA (Fig. 3D) detected antibodies to *B. bigemina* from 13 to 274 days post-infection. No cross-reaction was observed between BoICT and BiICT. In addition, 10 sera from non-infected cattles, 5 *N. caninum*- and 5 *C. parvum*-positive sera were negative in two ICTs.

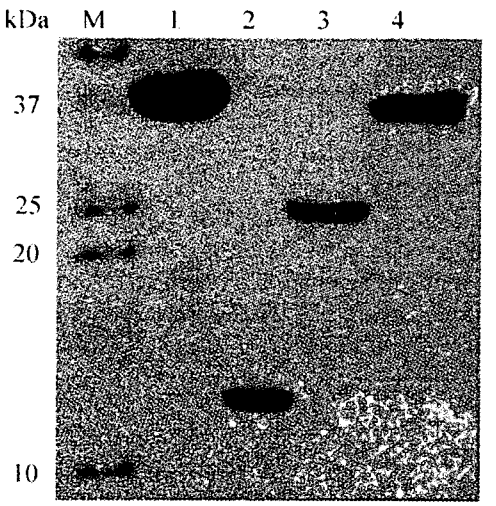

Fig. 2. SDS-PAGE analysis of recombinant *B. bigemina* and *B. bovis* rRAP-1/CTs. Purified recombinant products were subjected to SDS-PAGE with 18% polyacrylamide gel and then stained with Coomassie brilliant blue. (M) standard molecular masses; (lane 1) *B. bigemina* rRAP-1/CT-GST; (lane 2) *B. bovis* rRAP/CT after cleavage of GST; (lane 3) GST; (lane 4) *B. bovis* rRAP/CT-GST. The numbers to the left of the panel indicate the sizes of standard molecular masses.

3.3. Testing of field samples by diagnostic ICTs

To evaluate the field utilities of the two ICTs, we tested 186 field bovine sera collected from cattle living in Yanbian, China (100 samples) and in Mato Grosso do Sul, Brazil (86 samples) on the ICTs. The results of ICTs were compared to those of the diagnostic ELISA and IFAT that had been developed previously (Boonchit et al., 2004, 2006). Among 53 positive sera in BoICT, 41 and 39 sera were positive in ELISA and IFAT, respectively, while 131 sera in ELISA and 129 in IFAT of 133 negative sera in BoICT were negative. The overall concordances of BoICT were determined as 92.5% (172/186 sera) and 90.3% (168/186 sera) when the results of ELISA and IFAT were set

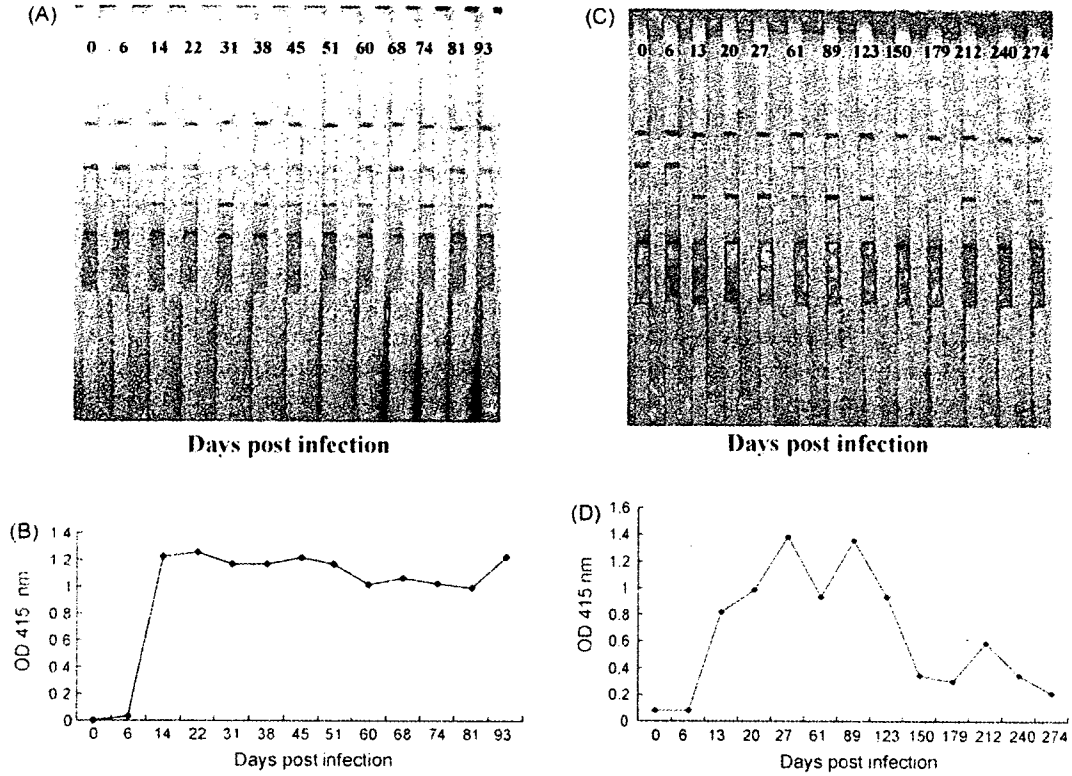

Fig. 3. Specific antibody responses in cattle experimentally infected with *B. bigemina*. (A) BoICT and (B) ELISA using *B. bovis*-rRAP-1/CT detected antibodies to *B. bovis* in sera collected from 14 to 93 days post-infection. (C) BiICT and (D) ELISA using *B. bigemina*-rRAP-1/CT detected antibodies to *B. bigemian* in sera collected from 13 to 274 days post-infection.

as the reference standards, respectively (Table 1). In contrast, 32 and 30 sera among 38 positive sera in BiICT were positive in ELISA and IFAT, respectively, while 148 sera in ELISA and 142 sera in IFAT of 148 negative sera in BiICT were negative. The overall concordances of BiICT showed 96.8% (180/186) and 92.5% (172/186) relative to the results of standard ELISA and IFAT, respectively (Table 2). None of the 186 field samples tested in this study showed a GST line on the NC membrane although there was a report of GST antibodies in field feline samples (Ferrer et al., 1998).

Table 1
Comparison among BoICT, ELISA, and IFAT for the detection of specific antibodies against *B. bovis* in field bovine sera collected from Yanbian, China and Mato Grosso do Sul, Brazil

BoICT		No. (%) with ELISA results		No. (%) with IFAT results	
Result	No. (%)	+	–	+	–
+					
China	8 (8)	2 (2)	6 (6)	0	8 (8)
Brazil	45 (52.3)	39 (45.3)	6 (7)	39 (45.3)	6 (7.0)
–					
China	92 (92)	2 (2)	90 (90)	0	92 (92)
Brazil	41 (47.7)	0	41 (47.7)	4 (4.7)	37 (43)
Total					
China	100 (100)	4 (4)	96 (96)	0	100 (100)
Brazil	86 (100)	39 (45.3)	47 (54.7)	43 (50)	43 (50)

Table 2
Comparison among BiICT, ELISA, and IFAT for the detection of specific antibodies against *B. bigemina* in field bovine sera collected from Yanbian, China and Mato Grosso do Sul, Brazil

BiICT		No. (%) with ELISA results		No. (%) with IFAT results	
Result	No. (%)	+	–	+	–
+					
China	4 (4)	0	4 (4)	0	4 (4)
Brazil	34 (39.5)	32 (37.2)	2 (2)	30 (34.9)	4 (4.7)
–					
China	96 (96)	0	96 (96)	1 (1)	95 (95)
Brazil	52 (60.5)	0	52 (60.5)	5 (5.8)	47 (54.7)
Total					
China	100 (100)	0 (0)	100 (100)	1 (1)	99 (99)
Brazil	86 (100)	32 (37.2)	54 (62.8)	35 (40.7)	51 (59.3)

4. Discussion

The ICTs were first described in the late 1960s, at when they were first constructed to assess the presence of serum proteins (Kohn, 1968). Over the past decade, many ICTs have been developed and at present, several ICTs have become commercially available for the diagnosis of many pathogens (Mills et al., 1999; Chandler et al., 2000; Richardson et al., 2002; Peruski and Peruski, 2003; Mohebali et al., 2004). There are a number of advantages of ICTs over conventional microbiological techniques, including ease of performance, stability at various temperatures, and comparatively low cost (Kohn, 1968; Mills et al., 1999; Chandler et al., 2000; Wongsrichanalai, 2001). Such tests could provide a useful diagnostic tool in a clinical setting, particularly, in resource-poor countries in which bovine babesiosis is seriously endemic.

The rhoptry-associated protein 1 (RAP-1) of *B. bovis* is a well-characterized protein (p60) (Suarez et al., 1991), and *B. bigemina* also has the RAP-1 homologous protein (p58) (McElwain et al., 1987; Molloy et al., 1998). Because the proteins show high identity in 300 amino acids located at the N-terminal regions in the *B. bovis* and *B. bigemina* (Boonchit et al., 2002), the C-terminal truncated recombinant antigens of *B. bovis* and *B. bigemina* RAP-1 were applied for the successful development of serologically diagnostic ELISAs for bovine babesiosis (Boonchit et al., 2004, 2006). Since highly specific recombinant protein in ELISA showed promise for its utilization in the diagnostic ICT (Huang et al., 2004a,b), we decided to use the recombinant *B. bovis* and *B. bigemina* RAP-1/CTs and evaluated their diagnostic potential in ICTs.

To evaluate the clinical and field utilities of ICTs (BoICT and BiICT), the results of ICTs were compared with those of standard ELISA and IFAT. The concordances were 92.3 and 90.3% between BoICT and the reference standards and 96.8 and 92.5% between BiICT and the reference standards, although the two ICTs accurately detected each of the experimentally positive sera without cross-reactivity. The reason for the slight discordance in the field sera is unknown, since we could not point out any clinical or technical factor. The discordance might be due to non-specific binding of unknown serum proteins to the antigens or accidental influence of serum storage, such as repeated freeze–thawing, or the storage length, problems which have been often reported in immunological methods (Faigel et al., 2000; Hujakka et al., 2001; Huang et al., 2004a,b). Because it is not known whether the discordant sera are really positive or negative for the babesiosis, verification on a blood smear or other molecular tools, such as a PCR, would be required to confirm the discordance.

Recent studies have evaluated the diagnostic potential of ICTs for other protozoan diseases, such as other babesiosis, malaria, cryptosporidiosis, and leishmaniasis, with high sensitivities ranging from 72 to 100% and high specificities from 61 to 100% (Mills et al., 1999; Chan et al., 2000; Reithinger et al., 2002). Taken together with previous results, our results may indicate that the BoICT and BiICT could also provide useful diagnostic tools in a clinical setting. Having commercially available ICTs, which are usually inexpensive, is certainly a valuable asset because of the possibility of quickly analyzing large numbers of sera in conventional laboratories and achieving an accurate diagnosis of infectious disease in remote areas in which laboratories are not readily accessible for a conventional assay. Its simplicity and accuracy may make this assay a useful diagnostic tool. The sensitivity and specific ICTs could provide very effective alternatives to currently available diagnostic tests and lead to a better knowledge of the prevalence and epidemiology of the disease.

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Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites

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Abstract

A loop-mediated isothermal amplification (LAMP) technique has been used as a novel nucleic acid detection method, whereby the target DNA can be amplified with high specificity and sensitivity under an isothermal condition using a set of four specific primers. In this study, we designed two sets of the LAMP primers for rhoptry-associated protein-1 genes of *Babesia bovis* and *B. bigemina*, in which a restriction enzyme cleavage site was inserted into two pairs of species-specific primers to construct a multiplex LAMP (mLAMP) method by combining these two sets totaling eight primers. The mLAMP method was distinguishable between *B. bovis* and *B. bigemina*, simultaneously, due to the subsequent restriction enzyme analysis. The sensitivities of the mLAMP method were 10^3 and 10^5 times higher on the detection limits for *B. bovis* and *B. bigemina*, respectively, than those of the classical PCR methods. Of 40 blood samples collected from cattle living in Ghana, 12 and 27% were positively detected by the mLAMP for *B. bovis* and *B. bigemina*, respectively. Furthermore, 14 and 23% of 90 blood samples from cattle in Zambia showed mLAMP-positive reactions to *B. bovis* and *B. bigemina*, respectively. These findings indicate that this mLAMP method is a new convenient tool for simultaneous detection of the bovine *Babesia* parasites.

Keywords: *Babesia bovis*; *Babesia bigemina*; LAMP; Multiplex; Restriction enzyme

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, 1994), generally, the disease caused by *B. bovis* is more severe than that by *B. bigemina* (Ristic, 1981.). Acute infections are usually diagnosed by a microscopic examination of blood smears, whereas subclinical infections should be identified serologically (Weiland and Reiter, 1988.). Differential diagnosis between the *B. bovis* and *B. bigemina* infections leads to a better understanding of their epidemiology, and the species-specific distribution in the field would provide useful information to control the diseases (de Vos and Potgieter, 1994).

The efficiency of the polymerase chain reaction (PCR) method has been verified on the epidemiological study of babesiosis by several investigators because of its high sensitivity and specificity (Almeria et al., 2001; Fahrimal et al., 1992; Figueroa et al., 1993; Oliveira-Sequeira et al., 2005; Smeenk et al., 2000). However, this method has not always been adapted for laboratory diagnosis for economic and practical reasons. Especially, automated and/or real-time quantitative thermal

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cyclers are required for the PCR methods, but they are not always affordable. Furthermore, the cyclers work erratically due to high ambient temperatures and humidity and/or dusty environments. Therefore, the development of cost-effective, simple, and rapid DNA amplification methods is needed for the diagnosis of early and advanced bovine babesiosis.

A loop-mediated isothermal amplification (LAMP) method allows a whole reaction process at an isothermal condition and finally makes it possible to easily detect the LAMP-amplified product due to a visual confirmation of the reacted tube (Notomi et al., 2000). Furthermore, since four LAMP-specific primers are designed to recognize six distinct regions on the target gene, the reacted DNA can be amplified with high specificity (Notomi et al., 2000). Since the LAMP method does not require any complicated equipment, it may provide a cost-effective, simple, and rapid DNA amplification method.

The aim of this study was to develop a new diagnostic method based on the LAMP technique for the simultaneous detection of *B. bovis* and *B. bigemina*. In this study, we designed two sets of each of the four specific LAMP primers for *B. bovis* and *B. bigemina*, in which a restriction enzyme cleavage site was inserted into two pairs of the species-specific primers to construct a multiplex LAMP (mLAMP) method by combining these two sets of total eight primers. The mLAMP method was constructed to distinguish between *B. bovis* and *B. bigemina* after the subsequent restriction enzyme analysis. In addition, the sensitivity of the mLAMP method was compared to those of conventional PCR and nested PCR methods. Finally, we investigated the species-specific distribution in cattle and/or wild animals living in Ghana and Zambia by using the mLAMP method and discussed the utility of the mLAMP method for a large-scale epidemiological study of bovine babesiosis.

2. Materials and methods

2.1. Parasites

The Texas strain of *B. bovis* (Hines et al., 1992) and the Argentine strain of *B. bigemina* (Hotzel et al., 1997) were maintained in purified bovine red blood cells (RBC) with a microaerophilic stationary-phase culture system (Avarzed et al., 1997). 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

B. bovis- and *B. bigemina*-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 5×10^{0} (parasitemia: 0.0000001%) to 5×10^{7} (1%) infected RBC/200 μl of the total RBC, separately, and then subjected to a DNA extraction with a QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan). The purified DNA samples were used as templates for the subsequent LAMP and PCR methods. DNA samples were 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 by using a QIAamp DNA Mini Kit (QIAGEN). As negative controls, extracted DNA samples of normal bovine blood and other cultured parasites (*Theileria parva*, *Trypanosoma evansi*, and *Neospola caninum*) were prepared as described above.

2.3. LAMP reaction and restriction enzyme-digestion of the amplified DNA products

The non-multiplex LAMP method requires a set of four specific primers: a forward inner primer (FIP), a backward inner primer (BIP), and two outer primers (F3 and B3), which recognize a total of six distinct nucleotide sequences (B1, B2, B3, F1, F2, and F3) on the target gene (Notomi et al., 2000). The specific primers for *B. bovis* and *B. bigemina* were designed against the species-specific rhoptry-associated protein-1 (RAP-1) gene sequences (Gene Bank accession numbers: *B. bovis*, genbank: AF027149; *B. bigemina*, M60878). For the construction of a multiplex LAMP (mLAMP), we modified the FIPs and BIPs by inserting a restriction enzyme (*Eco*RI) cleavage site between the F1 complementary and F2 and between the B1 complementary and B2, respectively, as shown in Table 1. The LAMP reaction was conducted as described previously (Notomi et al., 2000). Briefly, the reaction was performed in 25 μl of a mixture containing 0.5 μl with different DNA weights (5×10^{0} ng to 5×10^{-7} ng/test) of the extracted DNA template, 40 pmol each of the FIP and BIP primers, 5 pmol each of the F3 and B3 primers, and 1 μl of *Bst* DNA polymerase (Eiken Chemical Co., Ltd.,

Table 1
LAMP primers for *Babesia bovis* and *Babesia bigemina* detection

Primer	Type	Sequence (5′–3′)[a]
Bovis-F3	F3	ACCAAAAACTATCTGAAAGCCAATG
Bovis-B3	B3c	GGAGCCTCCCTGAAGAACT
Bovis-FIP	F2-F1c	AGGTTCGGCTACATTCTCTTTCA*GAATTC*TGAGCCCACTAAAAAGTTTATGC
Bovis-BIP	B1-B2c	GCCAACCCACCAAGGAGTTTTTCA*GAATTC*TTGGTTGGTTGACCGATGTT
Bigemina-F3	F3	CGGCGGCTAAGTTCTTCAA
Bigemina-B3	B3c	GAACGAGGTCATCGCAGG
Bigemina-FIP	F2-F1c	CCTAACCAAACGCTTCAACGCC*GAATTC*TTGCTTTCACAACTCGCCTG
Bigemina-BIP	B1-B2c	AGCAACCTTCCCGTTGACCTT*GAATTC*CATCATGTACTCGCCGTAGC

[a] Underlining indicates a restriction enzyme site of *Eco*RI.

Tokyo, Japan) in a LAMP buffer [20 mM Tris–HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, and 1.4 mM each of deoxynucleoside triphosphates (dNTPs); SIGMA, Tokyo, Japan]. The mLAMP reaction was performed as described above with each of the specific primers (a total of eight primers; FIPs, BIPs, F3s, and B3s for each of the parasites) included in the same reaction tube. In a conventional heat block, the mixture was incubated at 64 °C (57–70 °C was also demonstrated) for 90 min for the LAMP reaction and subsequently at 80 °C for 5 min for the termination. To distinguish the amplified DNA products between *B. bovis* and *B. bigemina*, 1 μg/μl of the products was digested with the *Eco*RI at 37 °C for 1 h. The LAMP products and digested DNA products were subjected to electrophoresis on a 2% agarose gel and then visualized under an ultraviolet (UV) light after staining with ethidium bromide (SIGMA).

2.4. PCR amplification

PCR and nested PCR (nPCR) amplifications were carried out with the published pairs of species-specific primers for the detections of *B. bovis* and *B. bigemina* (Figueroa et al., 1993). PCR was performed in 25 μl of a mixture containing 0.5 μl with different DNA weights (5×10^{0} to 5×10^{-7} ng/test) 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 reaction was performed at 35 cycles under the following conditions: 5 min at 95 °C to activate the Taq Gold DNA polymerase, 1 min of denaturation at 94 °C, 1 min of annealing at 70 °C (*B. bovis*) or 58 °C (*B. bigemina*), 1 min 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 61 °C (*B. bovis*) or 65 °C (*B. bigemina*). The PCR and nPCR products were subjected to electrophoresis and then visualized as described above.

2.5. DNA sequencing

Digested LAMP DNA products were purified after 2% agarose gel electrophoresis and then cloned into a pCRII cloning vector using a 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 Development Co., Ltd., Tokyo, Japan) was used to align the determined sequences.

2.6. Field samples

Forty and 30 field blood samples were collected on FTA cards from cattle living in Ghana and Zambia, respectively. Furthermore, additional 60 blood samples were collected from other cattle in Zambia, and the DNA samples were extracted with the SepaGene (Sanko Junyaku Co., Ltd., Tokyo, Japan). Additionally, a total of 36 field blood samples of wild animals were also collected on FTA cards from 20 wildebeests, 8 impalas, and 8 African buffaloes living in Zambia.

3. Results

3.1. Specificities of non-multiplex and multiplex LAMP methods

Four LAMP species-specific primers either for *B. bovis* and *B. bigemina* specifically produced the positive LAMP amplicons of typical ladder patterns from the target genes of the respective parasites in the non-multiplex species-specific LAMP methods (Fig. 1, Panel A: Lane 1, *B. bovis*; Lane 2, *B. bigemina*, Panel B: Lane 1, *B. bigemina*; Lane 2, *B. bovis*) but not from the DNAs of normal bovine blood and other protozoan species (*T. parva*, *T. evansi*, and *N. caninum*), as shown in Fig. 1 (Panels A and B, lanes 2–6). The sizes of non-multiplex LAMP fragments digested by *Eco*RI were consistent with the predicted sizes as two major bands for each parasite, as shown in Fig. 2 (lanes 2 and 4), as well as the results of the multiplex LAMP (mLAMP) method for each parasite (data not shown); 163, 169, and 221 bp for *B. bovis* and 83, 103, and 143 bp for *B. bigemina*. Additionally, the mLAMP method with all of the eight primers simultaneously produced the positive LAMP products from a mixture of *B. bovis* and *B. bigemina* DNAs (Fig. 2, lane 5) and showed 4 major bands (103–83, 143, 169–163, and 221 bp) after subsequent digestion with *Eco*RI (lane 6). To evaluate the accuracy and robustness of our mLAMP method, the LAMP reactions were carried out on the

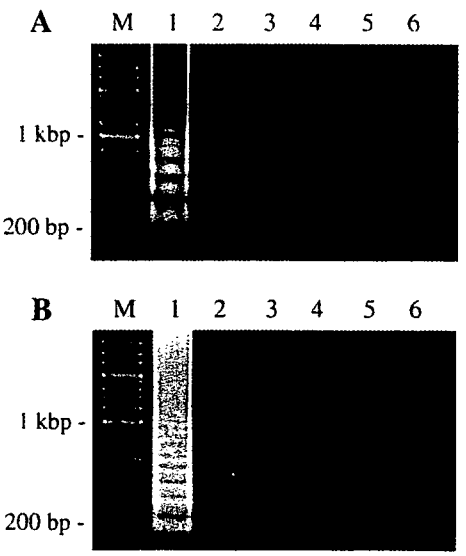

Fig. 1. Specificities of non-multiplex LAMP methods. Each LAMP reaction was carried out using four primers (FIP, BIP, F3, and B3) for the detection of *B. bovis* (Panel A) or *B. bigemina* (Panel B) with each extracted DNA. In Panel A, Lane 1, *B. bovis*; lane 2, *B. bigemina*; lane 3, *T. parva*; lane 4, *T. evansi*; lane 5, *N. caninum*, and lane 6, normal cattle DNA. In Panel B, Lane 1, *B. bigemina*; lane 2, *B. bovis*; lane 3, *T. parva*; lane 4, *T. evansi*; lane 5, *N. caninum*; and lane 6, normal cattle DNA. Lane M shows 200-bp ladder size markers.

Fig. 2. Comparison of species-specific ladder pattern of each LAMP product and its restriction enzyme-digestion. LAMP-amplified products using each combination of the multiplex LAMP mixture with *B. bovis* DNA (Lane 1), *B. bigemina* DNA (Lane 3), or both the DNAs (Lane 5) and the *Eco*RI-digested DNA fragments of each LAMP-amplified product obtained from *B. bovis* (Lane 2), *B. bigemina* (Lane 4), or both (Lane 6) were subjected to electrophoresis on a 2% agarose gel. Lane M shows 200-bp ladder size markers.

Table 2
Comparison of the positive numbers among conventional PCR, nested PCR, and multiplex LAMP methods for *B. bovis* and *B. bigemina* detections from field bovine blood samples collected from Ghana and Zambia

	Number	Parasites	Positive numbers(%)		
			PCR	Nested PCR	mLAMP
Ghana cattle	40	*B. bovis*	0 (0)	1 (2.5)	12 (30)
		B. bigemina	0 (0)	10 (25)	27 (67.5)
Zambia cattle[a]	30	*B. bovis*	0 (0)	1 (3.3)	6 (20)
		B. bigemina	0 (0)	0 (0)	5 (16.7)
Zambia cattle[b]	60	*B. bovis*	0 (0)	1 (1.7)	7 (11.7)
		B. bigemina	5 (8.3)	19 (31.7)	16 (26.7)
<Total of Zambia cattle>	90	*B. bovis*	0 (0)	2 (2.2)	13 (14.4)
		B. bigemina	5 (5.6)	19 (21.1)	21 (23.3)
<Total>	130	*B. bovis*	0 (0)	3 (2.3)	25 (19.2)
		B. bigemina	5 (3.8)	29 (22.3)	48 (36.9)

[a] DNA samples were extracted from FTA cards with a DNA blood mini kit.
[b] DNA samples were extracted from the blood with a SepaGene.

water bath at 57–70 °C separately. As a result, the positive ladder patterns were observed at 58–68 °C, and were observed strongly at 63–65 °C. The specificity of the mLAMP method was also established through control studies with DNAs of several control samples, as described above (data not shown). These findings demonstrated that each set of four species-specific primers was highly specific for the detection of the corresponded *Babesia* parasites in the non-multiplex LAMP methods and that the mLAMP method was also distinguishable between the *B. bovis* and *B. bigemina* LAMP amplicons due to the subsequent restriction enzyme analysis.

In order to confirm the nucleotide sequences of LAMP products, the amplified and digested DNA products were purified from the positive controls and cloned into a vector. The determined sequences of all DNA fragments were consistent with the reported ones of *B. bovis* and *B. bigemina* RAP-1 genes (Gene Bank accession numbers: *B. bovis*, AF027149; *B. bigemina*, M60878) (data not shown).

3.2. Sensitivities of the multiplex LAMP method

To evaluate the sensitivities of the mLAMP method, *B. bovis* or *B. bigemina* DNA samples were extracted from a 10-fold serial dilution of the culture and then subjected to the mLAMP method. In addition, the detection limits of the mLAMP method were compared to those of conventional PCR and nPCR methods. In PCR methods, positive bands of 356 bp (Fig. 3, Panel A) and 278 bp (Panel B) were detected from the diluted samples of 0.001% parasitemia (5×10^4 infected RBC) of *B. bovis* (lane 4) and 0.1% parasitemia (5×10^6 infected RBC) of *B. bigemina* (lane 2), respectively. Subsequently, in the nPCR methods, specific bands of 291 and 170 bp could be

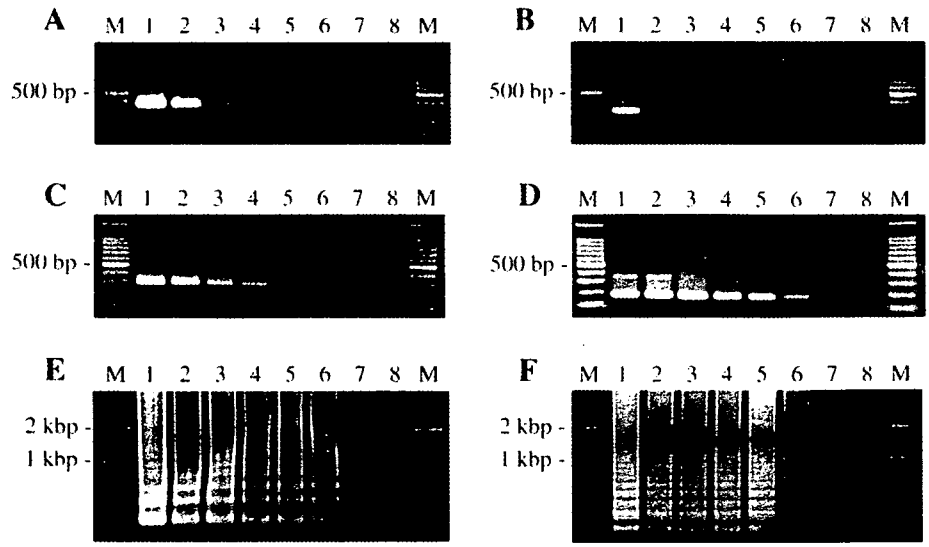

Fig. 3. Sensitivities of the mLAMP method and conventional PCR and nested PCR methods. LAMP, PCR, and nPCR methods were carried out using the extracted DNAs from dilutions of infected RBC in the *in vitro* culture. Lanes 1 to 8, 5×10^7, 5×10^6, 5×10^5, 5×10^4, 5×10^3, 5×10^2, 5×10^1, and 5×10^0 infected RBC; and lane M, 100-bp ladder size markers (PCR and nPCR) and 200-bp ladder size markers (mLAMP). Panel A, PCR reaction with *B. bovis* DNA; Panel B, PCR reaction with *B. bigemina* DNA; Panel C, nested PCR reaction with *B. bovis* DNA; Panel D, nested PCR reaction with *B. bigemina* DNA; Panel E, LAMP reaction with *B. bovis* DNA; Panel F, LAMP reaction with *B. bigemina* DNA.

observed from the diluted samples of 0.0001% parasitemia (5×10^3 infected RBC) of *B. bovis* (Panel C, lane 5) and 0.00001% parasitemia (5×10^2 infected RBC) of *B. bigemina* (Panel D, lane 6), respectively. In contrast, the ladder LAMP amplicons were detectable in the mLAMP method from the diluted samples of 0.000001% parasitemia (5×10^1 infected RBC) of *B. bovis* (Panel E, lane 7) and *B. bigemina* (Panel F, lane 7). The non-multiplex LAMP methods also showed the same sensitivities to those obtained in the mLAMP method (data not shown). The sensitivities of the mLAMP method were 10^3 and 10^5 times higher than those of the classical PCR methods and 10^2 and 10^1 times higher than those of the nPCR methods on the detection limits for *B. bovis* and *B. bigemina*, respectively.

3.3. Detection of B. bovis and B. bigemina from field samples by the multiplex LAMP method

Field blood samples collected from cattle living in Ghana and Zambia were surveyed by using the mLAMP method in order to demonstrate the field utility of mLAMP as a diagnostic tool for epidemiological studies. Forty and 30 field blood samples were collected on FTA cards from Ghana and Zambia, respectively, while 60 other DNA samples were extracted with a SepaGene from cattle in Zambia. As shown in Table 2, no PCR detection for *B. bovis* was observed in either Ghana or Zambia, while the detection rates for *B. bigemina* were 0 and 5.6%, respectively. Subsequently, the nPCR detection rates for *B. bovis* were 2.5 and 2.2% in Ghana and Zambia, while those for *B. bigemina* were 25 and 21.1%, respectively. Mixed detection rates of *B. bovis* and *B. bigemina* were determined as 1.1 and 0% in Ghana and Zambia, respectively (data not shown). In contrast, the mLAMP detection rates for *B. bovis* were 30 and 14.4% in Ghana and Zambia, respectively, while those for *B. bigemina* were 67.5 and 23.3%, respectively. In addition, the mixed detection rates of *B. bovis* and *B. bigemina* were 22.5 and 4.4% in Ghana and Zambia, respectively (data not shown).

In addition, a total of 36 field blood samples of wild animals were also collected on FTA cards from 20 wildebeests, 8 impalas, and 8 African buffaloes living in Zambia, as shown in Table 3. The positive reaction of *B. bovis* was obtained from 4 wildebeests (20%) and 1 African buffalo (12.5%), while no reaction was detected from impalas (0%) in the mLAMP method. On the other hand, 7 wildebeests (35%), 2 impalas (25%), and 3 African buffaloes (37.5%) showed a positive reaction to *B. bigemina* using the method.

Table 3
Comparison of the positive numbers among conventional PCR, nested PCR, and multiplex LAMP methods for *B. bovis* and *B. bigemina* detection from field blood samples collected from wild animals in Zambia

	Number[a]	Parasites	Positive numbers (%)		
			PCR	Nested PCR	mLAMP
Wildebeest	20	*B. bovis*	0 (0)	0 (0)	4 (20)
		B. bigemina	0 (0)	6 (30)	7 (35)
Impala	8	*B. bovis*	0 (0)	0 (0)	0 (0)
		B. bigemina	0 (0)	2 (25)	2 (25)
African buffalo	8	*B. bovis*	0 (0)	0 (0)	1 (12.5)
		B. bigemina	0 (0)	0 (0)	3 (37.5)
<Total>	36	*B. bovis*	0 (0)	0 (0)	5 (13.9)
		B. bigemina	0 (0)	8 (22.2)	12 (33.3)

[a] DNA samples were extracted from FTA cards with a DNA blood mini kit.

In the field-applied experiments against cattle and wild animals, the mLAMP method exhibited higher detection abilities for the target *B. bovis* and *B. bigemina* infections than those of conventional PCR and nPCR methods.

4. Discussion

In this report, we described the successful development of an mLAMP method for the simultaneous detection of *B. bovis* and *B. bigemina*. In the method, sets of a designed primer specifically amplified the target DNAs derived from the respective RAP-1 genes. Our findings regarding high specificity are in agreement with previous reports of other LAMP methods (Ihira et al., 2004; Ikadai et al., 2004; Kuboki et al., 2003). Furthermore, the mLAMP method was highly sensitive, as DNA with as few as six target gene copies was detectable in the LAMP reaction. Moreover, the cycling reaction of LAMP was reported to continue for 1 h with an accumulation of 10^9 copies of the target DNA (Notomi et al., 2000). Besides, the high amplification efficiency yields a white precipitate in the LAMP reaction tube (Mori et al., 2001), which is a potent advantage to develop a simple and rapid diagnostic tool.

Successful amplification of the LAMP method relies on the specificities of designed primers. In the present study, the LAMP primers were designed on the basis of species-specific sequence of each *Babesia* parasite (Hotzel et al., 1997; Mishra et al., 1992). The difference of *B. bigemina* and *B. bovis* RAP-1 gene sequences was described in the 3′-terminal regions (Boonchit et al., 2002; Suarez et al., 1991). In an earlier report, no cross-reactivity of the C-terminal regions of RAP-1 was shown in the serodiagnostic ELISA between *B. bovis* and *B. bigemina* infections (Boonchit et al., 2006). In order to develop species-specific LAMP methods for the specific detection of *B. bovis* and *B. bigemina*, we successfully designed specific primer sets by targeting the 3′-terminal regions of RAP-1 genes.

The LAMP reaction has been carried out as the standard by using two inner primers containing the TTTT linker in the middle sequence (Notomi et al., 2000). Recently, several LAMP methods combining the technique of restriction enzyme cleavage have been constructed using an original cleavage site within the amplified DNA products rather than within the designed primers (Dukes et al., 2006; Pham et al., 2005). The strategy has been used to confirm whether or not the amplified DNAs are derived from the target genes. In contrast to these approaches, we artificially inserted a restriction enzyme cleavage site (*Eco*RI site: GAATTC) into two inner primer sequences instead of the TTTT sequence. As a result, the mLAMP method produced different sizes of species-specific bands for *B. bovis* and *B. bigemina* due to the subsequent digestion of *Eco*RI after the LAMP reaction. Our findings demonstrated that the mLAMP method could amplify the target DNAs effectively and simply discriminate among the *B. bovis*, *B. bigemina*, or a mixed infection.

The mLAMP method positively detected both *B. bovis* and *B. bigemina*, including the mixed infections, from some field

blood samples collected from Ghana and Zambia, with higher sensitivity than those of conventional PCR and nPCR methods. Accordingly, this is the first report in which the bovine *Babesia* parasites were investigated in these countries using molecular techniques, although the identification of these species had been attempted on a morphological examination with microscopy in Zambia in previous reports (Jongejan et al., 1986, 1988). According to our findings, the rate of *B. bigemina* infection was substantially higher than that of *B. bovis* infection in both countries. Interestingly, the mixed infection rate in Ghana (22.5%) was significantly higher in cattle than that in Zambia (4.4%). In addition, the positive rates of *B. bovis* and *B. bigemina* in Ghana were 2 to 3 times higher than those in Zambia in the mLAMP survey. A further epidemiological study with a much larger number of field bovine blood samples will be interesting to identify the correct species-distribution in Zambia and Ghana using the mLAMP method.

Carmichael and Hobday (Carmichael and Hobday, 1975) reported that they found no *Babesia* spp. from the blood smears of 190 African buffaloes in Northern Botswana. However, Karbe et al. speculated that the African buffalo might play an important role in the infectious carrier of *Babesia* parasites (Karbe et al., 1979). In this study, we first conducted a molecular epidemiological survey for wild animals (wildebeests, impalas, and African buffaloes) in two African countries. Our results suggested that *B. bigemina* could infect these three types of animals, while *B. bovis* could infect only impalas. Although the mLAMP method can be used for epidemiological studies in endemic countries, current knowledge of the distribution of bovine *Babesia* parasites is still incomplete because of the low sample number from small regions of each country.

In conclusion, our mLAMP method is a new, convenient tool for simultaneously identifying the bovine *Babesia* parasites. This powerful tool should be examined to perform the amplification and detection analysis in developing countries. In the present study, positive reactions could be achieved with our LAMP method at 58–68 °C, therefore, these results strongly suggest that our LAMP method could be applicable in poorly equipped facilities. Additionally, further epidemiological studies using the mLAMP method in a larger scale are highly required for the identification of the correct species-distribution in the world.

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ANTIBODY ISOTYPE RESPONSES TO PARAMYOSIN, A VACCINE CANDIDATE FOR SCHISTOSOMIASIS, AND THEIR CORRELATIONS WITH RESISTANCE AND FIBROSIS IN PATIENTS INFECTED WITH *SCHISTOSOMA JAPONICUM* IN LEYTE, THE PHILIPPINES

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Abstract. We examined whether antibody isotype responses to paramyosin (PM), a vaccine candidate for schistosomiasis, are associated with age-dependent resistance and pathology in liver fibrosis using human sera collected from 139 individuals infected with *Schistosoma japonicum* in Leyte, The Philippines. We report that IgA and IgG3 responses to PM showed a positive correlation with age and that the epitopes responsible were localized predominantly within the N-terminal half of PM. In addition, the IgG3 response to PM was associated with serum level of procollagen-III-peptide (P-III-P), an indicator of progression of liver fibrosis. These results imply that IgG3 against PM may not only provoke age-dependent resistance to *S. japonicum* infection but also enhance liver fibrosis. In contrast, levels of IgE to PM and to multiple PM fragments showed a negative correlation with P-III-P level. Thus, in contrast to IgG3, increases in PM-specific IgE may contribute to suppression of liver pathogenesis in schistosomiasis.

INTRODUCTION

A number of epidemiologic studies have suggested the occurrence of age-dependent, acquired resistance to reinfection with *Schistosoma mansoni*,[1] *S. haematobium*,[2] and *S. japonicum*.[3,4] Age-dependent resistance is correlated with specific antibody isotype responses to the schistosome antigens, especially IgE responses to adult worm antigen (AWA).[5–8] In addition, IgA specific to parasite antigens was shown to be associated with resistance.[9,10] Thus, IgE and IgA may play a role in mediating protective immunity. Conversely, IgM, IgG2, and IgG4 have been suggested to block killing by antibody-dependent cellular cytotoxicity (ADCC) of the parasites, acting as a blocking antibody.[6,11] Nevertheless, the responses of various isotypes are controversial in their ability to provoke an immune effector mechanism.

Paramyosin (PM) is an invertebrate myofibrillar protein and is one of six candidate vaccines against schistosomiasis.[12] Vaccination with recombinant PM induced a significant reduction in worm recovery after challenge infection with *S. japonicum* in mice, pigs, and water buffaloes as experimental animal models.[13,14] Immunohistochemical and immunoelectron microscopic analyses indicated that PM is localized on the surface of cercaria, schistosomula, and adult *S. japonicum*, as well as in the muscle layers, suggesting that the surface PM could evoke ADCC.[15,16] Passive transfer of PM-specific monoclonal IgE in mice at an early stage of challenge infection resulted in reduction of worm burden.[17]

In humans, antibody isotype responses against *S. japonicum* PM have been reported. A study in The Philippines showed that IgA titers to AWA are correlated with age and the major target of IgA was PM, suggesting a role of anti-PM IgA in acquired immunity.[9] In contrast, antibody responses to PM were not correlated with susceptibility in another study in China.[18] These discrepancies may have been due to geographic differences of both human and parasite populations and differences in the PM epitopes recognized by the specific antibody isotypes, some of which would be protective with others acting as blocking antibodies.

The major pathologic lesion of *S. japonicum* infection is periportal fibrosis, which is a consequence of prolonged granuloma formation surrounding the deposited parasite eggs in the liver. From the practical view of vaccine development, schistosome vaccines are required not only to reduce worm burden but also to ameliorate liver fibrosis. With regard to the roles of isotype responses to parasite antigens in fibrosis, analyses of IgE-deficient mice infected with either *S. japonicum* or *S. mansoni* indicated that IgE modifies granuloma formation.[19,20] In addition, increased levels of IgG4 to parasite egg antigens in schistosomiasis mansoni patients with and liver fibrosis have been demonstrated.[21] Interestingly, PM has been suggested to be involved in granuloma formation in mice infected with *S. mansoni*.[22,23] Thus, it is important to examine the role of isotype responses to PM in liver fibrosis for schistosome vaccine development.

The present study was performed to determine whether isotype responses against PM are involved in age-dependent resistance and liver fibrosis in human *S. japonicum* infection. We demonstrate that IgG3 and IgA against PM were correlated positively with age, and the epitopes recognized varied among isotypes. In addition, we observed a positive correlation between IgG3 responses to PM and serum level of procollagen-III-peptide (P-III-P), an indicator of progression of liver fibrosis. Surprisingly, IgE specific to PM showed a negative correlation with P-III-P level, suggesting the involvement of IgE-PM interactions in liver fibrosis. The possibility of using PM as a schistosome vaccine is also discussed.

MATERIALS AND METHODS

Study design and evaluation of liver fibrosis. The study was carried out in villages in Leyte, The Philippines, where schis-

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tosomiasis japonica is endemic. In this area, mass screening by semi-quantitative stool examination using Kato-Katz method, followed by treatment with praziquantel against *S. japonicum* infection, was conducted from 1981 to 1999, as part of the National Schistosomiasis Control Program of the Philippines. In July and August 1999, outpatients from Schistosomiasis Research Hospital, who were diagnosed as having *S. japonicum* infection by detection of the parasite eggs in their feces, were enrolled in the present study. The purpose and protocols of the study were explained and written consent obtained from all the patients. All enrolled patients underwent serologic and ultrasonographic examinations. Patients positive for hepatitis B surface antigen by radioimmunoassay (cut-off index > 2.0) and/or antibody to hepatitis C virus (second generation) and persons with alcoholism with bright liver by ultrasonography (alcohol consumption > 80 mL/day for ≥ 5 years) were excluded from the study.

A total of 139 patients were selected for further analyses. The degree of liver fibrosis was estimated by ultrasonography and classified into four stages (type 0: normal pattern; type 1: linear pattern; type 2: tubular pattern; type 3: Network pattern) as described.[24,25] Serum levels of P-III-P, type IV collagen, and total bile acids (TBAs) were measured in only 133 of the 139 blood specimens because the other six specimens were lost during analyses. Eight control sera were collected from healthy adult volunteers who lived in Japan and were free from *S. japonicum* infection.

Schistosome antigens and recombinant paramyosins. Soluble AWA was extracted from adult worms of the Yamanashi strain of *S. japonicum* by repeated freezing and thawing.[17] After centrifugation at 10,000 × *g* for 30 minutes at 4°C, the supernatant was recovered and cryopreserved at −80°C until use. Full-length *S. japonicum* PM and six truncated forms were designated as PM (1–866 amino acids), PM1 (1–164 amino acids), PM2 (157–302 amino acids), PM3 (297–451 amino acids), PM4 (447–602 amino acids), PM5 (597–742 amino acids), and PM6 (734–866 amino acids). The PM cDNAs were amplified by a polymerase chain reaction using the *S. japonicum* PM cDNA[16] as a template and the following primers: PM, 5′-CGGGATCCCATATGATGAATCACGATACAG-3′ and 5′-GCGGATCCTACATCATACTTGTTGC-3′; PM1, 5′-CGGGATCCCATATGATGAATCACGATACAG-3′ and 5′-CGGGATCCCCGGGTACCGAGCTCGACTTTTGATTCAGCTGATTG-3′: PM2, 5′-CGGGATCCATATGGTCGACGAATTCGCTAAGCAATCAGCTGAATC-3′ and 5′-CGGGATCCCTCGAGAAGCTTGAATTCCTCTGTTTTACTC-3′; PM3, 5′-CGGGATCCGAGTAAAACAGAGGAATTC-3′ and 5′-CGGGATCCCAGCTTCTAATTGAGACCA-3′; PM4, 5′-CGGGATCCGTCTCAATTAGAAGCTGAA-3′ and 5′-CGGGATCCCAACTTCATTTGCCAGCTG-3′. The amplified cDNAs were digested with *Nde* I/*Bam* HI (PM, PM1, and PM2) or *Bam* HI (PM3 and PM4) and subcloned into the expression vector pET14b. cDNA for PM5 was derived by *Pvu* II/*Eco* RI digestion of the PM cDNA, end-filled, and subcloned into the *Eco* RV site of the pT7Blue-T vector (Novagen Inc., Madison, WI). The *Nde* I/*Bam* HI fragment carrying the PM5 cDNA was subcloned into pET14b. The cDNA of PM6 was derived by *Pst* I/*Bam* HI digestion of the PM cDNA, end-filled, and subcloned into the end-filled *Xho* I site of pET14b. Transformation of bacteria, induction of expression, and purification of recombinant PMs with an N-terminal histidine 6-tag were carried out as described.[13] The PM was found to contain many degraded forms and was purified further by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by electro-elution. The recombinant PMs were stored in 10 mM sodium phosphate (pH 7.2), 1 M NaCl, and 4 M urea at -80°C until use.

Measurement of antibody titer specific to the schistosome antigens in human sera. An enzyme-linked immunosorbent assay (ELISA) was carried out using AWA, the full-length PM, and a series of recombinant PMs. Briefly, 96-well microtiter plates were coated with 5 μg/ml of AWA or 1 μg/ml of PMs. After washing out the unbound antigens three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), the plates were blocked with blocking solution containing 0.5% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO) in PBST for 30 minutes at room temperature. The plates were then washed three times with PBST. Human sera were diluted 1:100 with blocking solution for detection of IgG, IgG1, IgG2, and IgG3 and 1:50 for detection of IgG4, IgE, and IgA, and then incubated overnight at 4°C. The plates were washed five times with PBST and incubated with horseradish peroxidase–conjugated anti-human IgG1, IgG2, IgG3, IgG4, and IgA (anti-IgG; EY Laboratories, Inc., San Mateo, CA; IgG1, IgG2, IgG3, and IgG4; Southern Biotechnology Associates Inc., Birmingham, AL; and IgA; ICN Biomedicals, Costa Mesa, CA) or biotinylated anti-human IgE (Vector Laboratories Inc., Burlingame, CA) at a dilution of 1:1,000 for one hour at room temperature. The plates were then washed five times with PBST. For detection of IgE, the plates were further treated with a VECTASTAIN® Elite ABC standard kit under the conditions recommended by the manufacturer (Vector Laboratories Inc.). The assays were developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and the optical density was measured at 405 nm using a microplate reader (Model MTP-22; Corona Electrics Co. Ltd., Ibaraki, Japan) with a reference measured at 492 nm.

Statistical analysis. StatView™ version 4.0 (Abacus Concepts Inc., Berkeley, CA) and HALWIN version 6.2 (Gendai-Sugakusha Co. Ltd., Kyoto, Japan) were used for data analyses. Optical densities of serum concentrations of P-III-P and type IV collagen and the antibody titers were log-transformed before analyses. We used Student's *t*-test to evaluate differences of log-transformed means of antibody titers between the study and control groups. Pearson's correlation coefficient was used to quantify associations between age, ultrasonographic evaluation, and log-transformed data for P-III-P, type IV collagen, and antibody titers. In the present study, no correction has been made for multiple comparisons between levels of antibodies to AWA and PM in correlation analyses with epidemiologic indicators because PM is present in the AWA preparation and, therefore, anti-AWA responses include the responses to PM. Multiple regression analysis was used for comparisons of isotype response levels against the PM fragments and their correlations with age and markers of fibrosis.

RESULTS

Epidemiologic outcomes. The cohort of 139 subjects ranged in age from 9 to 69 years old, and the male:female sex ratio was 92:47. Table 1 shows the relationships between age

TABLE 1

Correlations between age and markers of fibrosis in schistosomiasis japonica patients in Leyte, The Philippines*

Markers	Correlation coefficient (R)		
	US score	P-III-P†	Type IV†
Age	0.488 (< 0.001)	0.039 (0.655)	0.126 (0.147)
US score		0.306 (0.003)	0.278 (0.001)
P-III-P			0.670 (< 0.001)

* US = ultrasound. Values in parentheses are P values.
† Transformed into $\log_{10}$.

and markers of fibrosis in our patient population. We adopted four indicators to estimate the degree of liver fibrosis: ultrasonographic score and serum levels of P-III-P, type IV collagen, and TBA.

Age showed a strong correlation with ultrasonographic score (R = 0.488, P < 0.001), but was not correlated with P-III-P or type IV collagen levels. In addition, ultrasonographic score was correlated with P-III-P and type IV collagen levels (R = 0.306, P = 0.003 and R = 0.278, P = 0.011, respectively). In contrast, the TBA level was not correlated with age, ultrasonographic score, or other serologic markers.

Relationships between age and antibody isotype responses against PM. To determine whether isotype responses against PM are associated with age-dependent resistance in Filipino patients, we measured the serum levels of IgA, IgE, IgG1, IgG2, IgG3, and IgG4 against PM and AWA (Figure 1). Because the reactivity of secondary antibodies used for the ELISA varied, it was difficult to determine the amounts of antibody among the isotypes. With the exception of IgG2, the levels of all antibody isotypes against PM and AWA increased significantly in patients infected with *S. japonicum*. The unresponsiveness of IgG2 production against AWA in Filipino patients was consistent with previous findings in Chinese patients with schistosomiasis japonica.[18]

We selected IgA, IgE, IgG1, IgG3, and IgG4 isotypes to examine the relationships between age and their responses against AWA and PM (Table 2). Age showed a positive correlation with serum levels of IgG3 against both AWA (R = 0.216, P = 0.014) and PM (R = 0.325, P = 0.001) and with the level of IgA against PM (R = 0.226, P = 0.007). This was in part consistent with the findings of a previous report, in which the anti-AWA IgA level was correlated with age and PM was a major target of the IgA response in The Philippines.[9] The IgE, IgG1, and IgG4 responses did not show such correlations with age.

Relationships between fibrosis and antibody isotype responses against PM. To determine whether isotype response levels against PM are associated with fibrosis, we examined the relationships between fibrosis and levels of IgA, IgE, IgG1, IgG3, and IgG4 against PM in patients with schistosomiasis japonica. We observed that correlations of isotype responses with fibrosis were different among the indicators of fibrosis, ultrasonographic score and serum levels of P-III-P and type IV collagen (Table 3). With ultrasonographic score, positive correlations were observed for IgA, IgG3, and IgG4 levels against PM. In contrast, the P-III-P level showed a positive correlation only with IgG3 to PM, and type IV collagen level was not associated with isotype responses to PM.

Unexpectedly, IgE levels against PM showed a negative correlation with serum P-III-P level (R = −0.260, P = 0.003; Table 3), in which individuals with high IgE titers developed lower levels of serum P-III-P. Such trends were also observed between ultrasonographic score and level of IgE to PM (R = −0.069, P = 0.441), and between type IV collagen and IgE levels (R = −0.107, P = 0.228).

The IgG1 responses to AWA showed a positive correlation with the ultrasonographic score (R = 0.320, P < 0.001), but no positive correlations were observed against PM. These results suggest that fibrosis associated with IgG1 responses may be attributable to other parasite antigens.

Epitope analyses of PM recognized by isotypes. In light of these findings, we attempted to identify the epitopes recognized by IgA, IgE, IgG3, and IgG4 isotypes that are associated with age-dependent resistance or fibrosis in *S. japonicum* infection. We constructed a series of deletion mutants, PM1 (1–164 amino acids), PM2 (157–302 amino acids), PM3 (297–451 amino acids), PM4 (447–602 amino acids), PM5 (597–742 amino acids), and PM6 (734–866 amino acids). These truncated mutants had an average length of 150 amino acids and provided a sequential overlap of at least five residues (Figure 2).

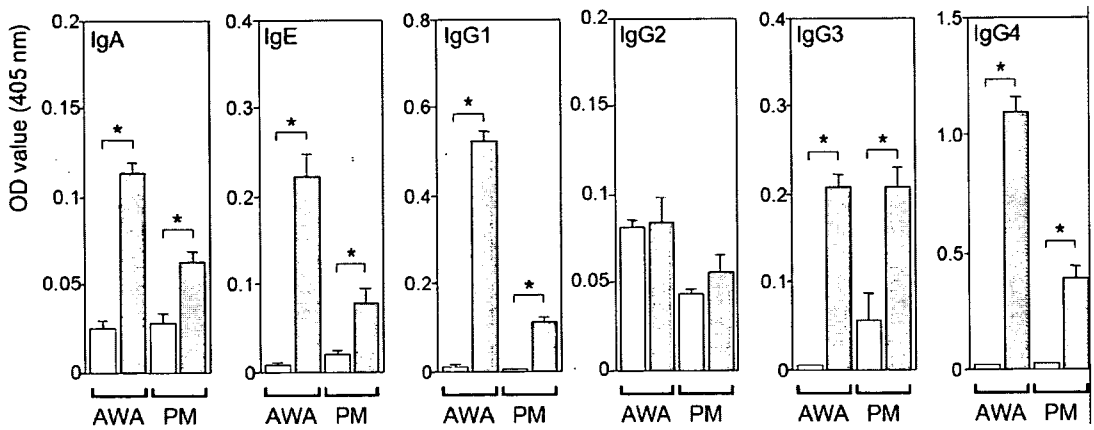

FIGURE 1. Antibody isotype levels (geometric mean ± SE) against *Schistosoma japonicum* adult worm antigens (AWA) and paramyosin (PM) in 8 healthy (open bars) and 139 infected (gray bars) individuals OD = optical density. *P < 0.01.

TABLE 2
Correlations between antibody isotype levels (log_{10}) to *Schistosoma japonicum* antigens and age*

Antibody	Correlation coefficient (R) by antigen							
	AWA	PM	PM1	PM2	PM3	PM4	PM5	PM6
IgA	0.138	0.226	0.150	0.234	0.335	0.117	0.113	0.004
	(0.105)	(0.007)	(0.077)	(0.006)	(< 0.001)	(0.169)	(0.185)	(0.966)
IgE	−0.064	0.031	0.113	0.041	−0.004	−0.060	−0.027	0.016
	(0.473)	(0.731)	(0.204)	(0.648)	(0.964)	(0.502)	(0.762)	(0.860)
IgG1	0.093	0.113						
	(0.287)	(0.198)						
IgG3	0.216	0.325	0.268	0.254	0.183	0.030	0.223	0.090
	(0.014)	(< 0.001)	(0.002)	(0.003)	(0.037)	(0.733)	(0.011)	(0.307)
IgG4	0.092	0.152	0.126	0.108	0.231	0.114	0.239	0.084
	(0.306)	(0.088)	(0.158)	(0.226)	(0.009)	(0.201)	(0.007)	(0.346)

* Values in parentheses are P values. R values for IgG1 responses to the truncated PMs are not shown. AWA = adult worm antigen; PM = paramyosin.

Box and whisker plots of isotype responses demonstrated the presence of low responders and high responders for antibody production against the full-length PM and its deletion mutants (Figure 3). Among the deletion mutants, PM6 hardly evoked any antibody production for any antibody isotype. The IgA and IgG3 isotypes reacted predominantly with the PM1, PM2, and PM3 mutants. In contrast, IgG4 appeared to react predominantly with PM2, PM3, PM4, and PM5. IgE did not show such specificity.

The IgA and IgG3 response levels against PM1, PM2, and PM3, and IgG3 levels against PM5 showed a positive correlation with age (Table 2). Multiple regression analysis was carried out to specify the responsible PM epitope(s) and showed correlations of age with IgA levels against PM3 (R = 0.356, $P < 0.001$) and with IgG3 levels against PM2 and PM3 ($R = 0.318$, $P < 0.001$ and $R = 0.307$, $P < 0.001$, respectively). These results suggest that levels of anti-PM3 IgA and levels of anti-PM2 and anti-PM3 IgG3 are likely to be associated with age-dependent resistance.

With regard to fibrosis, IgG3 levels against any of the de-

TABLE 3
Correlations between antibody isotype levels (log_{10}) to antigens of *Schistosoma japonicum* and various markers of fibrosis*

Antibody	Correlation coefficient (R) by antigen							
	AWA	PM	PM1	PM2	PM3	PM4	PM5	PM6
US score								
IgA	0.143	0.180	0.109	0.280	0.180	0.128	0.124	0.111
	(0.094)	(0.034)	(0.204)	(< 0.001)	(0.034)	(0.134)	(0.146)	(0.193)
IgE	0.035	−0.069	0.031	−0.020	−0.111	−0.124	−0.130	−0.204
	(0.693)	(0.441)	(0.726)	(0.821)	(0.210)	(0.163)	(0.141)	(0.020)
IgG1	0.320	0.081						
	(< 0.001)	(0.352)						
IgG3	0.313	0.241	0.017	0.151	0.156	0.084	0.142	−0.084
	(< 0.001)	(0.006)	(0.845)	(0.086)	(0.076)	(0.344)	(0.107)	(0.344)
IgG4	0.198	0.246	0.196	0.137	0.106	0.186	0.256	0.128
	(0.026)	(0.005)	(0.027)	(0.124)	(0.235)	(0.186)	(0.004)	(0.151)
P-III-P								
IgA	0.027	−0.048	−0.025	0.263	0.194	−0.013	0.295	0.238
	(0.756)	(0.587)	(0.77)	(0.002)	(0.025)	(0.880)	(< 0.001)	(0.006)
IgE	−0.049	−0.260	0.051	−0.056	−0.178	−0.304	−0.260	−0.194
	(0.581)	(0.003)	(0.564)	(0.531)	(0.043)	(< 0.001)	(0.003)	(0.027)
IgG1	0.097	−0.156						
	(0.267)	(0.074)						
IgG3	0.215	0.292	0.025	0.188	0.216	0.011	0.286	0.182
	(0.014)	(< 0.001)	(0.776)	(0.033)	(0.014)	(0.900)	(< 0.001)	(0.038)
IgG4	0.028	0.076	0.120	0.056	0.025	0.028	0.085	0.085
	(0.751)	(0.397)	(0.178)	(0.532)	(0.777)	(0.755)	(0.342)	(0.342)
Type IV								
IgA	−0.079	−0.067	0.032	0.038	−0.014	0.027	0.088	0.084
	(0.306)	(0.447)	(0.719)	(0.666)	(0.869)	(0.755)	(0.316)	(0.337)
IgE	−0.030	−0.107	−0.041	−0.093	−0.062	−0.140	−0.072	0.002
	(0.734)	(0.228)	(0.645)	(0.296)	(0.488)	(0.114)	(0.416)	(0.978)
IgG1	0.072	−0.057						
	(0.413)	(0.514)						
IgG3	0.174	0.150	0.073	0.053	0.020	−0.037	0.088	0.177
	(0.047)	(0.088)	(0.413)	(0.551)	(0.825)	(0.675)	(0.321)	(0.044)
IgG4	−0.032	−0.035	0.100	−0.031	−0.034	−0.007	0.032	−0.013
	(0.719)	(0.693)	(0.266)	(0.731)	(0.706)	(0.940)	(0.718)	(0.884)

* Values in parentheses are P values. R values for IgG1 responses to the truncated PMs are not shown. US = ultrasound; AWA = adult worm antigen; PM = paramyosin.

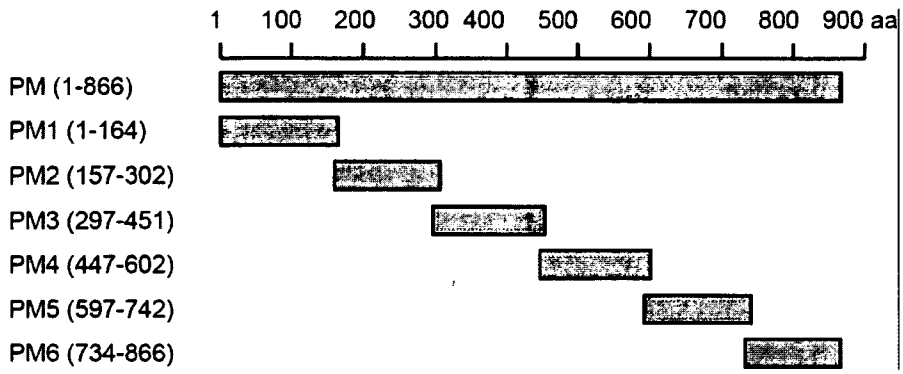

FIGURE 2. Schematic representation of recombinant *Schistosoma japonicum* paramyosin (PM) and its deletion derivatives used in this study. The scale and numbers indicate the amino acid (aa) positions. The full-length PM consists of 866 amino acids. The deletion mutants and their amino acid positions were as follows; PM1 (1–164 amino acids), PM2 (157–302 amino acids), PM3 (297–451 amino acids), PM4 (447–602 amino acids), PM5 (597–742 amino acids), and PM6 (734–866 amino acids).

letion mutants showed no significant correlations with ultrasonographic score and P-III-P levels, despite the positive correlation of anti-PM IgG3 levels with these markers (Table 3). Similarly, multiple regression analysis did not show significant correlations of IgG3 levels against any PM fragments with ultrasonographic score and P-III-P levels. It is likely that the multiple PM epitopes were associated with fibrosis and recognized differently by the patients.

The PM epitopes associated with fibrosis were recognized differently by IgA and IgG4. The IgA levels to PM2 and PM3 and IgG4 levels to PM1, PM4, and PM5 showed positive correlations with ultrasonographic score (Table 3). Multiple regression analysis showed correlations of ultrasonographic score with IgA levels against PM2 ($R = 0.292$, $P < 0.001$) and with IgG4 against PM1 and PM5 ($R = 0.196$, $P = 0.028$ and $R = 0.258$, $P = 0.004$, respectively).

Negative correlations between IgE titers and P-III-P levels were observed for PM3, PM4, PM5, and PM6, but the IgE responses against these deletion mutants were weak. Multiple regression analysis showed a correlation of P-III-P levels with anti-PM4 IgE levels ($R = -0.309$, $P < 0.001$). These results were consistent with the relationship between IgE levels and full-length PM, and suggested that PM4 recognized by IgE has a role in suppression of the progression of fibrosis.

DISCUSSION

Schistosome vaccines are expected to show both effects against infection and disease. In the present study, we addressed the relationships of antibody isotype responses to PM, not only with age-dependent resistance but also with fibrosis, because liver fibrosis is the most important lesion in schistosomiasis japonica.

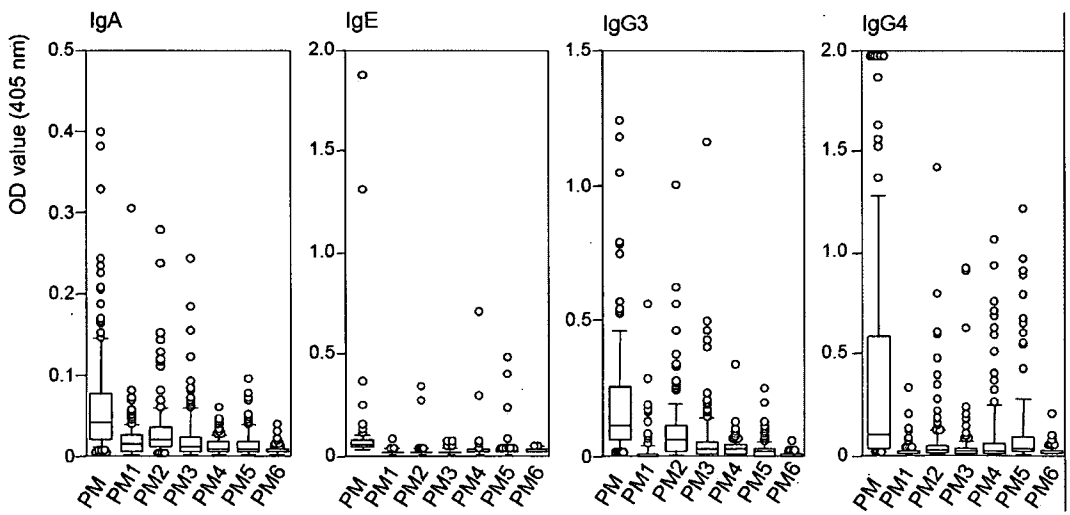

FIGURE 3. Box and whisker plots of IgA, IgE, IgG3, and IgG4 isotype responses to *Schistosoma japonicum* paramyosin (PM) and its deletion mutants. The full-length PM and a series of deletion mutants (PM1, PM2, PM3, PM4, PM5, and PM6) were used for an enzyme-linked immunosorbent assay. The box indicates the area ranging from the first to the third quartiles of each dataset and the median is indicated by the black centerline. The vertical bar represents 1.5 times the interquartile range (IQR) from the upper or lower quartile. Points at a greater distance from the IQR are plotted individually as circles. OD = optical density.

To determine the epidemiologic states of patients with schistosomiasis japonica in The Philippines, we first examined the relationships between age and fibrosis (Table 1). We observed a positive correlation between age and ultrasonographic score but not between age and any serologic markers of fibrosis. Correlations between age and ultrasonograpic score appear to reflect accumulation of fibrosis along with age rather than the current progression of fibrosis. For example, cases of schistosomiasis japonica in older persons in Japan, which showed advanced liver fibrosis by ultrasonographic scoring, did not excrete eggs.[26]

Positive correlations of ultrasonograpic score with P-III-P and type IV collagen levels are consistent with the previous findings for schistosomiasis japonica.[24,27] It is noteworthy that P-III-P level reflects mainly the progress of collagen synthesis, while the type IV collagen level reflects collagen degradation.[28] Thus, correlations between ultrasonographic score and these serologic markers may reflect the current pathologic progress.

The TBA level has been suggested to be a good indicator of hepatic fibrosis.[25] In the present study, however, the TBA level did not show any correlations with other indicators of fibrosis. This discrepancy may have been due to the difference in duration between the previous study design[25] and the present study design, in that the subjects in the present study had received mass treatment with praziquantel in the previous 10 years, which may have influenced the serum level of TBA.

The IgG3 levels to both AWA and PM showed a positive correlation with age (Table 2). A similar age-related trend with these IgG3 responses was reported previously in the human population in The Philippines.[7,29] In addition, multiple regression analysis showed a correlation of age with IgG3 levels against PM2 and PM3. These findings suggest that IgG3 responses to PM, especially anti-PM2 and anti-PM3 IgG3 levels, may be involved in protective immunity to *S. japonicum* infection.

Likewise, IgA responses to PM showed a positive correlation with age (Table 2). This was consistent with the previous report of a positive correlation between IgA levels against PM and age in Filipino patients.[9] Multiple regression analysis suggested that the anti-PM3 IgA level is likely to be associated with age-dependent resistance. In contrast, there was no correlation between any antibody responses to PM and age in China.[18] Thus, the correlation between levels of IgA to PM and age is likely to be distinctive in The Philippines, possibly because of differences in epidemiologic and immunologic features between China and The Philippines.

We did not find any correlations between IgE levels and age, whereas AWA- and PM-specific IgE were present in the sera of Filipino patients. Similarly, we found no significant correlations between IgE levels and frequency of treatment, which is an indication of intensity of reinfection. These observations were consistent with the report that levels of IgE against AWA did not show correlations with age.[18,29] In contrast, levels of IgE to AWA were higher in subjects who were unsusceptible to reinfection two years post-treatment in China.[8] Another group has also reported an association between IgE response to AWA and age in The Philippines.[7] Since there is no direct evidence that human IgE in combination with effector cells mediates killing of the parasites, further analyses are necessary to explain this discrepancy by verifying the precise role of IgE in age-dependent resistance.

In the present study, the relationships between antibody response levels to PM and the degree of liver fibrosis were investigated. We observed positive correlations of antibody isotypic responses to PM with the degree of liver fibrosis as follows: the IgA, IgG3, and IgG4 levels with ultrasonographic score and the IgG3 level with the P-III-P level (Table 3). Multiple regression analysis showed positive correlations of ultrasonographic score with IgA levels against PM2 and with IgG4 against PM1 and PM5, suggesting that anti-PM2 IgA and anti-PM1 and -PM5 IgG4 are likely involved in progressive fibrosis.

The anti-PM IgG3 level showed a positive correlation with ultrasonographic score and P-III-P level. It is important to note that the ultrasonographic score likely represents accumulation of fibrous tissues in the liver, while the serum P-III-P level indicates the currently active state of fibrosis.[28] Therefore, the IgG3 response to PM maybe involved in both progression and the subsequent accumulation of fibrosis, but the PM epitope(s) associated with the degree of fibrosis is unclear.

In contrast to the IgG3 response to PM, a negative correlation between IgE response and serum P-III-P level was found in the Filipino patients (Table 3). In addition, multiple regression analysis showed a negative correlation of P-III-P levels with anti-PM4 IgE levels. In the experimental rodent model, there are controversial observations concerning the role of IgE in schistosome infection: reduced granuloma formation in mice lacking IgE[19,20] and enhanced granulomatous inflammation in FcεRI-deficient mice.[30] The discrepancy in the mode of IgE in granulomatous development may suggest that the roles of IgE in fibrosis are dependent on parasite antigens; IgE to PM may interfere with the progression of fibrosis, and other combinations may enhance fibrosis in humans.

Recent studies have demonstrated the roles of surface PM as immunomodulators. Paramyosin is capable of binding *in vitro* to collagen and the complement components C1, C8, and C9, which results in inhibition of complement activation and formation of membrane attack complex.[31–33] Likewise, PM can bind to the Fc domain of immunoglobulin *in vitro*.[34] The modes of isotype responses to PM in granuloma formation are unclear. However, it is possible that the immune complex of immunoglobulins and PM released from the parasite surface binds to the endothelial or fibroblastic matrix surrounding the embolized eggs through interaction of PM with collagen, which leads to enhanced inflammation and granuloma formation.

It is commonly assumed that granulomatous pathology is $CD4^+$ T cell-mediated in mice. It is noteworthy that B cell- or FcRγ-deficient mice exposed to *S. mansoni* developed larger granulomas in the chronic stage of the infection and displayed unaltered T cell responses, which suggests a suppressive role of immunoglobulin receptor–mediated responses in granuloma formation.[35] In the present study, it is possible to speculate that PM or PM-immunoglobulin complex modulate positively immune responses at the site of lesions, which leads to accumulation of fibrous tissues, the mechanism of which remains unknown.

In the present study, it was difficult to identify the epitope(s) responsible for age-dependent resistance or granu-

loma formation. Epitope mapping of PM recognized by the isotypes and their correlation analyses indicated some trends, in that age tended to be associated with the responses to the N-terminal half of PM (Table 2). In contrast, no such tendency was observed in relationships between liver fibrosis and the epitopes. These results suggest that multiple epitopes are involved in both age-dependent resistance and liver fibrosis.

A positive correlation between IgG3 responses to PM and P-III-P levels was an undesirable finding in the context of schistosome vaccine development. In contrast, IgE response to PM is likely to play a suppressive role in the development of fibrosis, a desired feature for a schistosome vaccine. These contrasting results clearly indicate that PM has complex roles in modulating human immune responses.

Although PM can induce protective immunity against challenge parasite infection in experimental animal models, there are marked immunologic and pathologic differences between humans and animals. Our findings provide insights into the importance of combinations between PM and isotype responses for schistosome vaccine development, in which the desired immune responses should be provoked to avoid exacerbating the pathology. Further studies to characterize the precise mode of PM in antibody-dependent killing of the parasite and in granuloma formation in humans are required prior to clinical trials.

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