phagocytosis-based immunity by introducing the possibility that phagocytosis is not limited merely to the digestion and autophagy of ingested pathogens but rather might act as a cellular barrier to block pathogen escape. The immune system in vertebrates has evolved the digestion mechanism as a means for antigen presentation, a prerequisite for induction of acquired immunity that is a major strategy to eradicate invaders, and that could conflict with an "encapsulatable" function. However, in the case of invertebrate arthropods lacking an antigen-based acquired immune system, it is likely that greater emphasis would be placed on the isolation and sequestration of engulfed pathogens from a host's vital tissues. Further delineation of the encapsulatable tolerance machinery will provide important insight into primitive immunity as well as host-pathogen interactions.

Pathogen-Sustainable Tolerance Properties in Infected Individuals

Phagocytic encapsulation enables host animals to carry live etiologic agents inside their bodies, thereby potentially providing additional time for eradication or simply acting as a sequestration method to avoid exposure to additional tissues sensitive to pathogenic effects. Furthermore, sequestration of agents capable of triggering immune responses would simultaneously avoid energetically expensive systemic immune responses that can limit life span. Conversely, it is likely that the "packing" event also plays a role in the survival of pathogens themselves. For example, an encapsulation-based cellular response can be exploited for the avoidance from host immune system for years or decades, as represented by *Mycobacterium tuberculosis*-induced latent infection that allows persistence of tubercule bacilli within macrophages in granulomatous lesions (Raupach and Kaufmann, 2001). Thus, phagocytic encapsulation probably contains two conflicting impacts on the host defense system: one whereby conserved tolerance may act as a comprehensive primitive defense system that prevents host animals from suffering damages prior to systemic resistance (e.g., in the case of acquired immunity), and the second in which an indirect effect of tolerance facilitates disease latency in individuals and consequent propagation in animal populations when resistance systems do not fully eliminate invaders.

While this study clearly demonstrates a role for *Dmp38b* in conferring tolerance to flies infected with *Salmonella*, a role for *Dmp38b* in resistance cannot be ruled out. Indeed, survival of an infected organism likely depends on a combination of both resistance to that organism and tolerance to the infection. Such a combination of mechanisms has been proposed to lead to nine different states of an organism (Ayres and Schneider, 2008) ranging from high tolerance coupled with low resistance to low tolerance coupled with high resistance and all states in between. The p38-mediated defense seen in this study potentially falls into the class represented by high tolerance and low resistance. In order to shed light on such complicated defense mechanisms, comprehensive work investigating the synergistic effects of both tolerance and resistance to host fitness will be required.

A provocative finding in this study involves a potential role for *Dmp38b* in the regulation of the melanization cascade, a mechanism involved in infection resistance (Ayres and Schneider, 2008). While previous reports have shown crucial negative regulatory mechanisms for both the Imd and Toll pathways in their regulation of AMP activity (Gordon et al., 2005; Foley and O'Farrell, 2004; Kim et al., 2005; Agaisse and Perrimon, 2004), negative regulation of the melanization cascade remains unclear. Intriguingly, *Dmp38b* appears to act as both a positive regulator for tolerance and a negative regulator of the melanization cascade (Figure S3C). Furthermore, in larvae, *Dmp38a* activity has been associated with a downregulation of AMP activity (Han et al., 1998). A shift from resistance to tolerance would be necessary for long-term survival of an organism, and control of such a shift by a bona fide tolerance mediator would guarantee tight association between these connected survival strategies. Given the conserved role of p38 MAP kinase as a mediator in pathogen-sustainable tolerance systems, our findings may provide a blueprint for host-pathogen interactions that is likely to influence the severity of infectious diseases in other metazoans, including vertebrates.

EXPERIMENTAL PROCEDURES

Fly Strains

Flies were raised on standard *Drosophila* medium at 25°C. *da-GAL4* line, KG01337 line, and other general strains were obtained from Bloomington Stock Center. All GS strains were provided from Kyoto *Drosophila* Genetic Research Center. *UAS-Dmp38b* line was a gift from Takashi Adachi-Yamada (Adachi-Yamada et al., 1999). *pxn-GAL4* line was a gift from Micheal J. Galko (Stramer et al., 2005). *UAS-DsRed* line was a gift from Makoto Sato (Sato and Kornberg, 2002). *Dmp38a* null mutant was a gift from Ross L. Cagan (Craig et al., 2004).

Bacterial Strains and Culture

Salmonella typhimurium (SL1344) and *Listeria monocytogenes* (10403S) were gifts from David Schneider (Brandt et al., 2004; Mansfield et al., 2003). *S. typhimurium* (TM232) was provided by Nobuhiko Okada (Miki et al., 2004). *Legionella pneumophila* (CR39) was provided by Hiroki Nagai (Nagai and Roy, 2001). *Staphylococcus aureus* was provided by Hiroshi Hamamoto and Kazuhisa Sekimizu (Hamamoto et al., 2004). The pMIG1 reporter plasmid was a gift from Stanley Falkow (Valdivia and Falkow, 1997). SL1344 and TM232 were cultured at 37°C in the dark without agitation in LB broth medium with 100 μg/ml streptomycin, and 100 μg/ml streptomycin and 50 μg/ml kanamycin, respectively. For pMIG1 plasmid maintenance, culture medium was supplemented with 100 μg/ml ampicillin. 10403S was cultured at 37°C in the dark without agitation in BHI broth medium with 100 μg/ml streptomycin. CR39 was cultured at 37°C in the dark on charcoal-yeast extract (CYE) plates with 100 μg/ml streptomycin as described previously (Nagai and Roy, 2001), and bacterial patch on the plate were suspended in sterile water for injection. *S. aureus* was cultured at 37°C in the dark with agitation in LB broth medium.

Microbial Infection to Fly and Cells

Seven- to ten-day-old male flies were used for all experiments. Before injection, the bacteria-containing medium was adjusted to appropriate concentration using Gene Quant *pro* (Amersham) with culture medium (SL1344, TM232, and CR39, 0.1 OD; 10403S, 0.01 OD; and *S. aureus*, 0.001 OD). Flies were anesthetized with CO_2 and injected with each strain of bacteria in 65 nl of medium. Injection was carried out by using an individually calibrated pulled glass needle attached to IM-300 microinjector (Narishige). Flies were always injected in the abdomen, close to the junction with thorax and just ventral to the junction between the ventral and dorsal cuticles. After injection, flies were transferred to fresh vials once a week. For inhibition of phagocytosis activity of hemocyte, FluoSpheres red fluorescent (F8763, Invitrogen) were injected into fly as previously described (Elrod-Erickson et al., 2000). For infection to cells, *Drosophila* S2 cells were cultured as previously described (Kanuka et al., 2005). Cells were incubated with bacteria for 1 hr at moi = 10 and treated with 100 μg/ml gentamycin for 1 hr to eliminate unengulfed bacteria.

Gain-of-Function Screening

To identify genes enhancing infection tolerance for bacterial pathogenicity we adapted the *P*-element-based Gene Search (GS) System (Toba et al., 1999). The GS vector contains the UAS enhancer adjacent to a core promoter. In this screen, genes are detected on the basis of phenotypic changes caused by the GAL4-dependent forced expression of the vector-flanking DNA. This system has greater efficiency than others that are presently used for gain-of-function screens (Toba et al., 1999). For the first screen, females of the w^{1118}; +/+; *da-GAL4* genotype were crossed to males from each line in the GS collection. F1 progeny were injected with *Salmonella* (approximately 5000 CFU), and survival rates were monitored daily. The infection phenotypes of GS lines (longer-lived and shorter-lived) were categorized into "suppressor" and "enhancer" of *Salmonella*-induced lethality, respectively. Genomic DNA regions flanking P element of the GS vector were recovered from these GS lines by standard inverse PCR protocols (http://www.fruitfly.org/about/methods/inverse.pcr.html). The recovered genomic fragments were sequenced and analyzed with BLAST at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) databases to identify the candidate genes (Table S1). In a secondary screen, bacterial load in each GS line identified as a "suppressor" strain was measured. Strains exhibiting both prolonged survival and normal bacterial load were kept and reexamined for reproducibility of phenotypes.

Dmp38b Mutant Fly

KG01337 (#14364) flies were crossed to transposase-expressing lines (w^{1118}; *Dr/TMS, Sb P*[ry^+, $\Delta 2-3$]), and male progeny carrying both elements were mated to w^{1118}; *Tft/CyO* females. Progeny with white eyes were collected and analyzed. Excision lines were screened by genomic PCR and sequenced using sets of primers that recognize genomic sequences flanking the *Dmp38b* locus. An imprecise excised line ($p38b^{ex9}$) was a null mutant line of *Dmp38b*, because RT-PCR analysis revealed that the amount of mRNA for *Dmp38b* was not confirmed in this strain (data not shown). Overexpression of *Dmp38b* rescued the null mutants phenotypes demonstrated in this report (data not shown). RT-PCR was carried out with the following primers sets: *Dmp38b*, 5′-CCGAGAGATCAATGTTGAGGCCGCGAAGTG-3′ and 5′-TTATGTTGTTCA GATCGGCGTCCATCAAGT-3′.

Colony-Forming Assay

Infected flies incubated for indicated durations were homogenized in 10 mM $MgSO_4$ solution, and the diluted series of the homogenized samples were prepared and plated on media containing 100 μg/ml streptomycin for whole-body CFU counts. For hemolymph CFU counts, *Salmonella*-infected flies incubated for indicated durations were injected with 260 nl PBS incubated 10 min before hemolymph was drawn, added to 100 μl 10 mM $MgSO_4$ solution, and plated on media containing 100 μg/ml streptomycin. The CFU in hemolymph was estimated supposing the hemolymph volume of individual flies is 200 nl.

Immunoblotting

For the detection of proteins, infected flies and S2 cells were collected and lysed in SDS sample buffer at each indicated time point. Immunoblotting was performed described previously (Kanuka et al., 2005), using a rabbit anti-active p38 antibody (1:1000, Cell Signaling Technology), rabbit anti-Dmp38b antibody (1:1000, Adachi-Yamada et al., 1999), mouse anti-β-tubulin (1:1000, Chemicon), anti-mouse IgG-HRP antibody (1:2000, Promega), and anti-rabbit IgG-HRP (1:2000, Cell Signaling Tchnology). The signals were visualized using Immobilon Western kit (Millipore). Immunoblotting of anti-β-tubulin antibody was used as a loading control.

Immunohistochemistry

Immunostaining of abdominal regions was carried out as previously reported (Kanuka et al., 2005) with some modification. The following antibodies and fluorescent materials were used for immunostaining: mouse P1 monoclonal antibody (1:10, a gift from Dr. Istvan Ando) (Kurucz et al., 2007), goat anti-mouse IgG-Alexa 568 (1:100, Invitrogen), goat anti-mouse IgG-Alexa 488 (1:100, Invitrogen), and FluoSpheres red fluorescent (F8763, Invitrogen). To analyze the topology of the infected hemocytes, optical sections were obtained along the z axis at 0.2 μm intervals, and the images of the X-Z and Y-Z planes were reconstructed by using Leica AF software (Leica). For quantitative analysis of fluorescence in the dorsal side of the abdomen, legs and wings were removed from anesthetized fly bodies and fixed on glass slides with double-sided adhesive tape. All fluorescent signals were examined using a MZ 16F fluorescence microscope for fly image and a TCS SP5 confocal microscopy for plasmatocytes image (Leica). Quantification of represented images was assessed by using ImageJ software (National Institutes of Health [NIH]).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, one table, seven figures, Supplemental References, and two movies and can be found with this article online at http://www.cell.com/cell-host-microbe/supplemental/S1931-3128(09)00258-3.

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Research brief

A single fluorescence-based LAMP reaction for identifying multiple parasites in mosquitoes

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ABSTRACT

Vector-borne diseases, such as malaria and lymphatic filariasis, are co-endemic in large parts of the world. To develop a multiplex amplification method for the simultaneous detection of multiple insect-borne infectious diseases, we used LAMP with fluorescently labeled primers to identify the *SPECT2* gene of *Plasmodium berghei* and the *cytochrome oxidase subunit I* gene of *Dirofilaria immitis* in mosquitoes. This technique could detect as few as 100 *P. berghei*-infected red blood cell-equivalents or one *D. immitis* microfilaria. Moreover, individual species of parasites in mosquitoes could be identified when a mixture of fluorescently labeled primer sets was used. These findings suggest that the multiplex LAMP assay is sensitive and specific enough to identify parasite-bearing mosquitoes in areas where several diseases occur simultaneously. This procedure could increase the efficiency and effectiveness of arthropod-borne disease elimination programs.

1. Introduction

Mosquitoes are important arthropod vectors for various disease-causing pathogens, including parasites, bacteria, and viruses, that infect a variety of hosts, including humans. Many of these diseases continue to pose an endemic threat to human populations, despite the ongoing efforts of elimination programs, such as the Global Malaria Program and Global Program to Eliminate Lymphatic Filariasis, run by the World Health Organization (WHO) (WHO, 2008a,b). These programs were devised to attempt "comprehensive vector-borne disease control" (WHO, 1995), because several of these important diseases are co-endemic (Muturi et al., 2008; Chadee et al., 2003; Ravindran et al., 1998) and transmitted by the same arthropod genus (Chadee et al., 2003; Burkot et al., 1990a). Moreover, in the case of mosquito-borne illness, some species of mosquito can carry and transmit more than one pathogen (Albuquerque and Ham, 1995), and occasionally, individual mosquitoes have been shown carry two pathogens simultaneously (Muturi et al., 2006; Burkot et al., 1990b). Thus, a method for detecting multiple pathogens in a single reaction would make parasite surveys more efficient and effective.

To this end, we examined the applicability of fluorescence-based loop-mediated isothermal amplification (LAMP) for the detection of *Plasmodium berghei* and *Dirofilaria immitis* in mosquitoes. LAMP is a widely used DNA amplification method for carrying out reactions under isothermal conditions (Notomi et al., 2000) using a single enzyme; this method has a higher reaction specificity than conventional PCR methods (Mori et al., 2001).

Malaria and filariasis are debilitating parasitic infectious diseases transmitted by mosquito vectors that affect more than one million people worldwide (WHO, 2007a,b). We recently demonstrated the applicability of LAMP for detecting parasites in mosquitoes with high sensitivity: a single *P. berghei* oocyst could be detected in *Anopheles stephensi* and a single *D. immitis* worm in *Aedes aegypti* (Aonuma et al., 2008, 2009). Here we report the development of a fluorescence-based detection assay for the combined survey of both pathogens using the same reaction conditions.

2. Materials and methods

2.1. Preparation of parasites and infected mosquitoes

To evaluate the detection of malarial parasites using our method, infected red blood cells (iRBCs) were collected from 7- to 8-week-old BALB/c mice that had been infected with the rodent malaria *P. berghei* ANKA strain. This strain expresses GFP driven by the *hsp70* promoter, permitting the easy detection of infected

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cells (a gift from Dr. M. Yuda; Ishino et al., 2006). The blood was stored at −20 °C until needed for DNA extraction. Female vector mosquitoes of the strain *A. stephensi* were allowed to feed on anaesthetized infected mice, and were then kept at 19 °C until their dissection for microscopic analysis. To evaluate the efficiency of LAMP performed on oocyst-carrying mosquitoes, each mosquito midgut was dissected in ice-cold PBS, and the number of oocysts was counted under a microscope. Each midgut was then collected along with the remaining carcass, and the tissues were stored at −20 °C until needed for DNA extraction.

For the filarial parasite-transmission model, we used *D. immitis*, and the vector mosquito *Ae. aegypti*. To culture microfilariae, adult *D. immitis* worms were isolated from an infected dog and cultured in RPMI 1640 medium. The microfilariae were paralyzed by cooling at 4 °C and collected by centrifugation at 130*g*, for 10 min. The isolated microfilariae were counted with a cytometer and stored at −20 °C until needed. *Ae. aegypti* mosquitoes were fed *D. immitis*-infected blood by loading sheets of Parafilm (Pechiney Plastic Packaging, Inc., Illinois, USA) with infected dog blood, and attaching the blood-filled Parafilm to the bottom of a culture flask containing warm water. The membrane feeder was then placed over a netted mosquito cup for 1 h. The infected mosquitoes were kept at 27 °C until 8 days post-infection, to allow the *D. immitis* to reach the L2 stage. The Malpighian tubules were then dissected from the infected mosquitoes in ice-cold PBS, examined microscopically to count the number of parasites, and the entire carcass, including the Malpighian tubules and parasites, of each mosquito was stored −20 °C until needed for DNA extraction.

2.2. DNA extraction

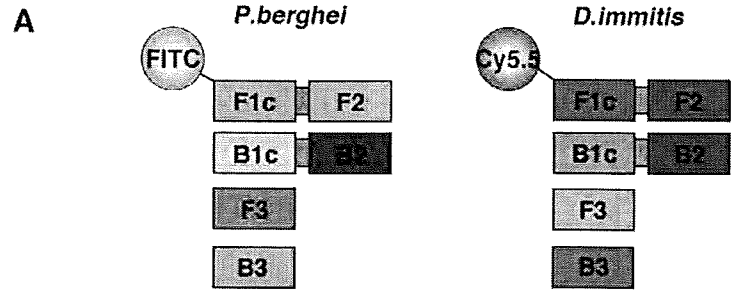
The genomic DNA of *P. berghei* iRBCs, *D. immitis* microfilariae, and infected mosquitoes was extracted by homogenizing the cells or organisms with a plastic homogenizer in Buffer A (0.1 M Tris, pH 9.0; 0.1 M EDTA; 1% SDS; and 0.5% DEPC) and incubating the homogenate for 30 min at 70 °C. Next, 22.4 μl of 5 M KoAc was added, and the mixture was cooled for 30 min on ice. After centrifugation at 20,000*g* for 15 min at 4 °C, the DNA-containing supernatant was transferred to a new tube and mixed with 45 μl isopropanol. The solution was centrifuged at 20,000*g* for 20 min at 4 °C, and the precipitated DNA was collected, rinsed with 70% ethanol, and dried. Each DNA pellet was diluted in TE, and the final DNA concentrations per 1 μl were equal to 1×10^5 iRBCs, 1×10^4 microfilariae, or one-fifth of a mosquito. Finally, 1 μl of each DNA solution was used as a template for the LAMP reaction.

2.3. LAMP reactions

The loci and sequences of the primers (F3, B3, FIP, and BIP) were described previously (Aonuma et al., 2008, 2009). The FIP primers were labeled with either the FITC (*P. berghei*) or Cy5.5 (*D. immitis*) fluorescent dye (Fig. 1). The LAMP reaction was performed according to the manufacturer's instructions (Eiken Chemical Co., Ltd., Tokyo, Japan). Briefly, the 12.5 μl reaction mixture contained 1 μl of extracted DNA, 2.5 pmol of each F3 and B3 primer, 20 pmol of each FIP and BIP primer, 6.25 μl of 2× Reaction Mix, and 0.5 μl of *Bst* DNA polymerase. The reaction mixture was incubated at 60 °C for 90 min using a Loopamp Real-time Turbidimeter (LA-200; Eiken Chemical Co., Ltd., Tokyo, Japan). The reaction was terminated by incubation at 95 °C for 2 min.

2.4. Analysis of LAMP products

Amplified DNA in the LAMP reaction causes turbidity due to the accumulation of magnesium pyrophosphate, a by-product of the reaction. The turbidity was monitored using both a Loopamp Real time Turbidimeter (LA-200; Eiken Chemical Co., Ltd., Tokyo, Japan) and the naked eye. Each LAMP product was subjected to electrophoresis in a 2% agarose gel at 100 V, and the fluorescent dye-conjugated fragments were examined with an image analyzer (LAS-3000; Fujifilm Corporation, Tokyo, Japan) before the gels were stained with ethidium bromide for examination under UV light.



B

Primer	Sequence (5'→3')
P. berghei	
F3	ATGGGAAACTCAAACGACAA
B3	GGTGATTTTACGTATGGGCTG
FITC-FIP(F1c-F2)	CTGCACCATGGAAATCATCTTTTGCTACCTTGTGTGTGTTGATGC
BIP(B1c-B2)	GACGGGGAATTAGTTCTAAGTTGCCGTTTCCCCTGTAAATCCAGTT
D. immitis	
F3	CCTGTTTTGGCTGGTTCTT
B3	AGCAAAAGTCATACTAGTCTGG
Cy5.5-FIP(F1c-F2)	ACCTCAGGATGTCCAAAAAACCAAAATGATGCTAATAAGGGGGGTAA
BIP(B1c-B2)	TACCGGTGTTTGGGATTGTTAGTGCCAAACAAACGATCCTTATCAGTC

Fig. 1. LAMP primer sets targeting the *P. berghei SPECT2* and *D. immitis cytochrome oxidase subunit I* gene. (A) Primers designed for the fluorescence-based LAMP reaction. FIP primers were conjugated with the fluorescent dye FITC (*P. berghei*) or Cy5.5 (*D. immitis*). (B) Sequences of primers used in the LAMP reactions.

3. Results and discussion

The sensitivity and specificity of LAMP reactions containing primer sets for both pathogens, including the fluorescent dye-labeled primers, were examined in reactions with *P. berghei* DNA equivalent to 1×10^0, 1×10^1, 1×10^2, and 1×10^3 iRBCs or *D. immitis* DNA equivalent to $1x10^0$, $1x10^1$, $1x10^2$, and $1x10^3$ microfilariae, as templates. Consistent with previous results (Aonuma et al., 2008, 2009) the LAMP reaction was sensitive enough to detect single parasite of *D. immitis*, albeit at slightly longer reaction times, suggesting that the conjugation of fluorescent dyes to the primers did not appreciably inhibit this LAMP reaction (Fig. 2A). The sensi-

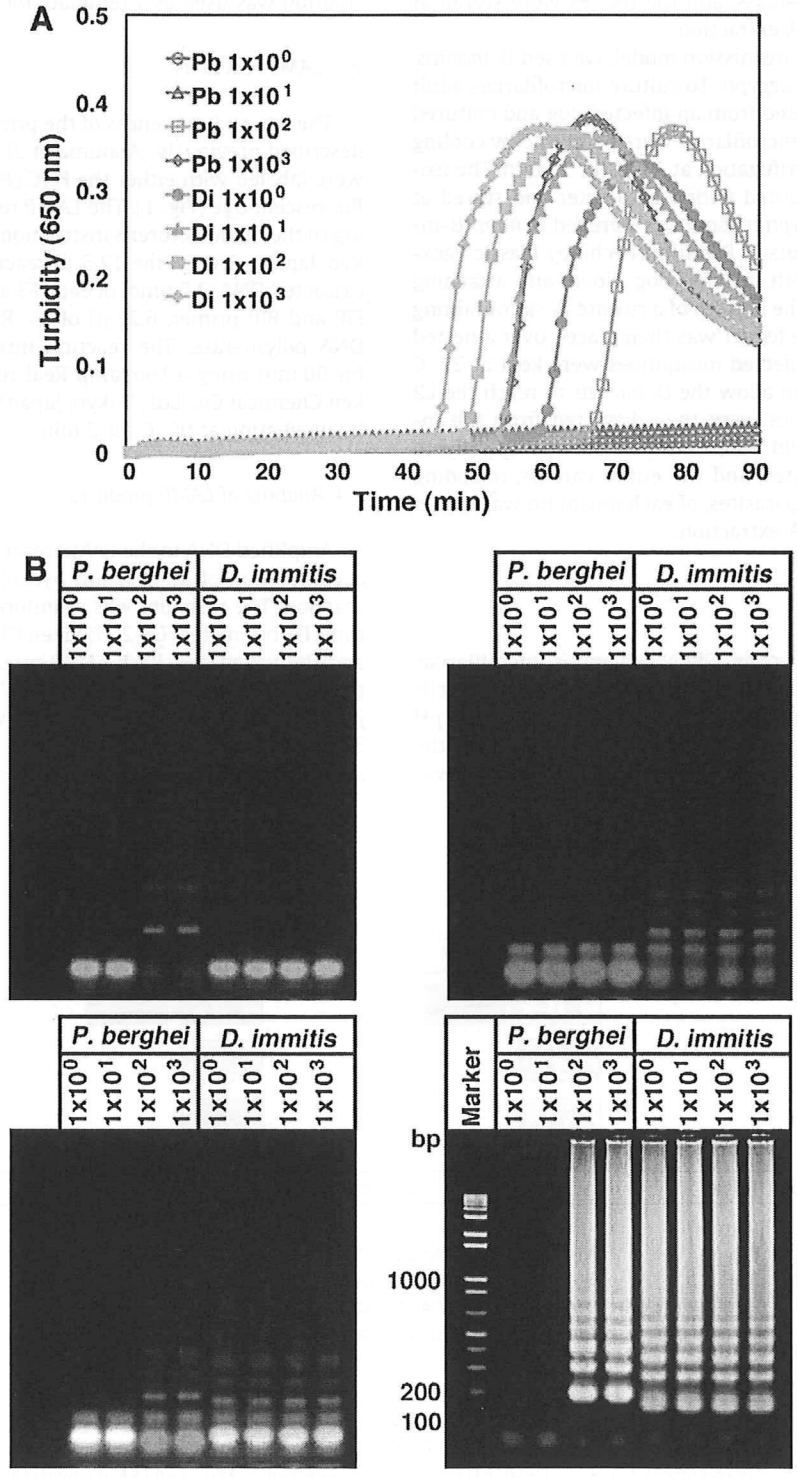


Fig. 2. Sensitivity of fluorescence-based LAMP detection of *P. berghei* and *D. immitis*. (A) Amplification of the target sequence with a primer set monitored by real-time turbidimeter (turbidity at 650 nm). *P. berghei* DNA (equivalent to 1×10^0, 1×10^1, 1×10^2, or 1×10^3 iRBCs) or *D. immitis* DNA (equivalent to 1×10^0, 1×10^1, 1×10^2, and 1×10^3 microfilariae) were used as templates for LAMP reactions of 90 min at 60 °C. (B) Agarose-gel electrophoresis of the LAMP-amplified products from (A). FITC fluorescence (green) indicates the amplified DNA sequence from *P. berghei* and Cy5.5 fluorescence (magenta) indicates that from *D. immitis* microfilariae. FITC (upper left), Cy5.5 (upper right), FITC and Cy5.5 merged image (left below), and ethidium bromide (right below). Numbers at left indicate the migration of molecular weight markers (bp).

tivity of *P. berghei* detection was slightly reduced, but low levels of parasitic load could still be detected. The optimized conditions were 60 °C for 90 min (data not shown). Using the optimized conditions, we next tested whether multiplex LAMP could be used to detect *P. berghei* or *D. immitis* simultaneously under the same reaction conditions. Both the FITC-labeled and Cy5.5-labeled primer sets were included in the reaction mixtures with DNA from either *P. berghei* or *D. immitis*. The fluorescence analysis of the amplified DNA demonstrated that each primer set specifically amplified the DNA of *P. berghei* (FITC) or *D. immitis* (Cy5.5) (Fig. 2B) under the same reaction conditions (Fig. 2B), indicating that LAMP performed using fluorescent dye-conjugated primers might enable the identification of two different parasitic species at the same time.

To evaluate the feasibility of a combined LAMP method designed to survey pathogen-carrying mosquitoes, we examined mosquitoes carrying *Plasmodium* or *Dirofilaria*. *A. stephensi* and *Ae. aegypti* were dissected, and the *P. berghei* oocysts and *D. immitis* second-stage larvae (L2), respectively, were counted. The number

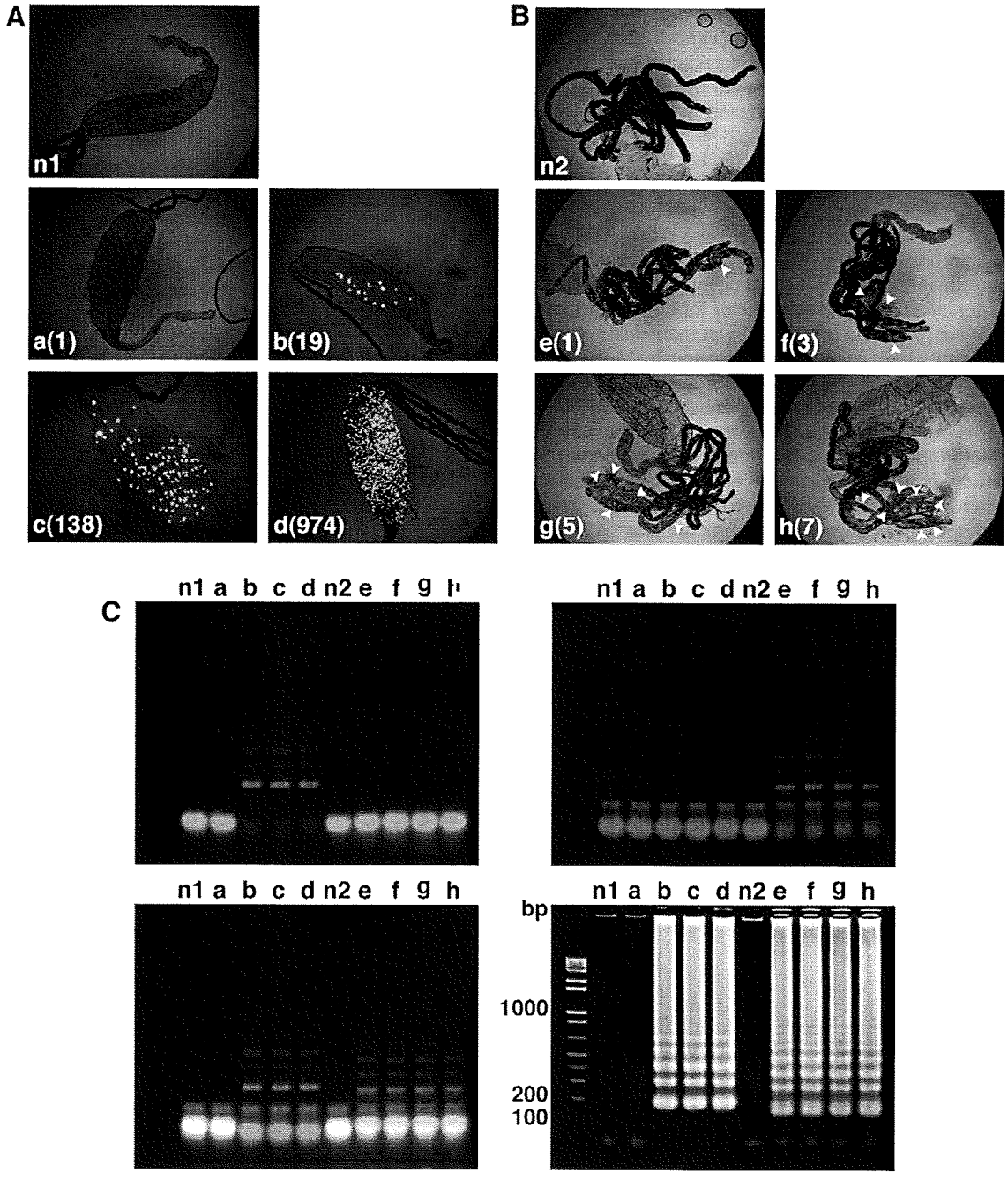


Fig. 3. LAMP-based identification of *P. berghei*- and *D. immitis*-carrying mosquitoes. (A) Each *P. berghei*-positive mosquito was observed under fluorescence microscopy to determine its state of infection. The punctate GFP signals (green spots) indicate *P. berghei* oocysts in the mosquito midgut (a–d). The number of oocysts is given in parenthesis. The uninfected mosquito shown in (n1) was used as a negative control. (B) Each *D. immitis*-carrying mosquito was observed under a microscope to determine its state of infection. Each white arrowhead indicates a *D. immitis* L2 larva in the mosquito Malpighian tubules (e–h). The number of L2 larvae is given in parenthesis. The Malpighian tubules of the uninfected mosquito shown in (n2) were used as a negative control. (C) LAMP detection of *P. berghei* oocysts and *D. immitis* L2 larvae in mosquitoes. Agarose-gel electrophoresis of the LAMP-amplified products from the samples in (A) and (B). FITC fluorescence (green) indicates *P. berghei* and Cy5.5 fluorescence (magenta) indicates *D. immitis* DNA. FITC (upper left), Cy5.5 (upper right), FITC and Cy5.5 merged image (left below), and ethidium bromide (right below). Numbers at left indicate the migration of molecular weight markers (bp).

of *P. berghei* oocysts per mosquito ranged from 1 to 974, and the *D. immitis* L2 larvae numbered between 1 and 7 (Fig. 3A and B). Next, the genomic DNA from each mosquito, along with the dissected midgut or malpighian tubules, was extracted and used for LAMP reactions. Notably, even small numbers of parasites, such as 1 *D. immitis* L2 larva or 19 *P. berghei* oocysts, could be detected in a single LAMP reaction tube containing the DNA of either parasite, but both sets of fluorescent dye-conjugated primers (Fig 3C).

The high sensitivity of the combined LAMP reaction, as demonstrated by the detection of a single parasite, was similar to that of individual LAMP reactions, demonstrated previously (Aonuma et al., 2008, 2009). Although the detection limit for *P. berghei* DNA in this study was limited to the equivalent of 50 iRBCs (data not shown), which is higher than in previous reports, infected mosquitoes contain thousands of sporozoites in their salivary glands. Therefore, this method is more than sufficiently sensitive for identifying infectious mosquitoes in the wild.

Our evaluation demonstrated that the LAMP procedure for detecting two different parasite species under the same reaction conditions was applicable for the identification of pathogen-carrying mosquitoes. Recently, several reports have shown that some species of mosquitoes act as vectors for both malarial and filarial parasites, sometimes carrying both pathogens at the same time. For example, *A. gambiae* is a potential vector for both *P. falciparum* and *Wuchereria bancrofti*, and occasionally an individual mosquito carries and transmits both parasites simultaneously (Muturi et al., 2006; Burkot et al., 1990b). We did attempt during this study to detect *P. berghei* and *D. immitis* simultaneously by LAMP in one reaction tube. However, the signals were poor, presumably due to some conflict in the enzymatic reactions (data not shown). We regard this study as a first step in the development of the new method for multiplex LAMP, and further investigation is needed to find the appropriate conditions for the synchronous LAMP reactions. Nonetheless, since the endemic areas for these two diseases largely overlap, the method developed in this study is expected to improve the effectiveness and efficiency of parasite surveys.

In this study, we demonstrated the utility of the multiplex LAMP method using fluorophore-conjugated primers to identify parasites in mosquitoes accurately and specifically. While *P. berghei* and *D. immitis* were used as model parasites in this study, this method should be easily amended for the diagnosis of other arthropod-borne parasitic diseases in humans, such as *P. falciparum, W. bancrofti,* and *Brugia malayi*. This method offers the possibility of a combined survey of multiple infectious diseases in areas where such diseases are co-endemic, thereby reducing the cost and time of diagnosis. Furthermore, the successful identification of infected mosquitoes using multiplex LAMP indicates that this method could provide a more efficient means to analyze the disease status of mosquitoes collected from field surveys.

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Detection of *Trypanosoma cruzi* and *T. rangeli* Infections from *Rhodnius pallescens* Bugs by Loop-Mediated Isothermal Amplification (LAMP)

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Abstract. We have developed two loop-mediated isothermal amplification (LAMP) assays for specific detection of *Trypanosoma cruzi* and *Trypanosoma rangeli* based on the 18S ribosomal RNA (rRNA) and the small nucleolar RNA (snoRNA) genes, respectively. The detection limit of the assays is 100 fg and 1 pg for *T. cruzi* and *T. rangeli*, respectively, with reactions conducted in 60 minutes. The two LAMP assays were used in detection of *T. cruzi* and *T. rangeli* infections in comparison with polymerase chain reaction (PCR) for DNA samples extracted from *Rhodnius pallescens* bugs collected from palm trees in Panama. Out of a total of 52 DNA samples from *R. pallescens* bugs 17 (33%) and 14 (27%) were *T. cruzi*-positive by LAMP and PCR, respectively, while, 7 (13%) and 4 (8%) were *T. rangeli*-positive by LAMP and PCR, respectively. Further evaluation of these LAMP assays is needed, especially with specimens collected from human patients as well as blood kept for transfusion purposes.

INTRODUCTION

Trypanosoma cruzi infects human beings and causes Chagas disease/American trypanosomiasis in Central and South America. It is transmitted by *Rhodnius* and *Triatoma* bugs through contaminated feces,[1,2] and can also be transmitted vertically by blood transfusion or during organ transplantation.[3] The non-pathogenic *Trypanosoma rangeli* shares the same geographical location and same insect vectors with *T. cruzi*,[2,4,5] hence, necessitating accurate differential diagnosis. *Trypanosoma rangeli* is capable of transmission both through feces and through the salivary glands.[6] Diagnosis of American trypanosomiasis relies on serological techniques, primarily using an indirect immunofluorescence assay (IFA) with *T. cruzi* epimastigote forms.[4] Xenodiagnosis is mainly used for direct detection of *T. cruzi* parasites, although sometimes it is not effective in patients with very low parasitaemia,[7] and furthermore, it is time consuming. Polymerase chain reaction (PCR) allows precise identification of the infecting trypanosome species from blood or tissue samples, and detection of trypanosomes in the vectors.[2,5,7] Despite its high specificity and sensitivity, the use of PCR is still not widespread in diagnostic laboratories of endemic areas,[4] mainly because of high costs and the requirement for specialized equipment.[8,9]

The loop-mediated isothermal amplification (LAMP) method is a gene amplification technique that uses 4 or 6 primers that detect DNA of pathogenic organisms with high sensitivity and specificity.[10,11] The major advantages of LAMP include: 1) the reaction is isothermal and detection can be conducted within 60 minutes, 2) it requires simple heating devices such as a water bath or laboratory heat block, and 3) the detection of reaction results can be seen immediately after incubation by the naked eye caused by turbidity occurring in positive amplification reactions[12] or by addition of fluorescent dyes after incubation such as SYBR green, ethidium bromide, or evagreen, which enable detection under UV light.[13] A more convenient loopamp fluorescent detection reagent (FD) (Eiken Chemical Co. Ltd., Tokyo, Japan) has been specifically developed for detection of LAMP products, whereby its addition to the reaction mixture before incubation enables detection of results immediately after incubation by the naked eye under UV without opening the reaction tube.[14] We have previously reported on LAMP primers developed for detection of *T. cruzi* infections,[15] although they were never tested with field samples from infected hosts or vectors. In this study, we have developed LAMP assays for detection of *T. cruzi* and *T. rangeli* by targeting the 18S ribosomal RNA (rRNA) and small nucleolar RNA (snoRNA) genes, respectively, and further used these assays for detection of *T. cruzi* and *T. rangeli* infections in triatomine bugs collected from royal palm trees (*Attalea butyracea*) in Viento Fronco, Chilibre district, Panama.

MATERIALS AND METHODS

Insect collection and DNA extraction. Fifty-two triatomine bugs were collected from royal palm trees (*Attalea butyracea*) growing nearby households in the community of Viento Fronco, Chilibre district, Colón province, Republic of Panamá. The bugs were transported to the laboratory with the permission of the Autoridad Nacional del Medio Ambiente (ANAM) from April to May 2006. In the laboratory the bugs were identified taxonomically and then dissected aseptically, and DNA was extracted from insect extracts (all internal organs of the bug) using the Puregene DNA Purification kit (Gentra Systems Inc., Minneapolis MN) according to the manufacturer's instructions. Briefly, cell lysis solution and proteinase K (100 μg/mL) were added to whole insect extracts. The mixture was pipetted up and down to lyse the cells and then incubated at 55°C overnight. Samples were cooled at room temperature, protein precipitation solution was added, and the mixture was then centrifuged. The DNA was finally precipitated with 100% isopropanol, washed with 70% ethanol, and then hydrated with 50 μL of DNA hydration solution.

For optimization, specificity, and sensitivity of the reactions, the phenol-chloroform method, as described by Sambrook and Russel,[16] was used for DNA extraction of *T. cruzi* (Tulahuan strain) epimastigotes from *in vitro* cultures and *Triatoma infestans* and *Rhodnius prolixus* from pathogen-free colonies of the National Research Center for Protozoan Diseases,

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Obihiro University and uninfected human blood DNA. The *T. rangeli* (Panama strain) DNA was kindly provided by Azael Saldaña, Parasitology Unit, College of Medicine, University of Panamá.

LAMP. Table 1 shows LAMP primer sets for *T. cruzi* and *T. rangeli* parasites targeting the 18S rRNA and the snoRNA-c11 genes, respectively, designed using the LAMP primer explorer software version 4 (http://primerexplorer.jp/e/). The LAMP reactions were performed as previously described by Notomi and others.[11] Briefly, a total volume of 25 μL containing 12.5 μL of 2× LAMP buffer (40 mM Tris-HCl [pH 8.8], 20 mM KCl, 16 mM $MgSO_4$, 20 mM $[NH_4]_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM of each deoxyribonucleotide triphosphates (dNTPs)), 1.3 μL primer mix (5 pmol each of F3 and B3, 40 pmol each of FIP and BIP and 20 pmol each of LF and LB), 8.2 μL distilled water, 1 μL (8 units) *Bst* DNA polymerase (New England Biolabs, Tokyo, Japan), and 2 μL of template DNA. In reactions whereby 1 μL FD was added to enable the detection by the naked eye, the volume of distilled water was adjusted appropriately. The reaction mixture was incubated at 63°C for 60 min using a Loopamp real-time turbidimeter (LA200, Teramecs, Tokyo, Japan).

PCR. The PCR reactions were performed with specific *T. cruzi* primers and *T. rangeli* primers reported previously.[17] The primer sequences are as follows:

For *T. cruzi*, Tcru1: 5′-AAA TAA TGT ACG GGK GAG ATG CAT GA-3′ and Tcru2: 5′-GGT TCG ATT GGG GTT GGT GTA ATA TA-3′ and for *T. rangeli*, TrINT1: 5′-CGC CCA TTC GTT TGT CC-3′ and TrINT2: 5′-TCC AGC GCC ATC ACT GAT C-3′. The PCR mixture (25 μL total volume) contained PCR Buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 2 mM each of the dNTPs, 5 pmol of each primer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Japan). The reaction mixtures were incubated in a PCR thermocycler (Applied Biosystems, Singapore) at 94°C for 10 min (initial denaturation step), and then subjected to 35 cycles consisting of 45 s at 94°C (denaturation step), 1 min at 58°C (annealing step), and 1 min at 72°C (extension), followed by terminal elongation for 7 min at 72°C. The PCR products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide solution for visualization under UV light.

RESULTS AND DISCUSSION

The loopamp real-time turbidimetry device enables observation of primer kinetics. Therefore, at the commencement of the study, LAMP reactions were conducted at 60, 63, 65, and 67°C to determine the optimal reaction temperature for both the *T. cruzi* and *T. rangeli* primer sets. The temperature at which the reaction would reach and cross the positive reaction threshold, which is 0.1 of released turbidity[18] in a short period of time or faster than others, was selected as the optimal reaction temperature (these reactions were done in five repetitions). As a result, the 63°C temperature was chosen as the optimal reaction temperature because the reaction threshold time for positive reactions was achieved faster at this temperature (data not shown). The *T. cruzi* 18S LAMP assay specifically amplified *T. cruzi* DNA without amplifying negative control DNA of *T. rangeli*, vector insects, and human host (Figure 1A). The detection limit of the assay was 100 fg of serially diluted *T. cruzi* DNA (Figure 1B and C). The *T. rangeli* snoRNA LAMP assay also specifically amplified *T. rangeli* DNA without amplifying the negative control DNA (Figure 2A) with a detection limit of 1 pg as determined from serially diluted DNA (Figure 2B and C). In this study, six LAMP primers were used for amplification of each target trypanosome DNA. In this way eight distinct regions were recognized on the target gene, thereby ensuring specificity, high sensitivity, and rapid reaction whereby amplification is achieved within 60 minutes.[10]

Rhodnius pallescens is considered to be the most important and widespread vector of *T. cruzi* and *T. rangeli* in Panama.[19] In the current study, we therefore evaluated detection performance of the newly developed *T. cruzi* and *T. rangeli* LAMP assays on DNA extracted from triatomine bugs (37 nymphae and 15 adults) collected from palm trees in Panama. Of the 37 DNA samples extracted from *R. pallescens* nymphae, 10 (27%) and 7 (19%) were *T. cruzi*-positive by LAMP and PCR, respectively. Of the 15 adult *R. pallescens* DNA samples, 7 (47%) were positive for *T. cruzi* infections by both LAMP and PCR assays (Table 2). Therefore, out of a total of 52 DNA samples from *R. pallescens*, 17 (33%) and 14 (27%) were *T. cruzi*-positive by LAMP and PCR, respectively.

However, for *T. rangeli*, 3/37 (8%) were positively detected by LAMP from *R. pallescens* nymphae DNA, while none were positive by PCR. Of the 15 adult *R. pallescens* DNA samples, 4/15 (27%) were positively detected for *T. rangeli* infections by both LAMP and PCR (Table 2). Therefore, out of a total of 52 DNA samples from *R. pallescens*, 7 (13%) and 4 (8%) were *T. rangeli*-positive by LAMP and PCR, respectively. The LAMP assays developed in this study have shown a slightly higher detection performance than PCR. This is in agreement with previous studies where LAMP and PCR were compared

TABLE 1
LAMP primer sets used in this study

Species	Accession no.	Size of target	Gene	Primer sequence
Trypanosoma cruzi	AF301912	187 bp	FIP:	5′-CGTGAGTTGAGGGAAGGCATGAGTTGTTGGCAGACTTCGGT-3′
			BIP:	5′-GCATCCAGGAATGAAGGAGGGTTCGTCTTGGTGCGGTCTA-3′
			F3:	5′-CCGTGTGGCACTGTTTGT-3′
			B3:	5′-TGAAGAATGCCTTCGCTGT-3′
			LF:	5′-CATGTGAGATGCGAAGGG-3′
			LB:	5′-CATGTGAGATGCGAAGGG-3′
Trypanosoma rangeli	AY028385	172 bp	FIP:	5′-TCATGCGTCGCAGCCGTACGCGAGAACGGGAGCA-3′
			BIP:	5′-TTGCAGTTTCCTGTCAGCCTGACGTTTCAGTGTGAGCTGAGT-3′
			F3:	5′-CGAGGACGGGCGAGAA-3′
			B3:	5′-AAAAGGGGGGAAAGCAAGT-3′
			LF:	5′-CCCGCCTTCTTCGCTCT-3′
			LB:	5′-GCGCGTGACGACACAAC-3′

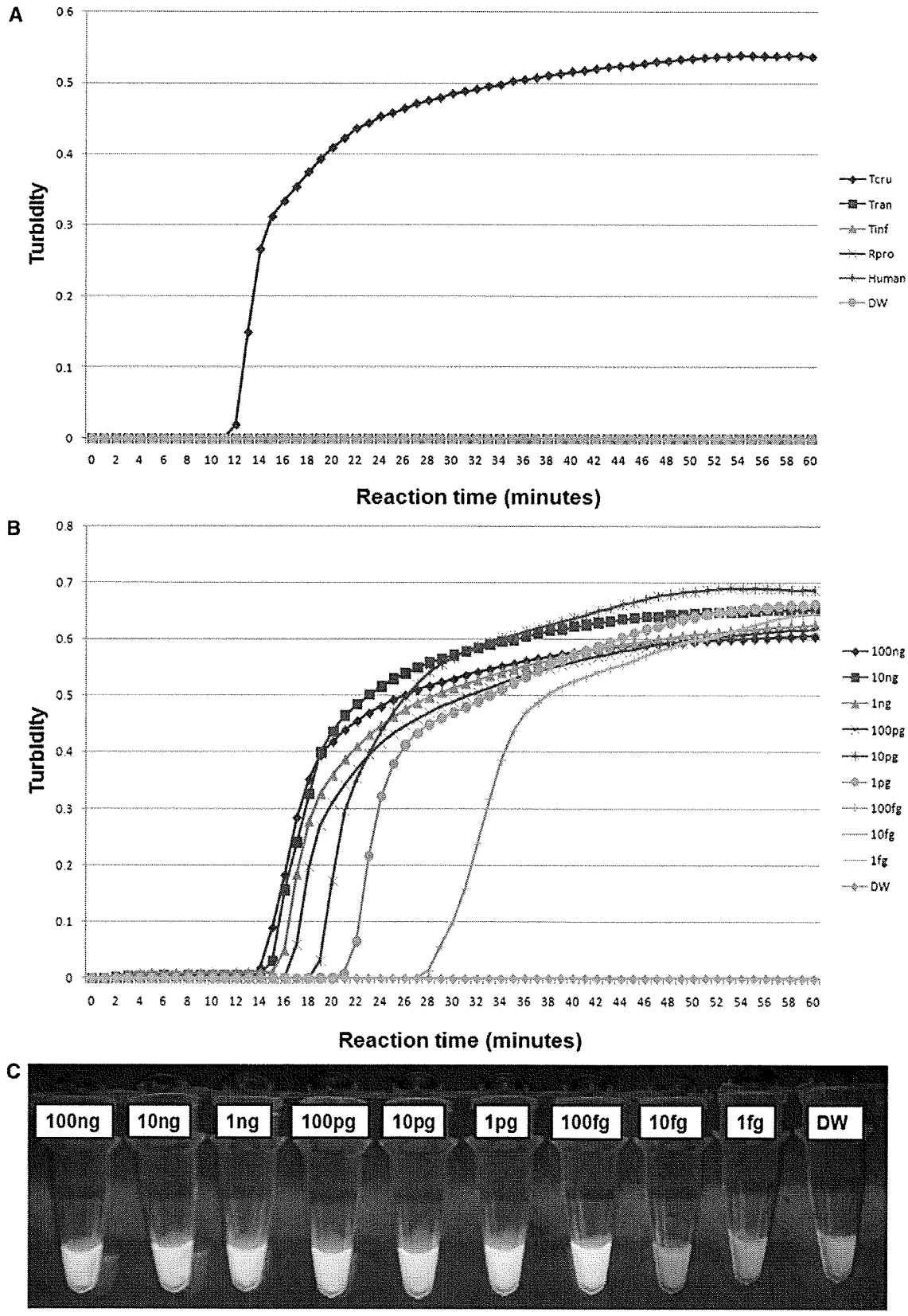

FIGURE 1. Loop-mediated isothermal amplification (LAMP) reactions with the 18S rRNA primer set for amplification of *Trypanosoma cruzi* DNA. Standard positive reaction threshold is 0.1 of the value of the turbidity released. (**A**) Specificity test of the LAMP assay using the real-time turbidimetry device. Tcru – *T. cruzi*; Tran – *Trypanosoma rangeli*; Tinf – *Triatoma infestans*; Rpro – *Rhodnius prolixus*; Human – DNA extracted from uninfected human blood; and DW – distilled water used as non-DNA negative control. Sensitivity test on serially diluted *T. cruzi* DNA from 100 ng down to 1 fg: (**B**) detection using the real-time turbidimetry device, (**C**) detection under UV light by the naked eye using FD reagent. The green/bright fluorescence indicates a positive reaction and the dark/less fluorescent color indicates a negative reaction. This figure appears in color at www.ajtmh.org.

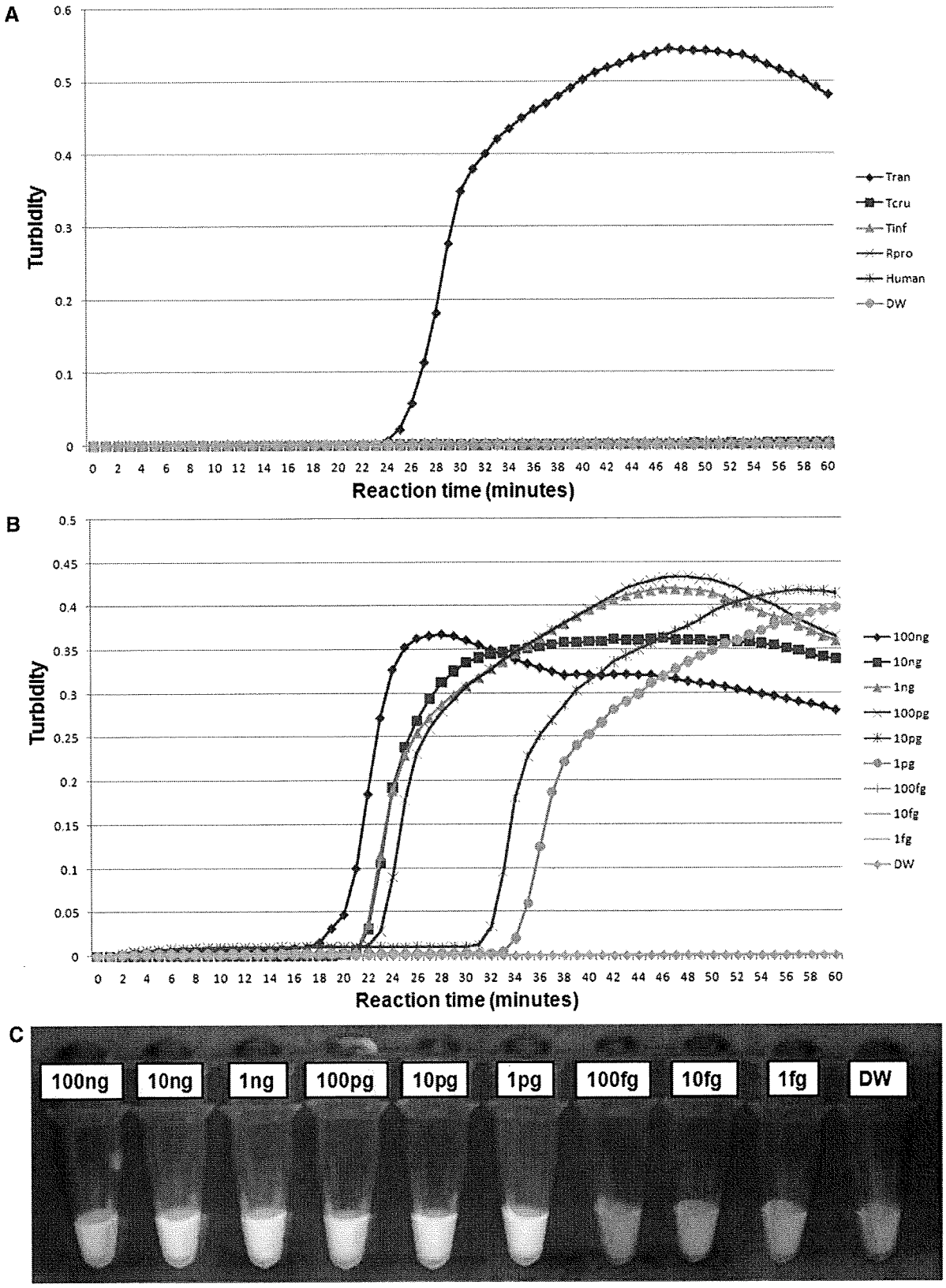

FIGURE 2. Loop-mediated isothermal amplification (LAMP) reactions with the snoRNA primer set for amplification of *Trypanosoma rangeli* DNA. Standard positive reaction threshold is 0.1 of the value of the turbidity released. (**A**) Specificity test of LAMP assay using the real-time turbidimetry device. Tran – *T. rangeli*; Tcru – *Trypanosoma cruzi*; Tinf – *Triatoma infestans*; Rpro – *Rhodnius prolixus*; Human – DNA extracted from uninfected human blood; and DW – distilled water used as non-DNA negative control. Sensitivity test on serially diluted *T. rangeli* DNA from 100 ng down to 1 fg: (**B**) detection using the real-time turbidimetry device, (**C**) detection under UV light by the naked eye using FD reagent. The green/bright fluorescence indicates a positive reaction and the dark/less fluorescent color indicates a negative reaction. This figure appears in color at www.ajtmh.org.

TABLE 2

Detection of *Trypanosoma rangeli* and *Trypanosoma cruzi* infections from triatomine bugs

Insect stage	Total no. of samples	*T. rangeli*		*T. cruzi*	
		PCR	LAMP	PCR	LAMP
		+ve*	+ve	+ve	+ve
Nymph	37	0 (0%)	3 (8%)	7† (19%)	10† (27%)
Adult	15	4‡ (27%)	4‡ (27%)	7‡ (47%)	7‡ (47%)
Total	52	4 (8%)	7 (13%)	14 (27%)	17 (33%)

*Positive detection.

†Both loop-mediated isothermal amplification (LAMP) and polymerase chain reaction (PCR) positively detected *T. cruzi* from same seven samples, whereas 3 samples were positive by LAMP only.

‡Four samples were detected as *T. cruzi* and *T. rangeli* mixed infections by both LAMP and PCR.

for detection of salivarian trypanosome infections.[8,20–22] Mixed infections were detected from four adult triatomine bug samples by both LAMP and PCR. This highlighted the importance of a species-specific assay for each trypanosome species.

In this study, we present LAMP assays based on 18S rRNA and snoRNA genes for detecting and differentiation of *T. cruzi* and *T. rangeli* infections. The *Bst* DNA polymerase used in the LAMP reaction is not affected by blood and tissue-derived components such as myoglobin, heme-blood protein complexes, and immunoglobin G.[8,23,24] This gives LAMP an advantage of greater detection efficiency in comparison to PCR for field-derived samples. Furthermore, the FD reagent that is added to the reaction tube before incubation enables detection of LAMP results by the naked eye immediately after the reaction without opening the reaction tube, thereby reducing the risk of contamination. This study brings LAMP to the fore as a possible alternative molecular diagnostic tool for confirmation of the presence of *T. cruzi* and *T. rangeli* infections in vectors, clinical samples, transfusion blood samples, and during organ transplantation.

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Immunogenicity and growth inhibitory efficacy of the prime–boost immunization regime with DNA followed by recombinant vaccinia virus carrying the P29 gene of *Babesia gibsoni* in dogs

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ABSTRACT

In recent studies, heterologous prime–boost approaches, employing plasmid DNA and viral vector pathogen-delivering sequences, have been considered an effective protection strategy for intracellular parasite infections. Here, we evaluated the efficacy of such a strategy against the canine *Babesia gibsoni* infection. The DNA (pCAGGS-P29) and recombinant vaccinia virus (vvP29) both encoding the P29 of *B. gibsoni* were used in this study. The dogs were immunized 3 times with priming DNA and boosted once with recombinant virus. The dogs immunized with P29 developed a significant level of IgG2 antibody against P29. The response was strongly boosted by the inoculation of vvP29. The peripheral IFN-gamma responses of the dogs immunized with P29 were significantly higher than those of controls after the parasite inoculation. Moreover, the P29 immunized group showed a significantly low level of parasitemia. In conclusion, this study supports the efficacy of a prime–boost strategy for dogs against canine *B. gibsoni* infection.

1. Introduction

Babesia gibsoni is an intra-erythrocytic, tick-transmitted protozoan that can cause clinical babesiosis in dogs. Infection is endemic in Asia, Africa, Europe, the Middle East and North America. Canine *B.* gibsoni infection is characterized by remittent fever, thrombocytopenia, sever anemia, and sometimes death (Boozer and Macintire, 2003; MacWilliams, 1987). In natural or experimental *B. gibsoni* infection, 60% of dogs are recovered from the acute stage and then shift to the chronic stage. In chronically infected dogs, the *B. gibsoni* parasite can be maintained for several years, and the dogs became a reservoir of the parasites for the next generation. Furthermore, it is known that a chronically infected bitch transmits the parasite in the uterus, which causes a fatal infection in her pups (Fukumoto et al., 2005a). For the reasons, the disease is frequently present in dogs and has recently become a serious clinical problem.

For the control and alleviation of *B. gibsoni* infection in dogs, vaccination is generally considered to be the most effective means. A traditional vaccine development study targeted the induction of the humoral immune response by the immunization of inactivated pathogens (Brown and Palmer, 1999). However, in the case of protection against intracellular parasites, the induction of the both humoral and cell-mediated immune response is considered to be important (Brown and Palmer, 1999; Tsuji and Zavala, 2003). Thus, the optimal immunization methods for inducing such type of response are required for the study. In recent years, it was reported that the heterologous prime–boost immunization regime with priming DNA followed by recombinant virus both expressing the same antigen has been shown to effectively trigger an immune response against several infectious intracellular pathogens (Amara et al., 2001; Gilbert et al., 2002; Hanke et al., 1998).

In our previous study, we identified a P29 gene expressed on *B. gibsoni* merozoites (Fukumoto et al., 2003). The P29 was considered to play roles at the parasite invasion of the host erythrocyte by the maintenance of physical strength. We also showed that the P29 was recognized as the immunodominant antigen of the dog infected with *B. gibsoni*. In this study, we determined the immunogenicity and growth inhibitory effect of heterologous immunization with priming DNA–boosting recombinant vaccinia virus, both carrying the P29 gene of *B. gibsoni*.

2. Materials and methods

2.1. Parasite

The NRCPD strain of *B. gibsoni* parasite (Fukumoto et al., 2000; Ishimine et al., 1978) was used in this study. The *B. gibsoni*-infected erythrocytes for challenges were collected from a dog experimentally infected with *B. gibsoni*.

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2.2. Construction of plasmid expressing the P29 gene

The entire P29 gene (Fukumoto et al., 2003) was inserted into the EcoRI restriction enzyme site under the control of the CAG promoter of the mammalian expression vector pCAGGS (Niwa et al., 1991; Tokui et al., 1997), designated pCAGGS-P29. pCAGGS-P29 was amplified in a DH5a strain of *Escherichia coli*, and the purification was performed using the QIAGEN Plasmid Mega Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The expression of the P29 was analyzed using rabbit kidney 13 (RK13) cells in vitro prior to an in vivo trial with dogs. RK13 cells were transfected with pCAGGS-P29 using a lipofectine reagent (Gibco BRL, Rockville, MD) by the standard method. The expression of the P29 was analyzed by the immunofluorescence antibody test (IFAT using an anti-P29 monoclonal antibody 2 days after transfection.

2.3. Construction of the recombinant vaccinia virus expressing the P29 gene

The entire P29 gene was inserted into the SalI site of the vaccinia virus transfer vector pAK8 (Yasuda et al., 1990). RK13 cells infected with the parent vaccinia virus LC16mO (mO) (Yasuda et al., 1990) strain were transfected with the pAK8-P29 using a lipofectine reagent (Gibco). Thymidine kinase-negative (TK−) viruses were isolated by a plaque assay of 143TK− cells in the presence of 5-bromo-2′-deoxyuridine at a concentration of 100 μg/ml (Yasuda et al., 1990). The plaque assay was done 3 times to clone the recombinant virus. The recombinant vaccinia virus expressing P29 (vvP29) was propagated in RK13 cells in Eagle's minimum essential medium supplemented with 7.5% fetal bovine serum (FBS). To analyze the expression of P29 in vitro, RK13 cells were inoculated with 5 plaque-forming units (PFU) of vvP29 or mO per cell. Two days after inoculation, the cells were harvested and then subjected to IFAT or Western blotting as described above.

2.4. Immunization of dogs

Purebred female specific pathogen-free beagle dogs (14–15 months) were used in this study. All dogs were purchased from Chugai medical animal institute (Nagano, Japan). All dogs were physically examined by the veterinarian of the Obihiro University of agriculture and veterinary medicine and received the routine vaccination including canine parvovirus, canine adenovirus (types 1 and 2) and distemper. Nine dogs were randomly divided into three groups ($n = 3$). The P29 immunized group received pCAGGS-P29 and vvP29. The control immunized group received pCAGGS empty plasmid and the parent vaccinia virus mO strain. The remaining group received no immunization treatment. The immunization regime used DNA priming three times and a vaccinia virus boosting once because this immunization schedule showed most effective result for the induction of a strong immune response against malaria infection than other vaccination regimes in humans (Dunachie and Hill, 2003). For the plasmid DNA immunization, dogs were injected intramusculary (IM) in the quadriceps muscle with a 1-ml syringe and a 21G needle. Each single dose consisted of 200 μg of DNA dissolved in 1 ml of PBS containing 25%(w/v) sucrose. The dogs were immunized three times at two-week intervals. Two weeks after the final DNA immunization, the dogs were boosted with 5×10^8 PFU of the recombinant vaccinia virus vvP29 or mO intravenously (IV). After the DNA or recombinant vaccinia virus immunization, dogs were examined every day for 14 days by a veterinarian and no side effect were observed.

2.5. Determination of antibody responses against P29 by enzyme-linked immunosorbent assay (ELISA)

The antibody responses of the immunized dogs were measured using the ELISA with GST-P29 as described in our previous paper (Fukumoto et al., 2003). The total IgG, IgG1, and IgG2 subclass antibody responses against P29 were analyzed. All serum samples were used in 1:200 dilutions for ELISA. All HRP-conjugated secondary antibodies were purchased from Bethyl laboratory (Montgomery, TX).

2.6. Determination of the total IgE response by ELISA

Total IgE of the immunized dogs were measured using a capture ELISA (Dog IgE ELISA Quantitation kit, Bethyl laboratories) to analyze the allergic reaction caused by the immunization of DNA and recombinant vaccinia virus. The ELISA was performed following the manufacture's instructions. The sera collected at pre-immunization and 2 weeks after the each immunization (day −56, −42, −28 and 0 of Fig. 4) were used for analysis. All serum samples were used in 1:200 dilutions. The concentration of total IgE was calculated from the standards (ranges: 10,000–7.8 ng/ml).

2.7. Parasite growth inhibition assay in dogs

Two weeks after the booster immunization, dogs were i.v. infected with 2×10^8 of *B. gibsoni*-infected RBCs collected from a dog experimentally infected with the *B. gibsoni* parasite (NRCPD strain). Parasitemia in peripheral blood, packed cell volume (PCV), RBC number, and hemoglobin concentration were monitored at one-day intervals.

2.8. Peripheral IFN-gamma response

The serum samples collected at days 0 and 8 after parasite inoculation were used for the assay. The samples were kept at −80 °C until use. IFN-gamma was measured using a capture ELISA (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

2.9. Statistical analysis

The parasitemia and antibody responses in the immunized dogs were statistically analyzed by the Student's *t*-test.

2.10. Animal experiment

All animal experiments in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

3. Results

3.1. Expression of the P29 in vitro by pCAGGS-P29

To investigate whether the pCAGGS-P29 plasmid expressed P29, we transfected the plasmid into RK13 cells and analyzed it by Western blotting and IFAT. In the Western blotting, the anti-P29 MAb specifically recognized a 29 kDa band of RK13 cells transfected with pCAGGS-P29 and not with empty plasmid pCAGGS. The molecular weight of P29 expressed by pCAGGS-P29 was similar to that of the native P29 from *B. gibsoni*. In the IFAT, the MAb specifically reacted to RK13 cells transfected with pCAGGS-P29 (Fig. 1).

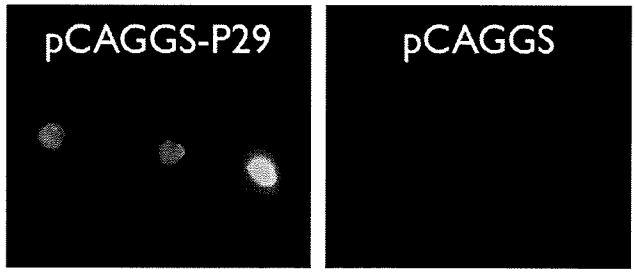

Fig. 1. Expression of the P29 in RK13 cells transfected with pCAGGS-P29. Expression of the P29 was analyzed by IFAT using anti-P29 monoclonal antibody. pCAGGS-P29, cells transfected with pCAGGS-P29; pCAGGS, cells transfected with control plasmid pCAGGS.

3.2. Expression of the P29 in vitro by the vvP29

RK13 cells were infected at 5 PFU/cell with a vvP29 or with a parent vaccinia virus mO. After incubation for 2 days, the cells were harvested and analyzed by Western blotting or IFAT using anti-P29 MAb. In Western blotting, a specific 29 kDa band was detected in the cells infected with vvP29 and not in mO (Fig. 2A). In IFAT, the anti-P29 antibody reacted specifically to the cells infected with vvP29 (Fig. 2B).

3.3. Antibody responses

To determine the profile of the immune response of the dogs immunized with the heterologous regime, the IgG response and its subclass were analyzed (Fig. 3). The total IgG responses against P29 of the P29 immunized dogs were under detectable level by each DNA immunization. However, two weeks after the booster immunization with vvP29, the antibody response was significantly increased (Fig. 3A, day −14 vs. day 0). The IgG subclass against P29 was also analyzed. The IgG1 antibody response maintained a low level (Fig. 3B). The IgG1 response of the dogs immunized with P29 did not show significance compared to that of the control groups. In contrast, the IgG2 antibody was significantly increased after the booster immunization with vvP29 (Fig. 3C, day −14 vs. day 0). When we compared IgG1 and IgG2, IgG2 was detected as major subclass all through the experimental period (Fig. 3B and C). The IgG2 response of the P29 group showed a significantly higher level (days 0–8, 16, 28, and 36) when compared to those of the control groups (Fig. 3C).

3.4. Allergic reaction of the immunized dogs

To determine the allergic reaction of the immunized dogs, serum total IgE concentration of immunized period (day −56 to 0 of fig. 3) was analyzed. All serum samples showed the concentration of less than 7.8 ng/ml (under detectable level) of serum total IgE responses (figure not shown).

3.5. Peripheral IFN-gamma response

The peripheral IFN-gamma response of the dogs after the challenge infection was analyzed by the capture ELISA. As shown in Table 1, at day 0 post-infection, all dog groups showed an undetectable level of IFN-gamma production. At 8 days post-infection, only the dog group immunized with P29 showed a significant level of the IFN-gamma response not detected in the control groups.

3.6. Inhibition of the parasite growth in the dogs

To determine the protective effect of the immunization with P29 by the heterologous prime–boost regime, the dogs were inoculated with *B. gibsoni* parasite on day 0 and parasitemia was monitored. The parasitemia was significantly inhibited (day 20) in the

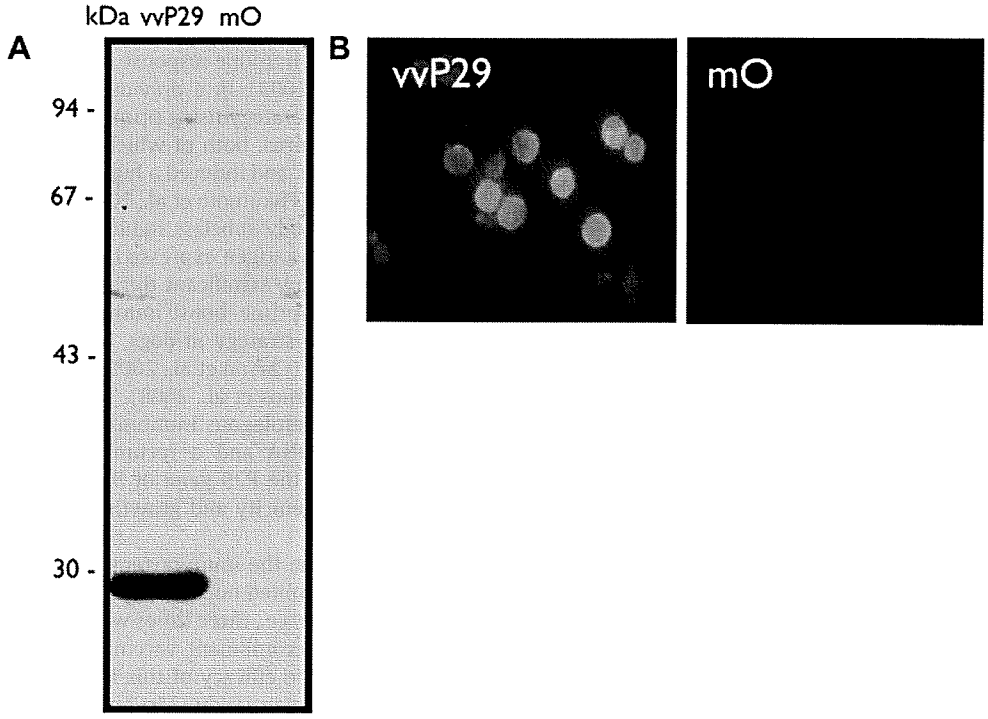

Fig. 2. Expression of the P29 in RK13 cells infected with vvP29. (A) Western blot analysis of the P29 expressed in RK13 cells. The expression of the P29 was detected using an anti-P29 monoclonal antibody. vvP29, lysates of recombinant vaccinia virus vvP29-infected cells; lane 2, control lysates of vaccinia virus mO-infected cells. (B) IFAT analysis of the P29 expressed in RK13 cells. The cells were stained with anti-P29 monoclonal antibody. vvP29, cells infected with the vvP29; mO, cells infected with the control parent virus mO.

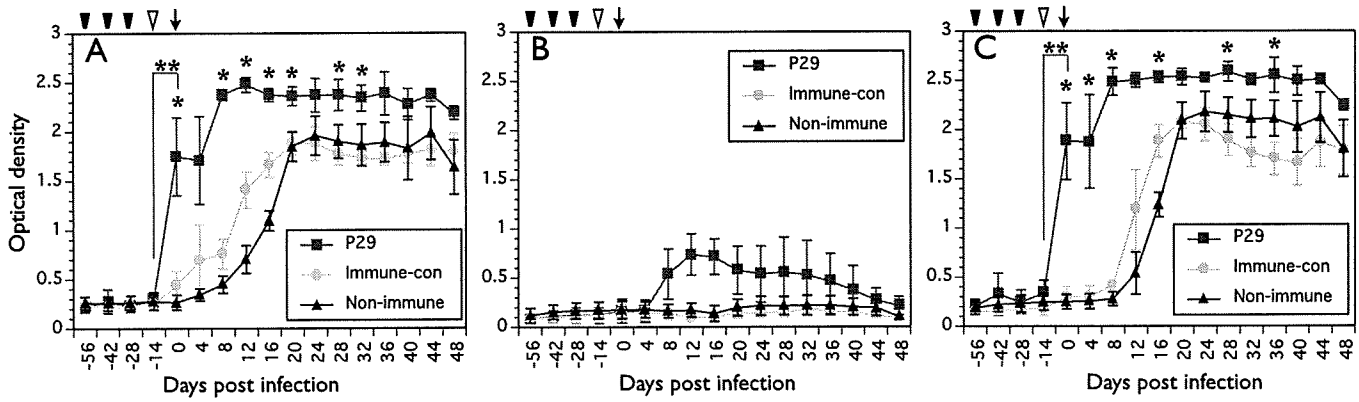

Fig. 3. Determination of the antibody response against P29 of dogs immunized with P29. Antibody response against each immunization was monitored at two weeks after each immunization. (A) Total IgG, (B) IgG1, and (C) IgG2. P29, sera collected from dogs immunized with pCAGGS-P29 and vvP29; immune-con, sera collected from dogs immunized with control plasmid pCAGGS and mO; non-immune, sera collected from non-immunized control dogs. The day at challenge infection of the parasites was designated as day 0 (solid arrow). The dogs were immunized with DNA at day −56, −42 and −28 (solid arrowhead), and immunized with vaccinia virus at day −14 (white arrow head). The asterisks (*) on the error bar show the significant difference ($P < 0.05$) between a dog group immunized with P29 and the control groups. The double asterisks (**) show the significant difference between day −14 and day 0. The results are shown as the mean values, and the error bars represent the standard deviations.

Table 1
Peripheral IFN-gamma responses of the immunized dogs after the *B. gibsoni* parasites inoculation.

Dog group ($n = 3$)	IFN-gamma response (pg/ml)	
	Day 0	Day 8
P29	UD[a]	64.64 ± 17.43[b]
Immune-con	UD	UD
Non-immune	UD	UD

[a] UD, under detectable level.
[b] Results was shown in mean ± standard deviation.

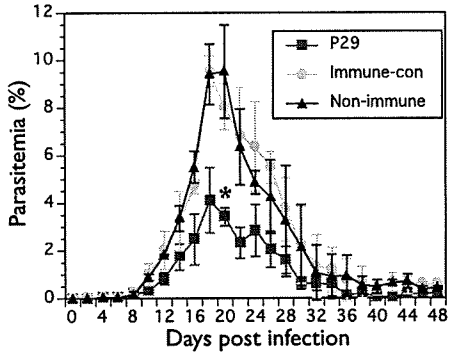

Fig. 4. Parasitemia of dogs after challenge infection with *B. gibsoni*-infected RBCs. P29, dogs immunized with pCAGGS-P29 and vvP29; immune-con, dogs immunized with control plasmid pCAGGS and mO; non-immune, non-immunized control dogs. The results are shown as the mean values, and the error bars represent the standard deviations. The asterisks show the significant difference ($P < 0.05$) between a dog group immunized with P29 and the control groups.

dog group immunized with P29 when compared to both of the control groups. There was no significant difference in the two control groups. At the peak of parasitemia, the ratio of the inhibitory effect was 54.5% compared to that of the immunized control group and 56.0% compared to that of the non-immunized group. There was no significant difference in clinical symptoms manifested as severe anemia (Fig. 5).

4. Discussion

This study demonstrates the immunogenicity and efficacy of a heterologous prime–boost immunization with priming DNA followed by recombinant vaccinia virus and the potential use of P29 as an immunogen against *B. gibsoni* infection.

For protection against animal babesiosis, the induction of an immune response, such as opsonizing IgG2 antibody and macrophages activated by the IFN-gamma produced by CD4 T cells, is considered to be important (Brown and Palmer, 1999). Individual immunization of the DNA (Bout et al., 2002; Kumar et al., 2002) or recombinant virus is known to induce immune responses of this type (Bender et al., 1996; Bennink et al., 1984). However, the inductivity of an immune response by individual immunization with DNA or a recombinant virus is limited and could induce moderate responses in mammals (Roy et al., 2000; Schneider et al., 1999, 2001; Swain et al., 2000), including dogs (Ramiro et al., 2003). To overcome these problems, in recent studies, it was shown that the combination of a heterologous prime–boost immunization with DNA followed by a recombinant vaccinia virus could induce a strong immune response in mammals (Amara et al., 2001; Ramiro et al., 2003). In a previous study, we demonstrated this immunization strategy using priming DNA followed by a recombinant vaccinia virus, both of which express a cell surface antigen P50 of *B. gibsoni*, and induced a high IgG2/IgG1 ratio of immune response in dogs (Fukumoto et al., 2007). On the other hand, the IgG1 and IgG2 antibody subclass induced by the immunization of recombinant P50 antigen expressed in insect cells with an adjuvant did not show any significant difference (Fukumoto et al., 2005b). Therefore, this prime–boost immunization regime also seems to be an effective strategy in dogs, but information regarding dogs is quite limited (Carson et al., 2009; Fukumoto et al., 2007; Ramiro et al., 2003; Ramos et al., 2008). To further demonstrate the efficacy of the heterologous immunization regime for dogs, we constructed the DNA and a recombinant vaccinia virus, both of which express the P29 gene of *B. gibsoni*, and we demonstrated their immunogenicity and growth inhibitory effects on *B. gibsoni* parasites.

To determine the immunogenicity of the heterologous regime of P29 with a limited number of dogs, we selected only one immunization regime, i.e., three times with priming DNA and a one-time boost with a recombinant vaccinia virus, both of which express P29, because the efficacy of this kind of regime had been shown in previous human study (McConkey et al., 2003), although dog groups immunized with DNA or vaccinia virus only was not determined. We analyzed the IgG response and the peripheral IFN-gamma as the marker for an immune response. The specific IgG against P29 was not detected in dogs (day −56 to −14) by the several

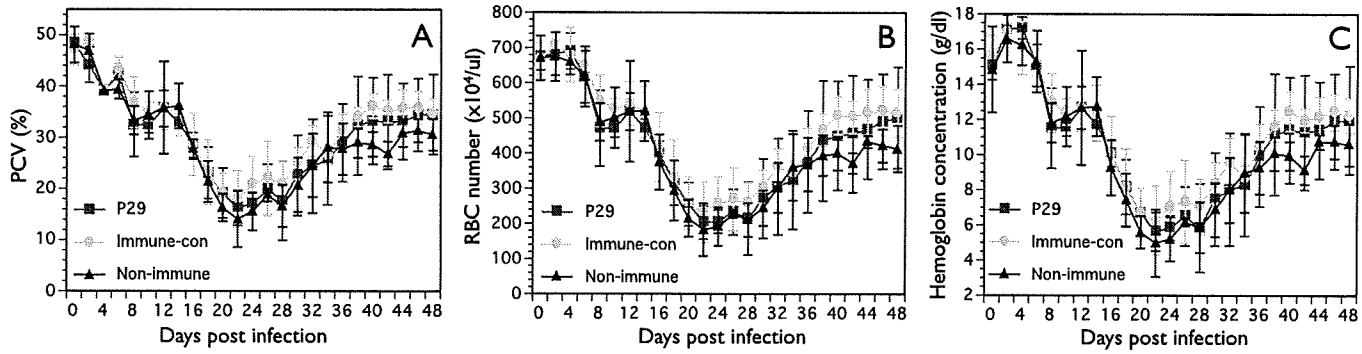

Fig. 5. Hematological parameters of the dogs after challenge infection with *B. gibsoni*-infected RBCs. (A) Packed cell volume (PCV), (B) RBC number, and (C) hemoglobin concentration. P29, dogs immunized with pCAGGS-P29 and vvP29; immune-con, dogs immunized with control plasmid pCAGGS and mO; non-immune, non-immunized control dogs. The results are shown as the mean values, and the error bars represent the standard deviations. Any significant difference was not observed between a dog group immunized with P29 and the control groups.

immunizations with DNA expressing P29. However, the specific IgG increased significantly (day −14 vs. day 0) with the booster immunization with the recombinant vaccinia virus vvP29. After the challenge infection of the *B. gibsoni* parasites, the IgG response against P29 in the dog group immunized with P29 significantly increased (day 8), and the IgG response maintained a significantly higher level throughout the experimental period than that in the control groups (days 8–48). To identify the immune response, we analyzed the IgG subclass against P29. The IgG2 subclass was detected as the major antibody subclass. Regarding the peripheral IFN-gamma response after the challenge infection with *B. gibsoni*, only the dog group immunized with P29 showed a detectable IFN-gamma response. These results suggested that the boosting effect of this type of immunization regime might be useful for the induction of IFN-gamma-producing $CD4^+$ T cell immune response for dogs.

To confirm the growth inhibitory effect of the heterologous prime–boost immunization with P29, a *B. gibsoni* parasite was inoculated 2 weeks after the vvP29 booster immunization. The peripheral parasitemia was monitored at one-day intervals. The parasite growth in the dog group immunized with P29 was significantly inhibited when compared to that of the control groups. The growth inhibitory rate of this parasite was quite similar to that in our previous study using the P50 gene as a target, and it did not show any significant difference between the P29 and P50 immunization experiments. In our previous study, P29 was suggested as an intracellular component (Fukumoto et al., 2003), and P50 was expressed on the parasite cell surface as a type-I transmembrane protein (Fukumoto et al., 2001). It was not clear how P29 interacted with the host and had a similarly protective action with P50; these results indicate that the activation of $CD4^+$ T cells and macrophage might be related to the inhibition of the parasite growth of P29, which could be a candidate antigen for further study of the control of canine *B. gibsoni* infection. However, individual antigen usage of P29 or P50 showed a limited growth inhibitory effect against the parasite and did not protect animals from clinical symptoms manifested as severe anemia.

These results indicated that further study of the detailed pathogenesis of this disease and the search for a more effective antigenic gene would be of value. In addition, the combined usage of multiple gene immunization in a prime–boost regime would be necessary for the development of an effective vaccine controlling canine *B. gibsoni* infection.

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