genome on the PlasmoDB (Version 11.0) website (http://plasmodb.org/plasmo/). To elucidate the involvement of these MCTs in lactate influx/efflux, we investigated the expression levels of the two genes by quantitative real-time PCR. The results exhibited that these MCT genes were differentially expressed at various developmental stages of the parasites (Supplementary Fig. 3). It is noteworthy that the expression levels of these genes are significantly enhanced at the trophozoite stage by lactate supplementation. This finding together with the fact that trophozoite-stage parasites exhibit the largest lactate production allows us to infer that these MCTs might be associated with lactate transport, probably functioning as an efflux pump even though further experiments are needed to address this point. Lactate dehydrogenase (LDH) is a final enzyme in glycolysis, converting pyruvate to lactate [13]. When lactate is present at a high concentration, LDH exhibits feedback inhibition and the conversion rate of pyruvate to lactate is decreased. Here we found that externally supplied lactate inhibited parasite growth *in vitro*. In this context, it is a logically acceptable notion that the excess amount of lactate may inhibit LDH activity, resulting in the retardation of parasite development. This assumption is supported by the fact that the expression levels of two MCT genes, putative lactate transporters, were enhanced by lactate supplementation at the trophozoite stage.

In the present study, we demonstrated that lactate is a critical factor for parasite development. It is therefore likely that a study focusing on the lactate transporter would accelerate the development of antimalarial drugs. To apply the lactate transporter to research as a drug target for controlling malaria, further biochemical analysis of the transporter such as MCTs is required. Similarly, it is very important to reveal the molecular mechanism by which lactate retards the development of the parasite.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2014.08.003.

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Succinctus

Evaluating experimental cerebral malaria using oxidative stress indicator OKD48 mice

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ABSTRACT

Cerebral malaria is a fatal complication of malaria. Conventional methods for evaluating experimental cerebral malaria have several drawbacks. Therefore, we aimed to develop an easy-to-use method for evaluating experimental cerebral malaria using OKD48 (Keap1-dependent Oxidative stress Detector, No-48-luciferase) mice to evaluate oxidative stress. OKD48 mice infected with *Plasmodium berghei* ANKA strain (PbA) suffered from experimental cerebral malaria and oxidative stress was successfully detected in the brains of living OKD48 mice developing experimental cerebral malaria. Oxidative stress in the brain was dependent on the development of experimental cerebral malaria, as prevention of experimental cerebral malaria did not elicit oxidative stress. We provide a novel evaluation method for experimental cerebral malaria using oxidative stress indicator OKD48 mice.

Malaria is an important infectious disease that causes approximately 200 million cases and 600,000 deaths annually. *Plasmodium falciparum*, one of five human parasite species, represents severe malaria causing fatal complications such as cerebral malaria (CM) and respiratory distress (non-cardiogenic pulmonary oedema). Pathological consequences of CM have been evaluated in animal models. Infection of C57BL/6 (B6) mice with a rodent malaria parasite, *Plasmodium berghei* ANKA strain (PbA), allows the development of neurological symptoms leading to death. This experimental system is widely used as a model of experimental CM (ECM) (Thumwood et al., 1988; Ishida et al., 2010).

Pathology in ECM is due to activation of the immune system, resulting in the sequestration of white blood cells (WBCs) on blood vessels in the brain, as well as causing the destruction of blood–brain barriers (BBBs) often associated with haemorrhage (Amante et al., 2010; Baptista et al., 2010). In general, ECM is evaluated by visually scoring neurological symptoms, observing destruction of BBBs and by microscopically observing sequestration and haemorrhage in histopathological brain sections (Thumwood et al., 1988; Ishida et al., 2010). However, these methods have several disadvantages. For instance, although clinical scoring by a SHIRPA protocol (Rogers et al., 1997; Lackner et al., 2006) which comprises a battery of 40 tests that provide a behavioural and functional profile by observational assessment, can be performed while mice are alive, it is time consuming. The histopathological analysis and observing BBB destruction are also time consuming and require complicated postmortem examinations. Therefore, we intended to develop a novel, easy-to-use method for evaluating ECM.

Oxidative stress plays a role in several biological phenomena (Sies, 1991), and is also important in the pathology of malaria (Becker et al., 2004; Percario et al., 2012). Previous reports have shown that oxidative stress is induced in the CNS of mice developing ECM (Reis et al., 2010; Ong et al., 2013). Recently, we succeeded in developing transgenic OKD48 (Keap1-dependent Oxidative stress Detector, No-48-luciferase) mice, which enabled us to evaluate oxidative stress in vivo (Oikawa et al., 2012). These mice have a transgene encoding a modified NF-E2-related factor 2 (Nrf2), an essential transcription factor for expression of anti-oxidative stress genes (Itoh et al., 1997). The Nrf2 modifications introduced to our OKD48 construct were as follows: (i) the DNA binding element was replaced by a luciferase gene, and (ii) three copies of antioxidant responsive element (ARE) were inserted in the 5′ region. Under normal physiological conditions, Nrf2 is associated with kelch-like ECH associating protein 1 (keap1) (Cullinan et al., 2004), resulting in ubiquitination and subsequent degradation by proteasomes.

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Alternately, a switch to oxidative conditions dissociates keap1 from Nrf2, which stabilises this transcriptional factor. Then, Nrf2 enters into the nucleus and binds to an ARE, resulting in transcriptional induction of antioxidant genes. In OKD48 mice, Nrf2-luciferase is also expressed, allowing oxidative stresses to be visualised as bioluminescence when luciferin is injected.

In this work, we successfully detected oxidative stress in the brains of living OKD48 mice that developed ECM with neurological symptoms following infection with PbA. This oxidative stress in the brain was highly specific for development of ECM. Our results propose a novel method for simple and quantitative evaluation of ECM using live animals.

C57BL/6J (B6) mice purchased from SLC (Hamamatsu, Japan) and B6 background OKD48 mice purchased from TransGenic Inc. (Kumamoto, Japan) were maintained under specific-pathogen-free conditions. Age and sex-matched groups of male and female mice (8–12 weeks old) were used in each experiment. All mouse experiments were reviewed by the Committee for Ethics on Animal Experiments in the Faculty of Medicine, and performed under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Gunma University, Japan, according to Japanese law (No. 105) and notification (No. 6) of the Government of Japan.

Blood-stage PbA parasites (uncloned line), a generous gift from Dr. M. Torii (Ehime University, Japan), *P. berghei* NK65 (PbNK) from Dr. M. Suzuki (Jobu University, Japan) and PbXAT, an attenuated variant of PbNK, from Dr. S. Waki (Gunma Prefectural College of Health Sciences, Japan) were used in this study. Parasitized red blood cells (pRBCs) were obtained after the fresh passage of frozen *P. berghei* stock through a donor B6 mouse, 4–5 days p.i. Mice were infected i.p. with 50,000 PbA or PbNK pRBCs, with 100,000 PbXAT pRBCs or with 25,000 *Plasmodium yoelii* 17XNL (PyNL) pRBCs. ECM was determined by observing clinical symptoms including paraplegia, head deviation, tendency to roll over on stimulation, ataxia, convulsion and death (Hearn et al., 2000).

Mice were i.v. injected with 0.2 ml of 1% Evans blue (Sigma, St. Louis, MO, USA). One hour later, mice were sacrificed and the brain was dissected after perfusion with PBS. To elute the dye, the brain was placed in 2 ml of formamide (Sigma) for 48 h at 37 °C. Evans blue concentration in the solution was measured as absorbance at 630 nm and calculated based on standard curves. Data are shown as μg of Evans blue per g of brain tissue.

Mice developing ECM were sacrificed and perfused with PBS, and the brains were removed and fixed with 4% paraformaldehyde. Tissue sections were stained with H & E. Samples were analysed using a Biorevo BZ-9000 microscope (Keyence, Osaka, Japan). The data were analysed with BZ-II software (Keyence).

Mice were imaged in an in vivo imaging system (Vivo Vision IVIS Lumina Xenogen, Alameda, CA, USA), under anaesthesia with isoflurane. Mice were i.p. injected with 150 mg/kg of D-luciferin (Promega, Tokyo, Japan) dissolved in PBS. Ten min after injection, parietal skin were resected to increase the detection of signal and the mice were placed in the in vivo imaging system (IVIS) imaging chamber. Data were collected with high sensitivity, 5 min exposure and analysed using LivingImage software (Xenogen).

To treat PbA-infected mice, pyrimethamine was orally administered in drinking water from 1 day p.i. (70 μg/ml, free drinking). To deplete $CD8^+$ T cells, 0.5 mg of anti-CD8 monoclonal antibody (clone 2.43) in 0.5 ml of PBS was injected i.p. 1 day before and 14 days after PbA infection. The successful depletion of $CD8^+$ T cells was confirmed by flow cytometry, which showed that >99% of the

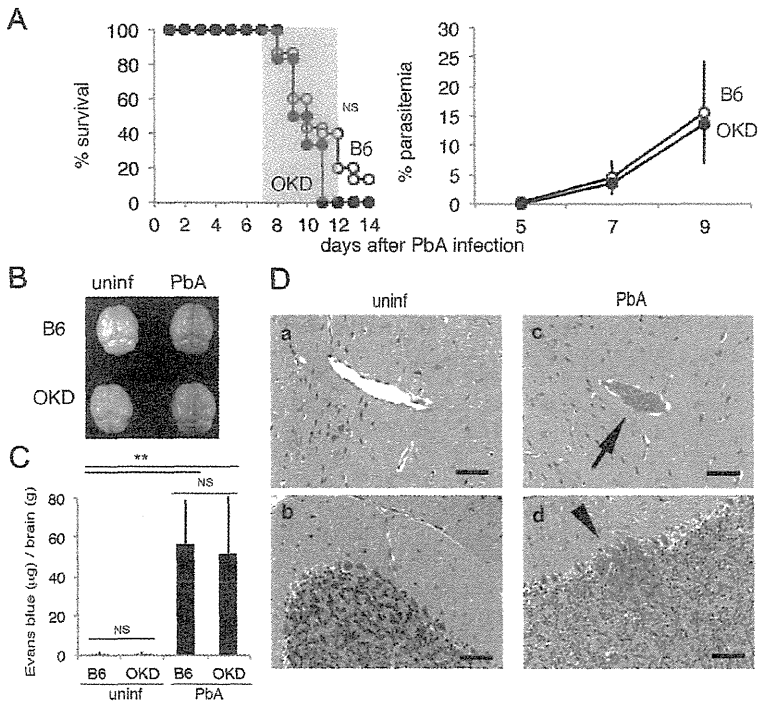

Fig. 1. Experimental cerebral malaria and its conventional evaluation. C57BL/6J (B6) and OKD48 (OKD) mice were infected with *Plasmodium berghei* ANKA strain (PbA). (A) Survival rate and parasitemia were monitored. The survival rate of B6 or OKD48 mice was calculated from five or two pooled individual experiments, respectively (B6, $n = 30$; OKD48, $n = 6$). A shaded area indicates the duration of experimental cerebral malaria symptoms shown by mice. Parasitemia values are means ± S.D. from 10 B6 or six OKD48 mice. NS, not significant. (B, C) Evans blue dye was injected i.v. into B6 and OKD48 mice developing experimental cerebral malaria or uninfected (uninf) mice to evaluate vascular permeability or destruction of the blood–brain barrier. Macroscopic appearance of the brain (B) and recovery of Evans blue dye from the brain (C) are shown. Values are means ± S.D. from six mice each. $^{**}P < 0.01$, Mann–Whitney *U* test. (D) Representative micrographs of H & E stained brain sections of mice developing experimental cerebral malaria (9 days after PbA infection) or in uninfected mice. Arrow and arrowhead indicate accumulation of mononuclear cells in the blood vessel and haemorrhage in cerebellum, respectively. Scale bars = 50 μm.

appropriate cell subset was depleted in the peripheral blood within 24 h after inoculation.

Brain was removed from mice, and the total RNA was purified using an Isogen (Wako, Osaka, Japan) kit. RNA was reverse-transcribed with Superscript First-strand (Invitrogen, Life Technologies Corporation, Tokyo, Japan) to synthesise the cDNA, according to the manufacturer's protocols. Quantitative PCRs (qPCRs) of HO-1: Mm00516005_m1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 4352339E (internal control) were performed using a TaqMan probe and TaqMan First Advanced Master mix (Applied Biosystems, Life Technologies Corporation, Japan) as described previously (Oikawa et al., 2012).

The differences between experimental groups were evaluated statistically with the Mann–Whitney *U* test. $P < 0.05$ was considered to be statistically significant. Significant differences in survival were tested with a log-rank test using Kaplan–Meier survival curves.

Almost all OKD48 mice infected with PbA developed ECM and died or remained symptomatic until approximately day 12 despite low parasitemia, similar to infected wild-type B6 mice (Fig. 1A). These data suggest that the genetic manipulation in OKD48 mice did not affect the development of ECM from PbA infection. Evaluation of ECM was performed not only by visual inspection of behavioural changes and neurological symptoms but also by pathological examinations of the extent of BBB destruction, cerebral blood vessel obstruction and haemorrhage in the brain. To evaluate BBB disruption, mice developing ECM after PbA infection were administered i.v. Evans blue dye. Increased permeability of blood vessels due to BBB disruption allowed leakage of the dye into cerebral parenchyma, resulting in a blue-stained brain and a large amount of Evans blue recovered from the brain (Fig. 1B and C). We also performed a histopathological examination of the brain (Fig. 1D). While neither occlusion of blood vessels nor haemorrhages were observed in uninfected mice (Fig. 1Da, b), haemorrhage and sequestered WBCs in microvessels were observed in mice developing ECM (Fig. 1Dc, d).

After establishing that the genetic manipulation in OKD48 mice did not affect the development of ECM following infection with PbA, we tested whether we could observe oxidative stress in the brains of these live mice by detecting bioluminescence with an in vivo imaging system. Although bioluminescence levels in the brains of OKD48 mice 3 days after PbA infection were comparable with those in uninfected mice (background levels), when ECM developed at approximately 8 days p.i. a significantly higher amount of bioluminescence could be detected in the infected mice (Fig. 2A). The bioluminescence signal in the brains of OKD48 mice developing ECM was approximately seven times greater than that in uninfected mice (Fig. 2B). These results indicate that OKD48 mice can be successfully monitored for levels of oxidative stress in the brain. Therefore, since the development of ECM results in oxidative stress, we suggest that these mice might be useful in detecting ECM.

Bioluminescence indicative of oxidative stress was successfully visualised in ECM using an in vivo imaging system. To confirm the presence of oxidative stress in mice infected with PbA, we used a more conventional biochemical method and performed quantitative reverse transcription (qRT)-PCR for mRNA encoding haeme oxiganase-1 (HO-1), an antioxidant induced under the control of Nrf2 (Alam et al., 1999). The levels of HO-1 mRNA in brain of B6 mice and OKD48 mice developing ECM were 10 times greater than those of uninfected mice (Fig. 2C). This result agreed with the bioluminescence observed in OKD48 mice, confirming induction of an oxidative stress response in infected brain.

Finally, to show that OKD48 mice could be useful as a new evaluation method for ECM, we investigated whether bioluminescence of these mice was detected specifically for ECM. Administration of

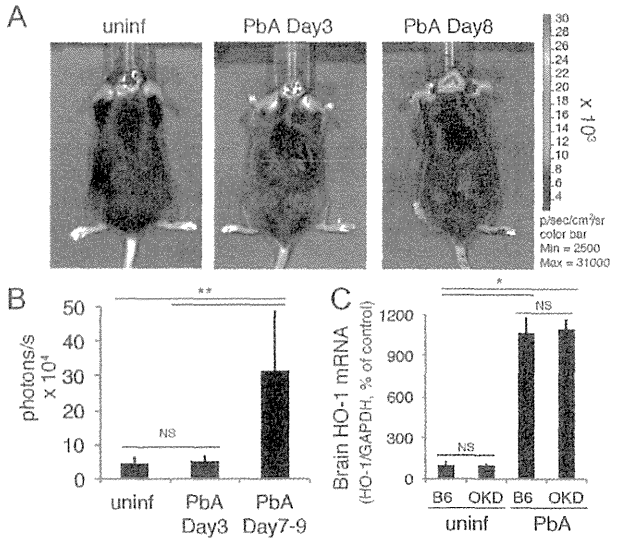

Fig. 2. Detection of oxidative stress to evaluate experimental cerebral malaria using *OKD48 (OKD) mice. (A, B) OKD48 mice were infected with Plasmodium berghei* ANKA strain (PbA) and were analysed for oxidative stress at the indicated days after infection in living animals. One macroscopic representative from five independent experiments is shown. The colour scale bar shows the photon counts (photon(p)/s/cm²/sr). Values in B are means ± S.D of photons/s from five pooled independent experiments (uninfected (uninf), $n = 6$; PbA day 3, $n = 6$; PbA day 7–9, $n = 7$). $^{**}P < 0.01$, Mann–Whitney *U* test. NS, not significant. (C) Detection of oxidative stress responses in experimental cerebral malaria mice in a conventional way. mRNA encoding haeme oxiganase-1 in brain from C57BL/6J (B6) mice and OKD mice developing experimental cerebral malaria or uninfected mice was reverse-transcribed and quantified by quantitative PCR. haeme oxiganase-1 mRNA was normalised by glyceraldehyde-3-phosphate dehydrogenase mRNA as an internal standard. Results are shown as the value relative to control uninfected B6 mice. One representative data from three independent experiments is shown. Data are means ± S.D. from mouse groups $n = 3$–6. $^{*}P < 0.05$, Mann–Whitney *U* test.

the anti-malarial drug pyrimethamine to PbA-infected mice killed parasites (Wilson and Edeson, 1953) (and thus no parasitemia was observed (Fig. 3Cc)) and prevented ECM development. Bioluminescence in the brains of untreated PbA-infected mice was detected as expected, but pyrimethamine-treated mice showed no bioluminescent signal (Fig. 3A and B). $CD8^+$ T cells play an essential role in the development of ECM (Yanez et al., 1996; Belnoue et al., 2002; Miyakoda et al., 2008) and in protection against blood-stage malaria (Imai et al., 2010). Indeed, PbA-infected mice depleted of $CD8^+$ T cells did not develop ECM and survived longer (Fig. 3C). We were unable to detect a significant bioluminescence signal in these mice, even in the presence of more parasites than in the control mice (Fig. 3A–C). In addition, bioluminescence was not significantly increased in mice infected with PyNL, PbXAT and PbNK strains, neither of which elicit ECM (Suzuki et al., 1987; Craig et al., 2012). These results indicate that enhancement of bioluminescence was specifically associated with development of ECM and that in vivo imaging with OKD48 mice is useful for evaluation of ECM.

We established a novel evaluation method for ECM. Oxidative stress was successfully detected in brains of OKD48 mice infected with PbA in association with the development of neurological symptoms. Bioluminescence in these brains was highly specific for ECM as no significant increase in bioluminescent signal was detected in mice infected with malaria parasites incapable of inducing CM or in PbA-infected mice that underwent interventions to prevent ECM. Therefore, usage of OKD48 mice is a specific and sensitive evaluation method for ECM.

In this method, oxidative stress can be quantitatively analysed based on transcriptional activation of Nrf2. It has been reported

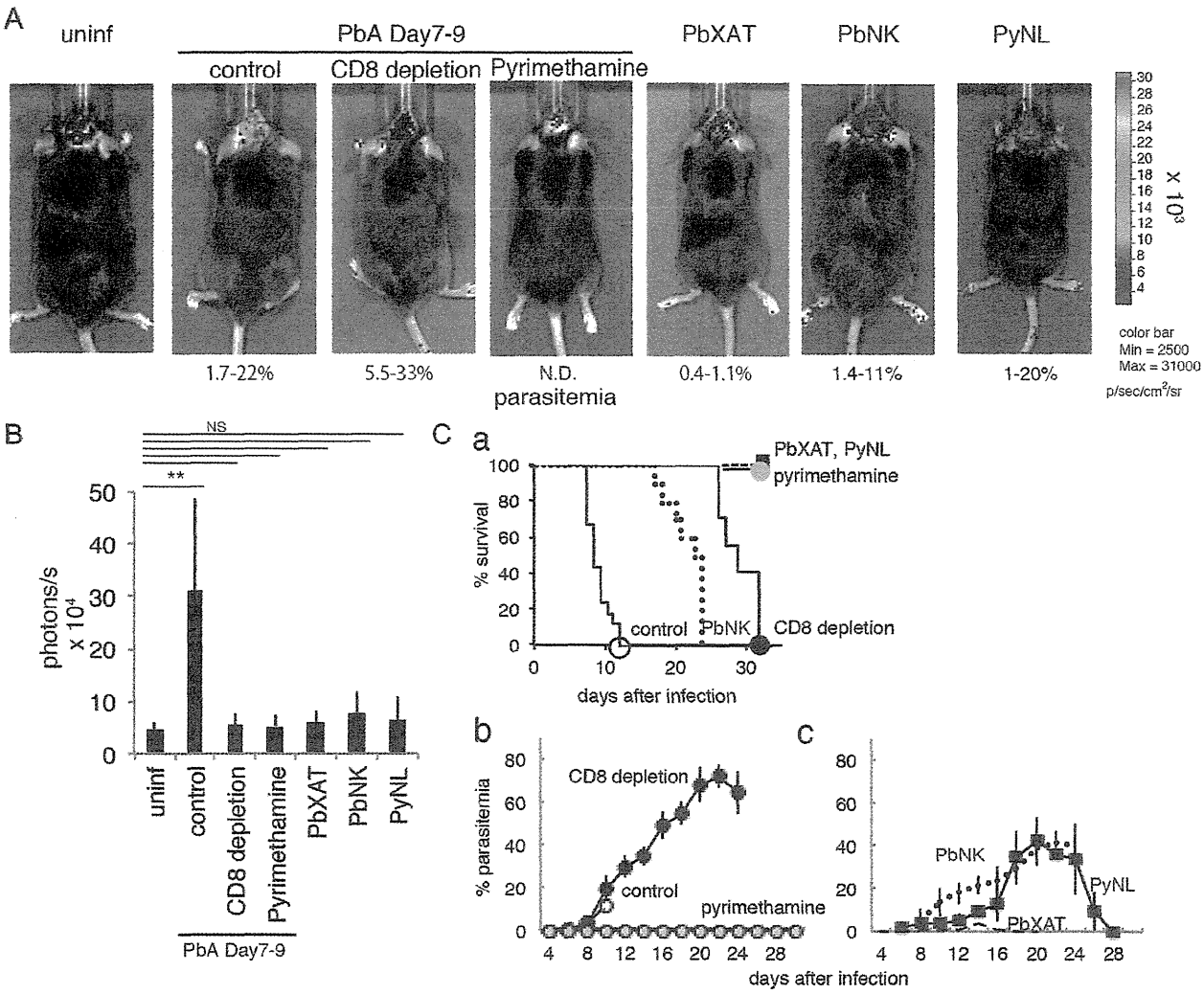

Fig. 3. Oxidative stress in the brain is associated with development of experimental cerebral malaria. OKD48 mice were infected with *Plasmodium berghei* ANKA strain (PbA), *P. berghei* NK65 (PbNK), an attenuated PbNK variant (PbXAT), and *Plasmodium yoelii* 17XNL (PyNL). PbA-infected mice were subjected to either treatment with the anti-malarial drug pyrimethamine, or to depletion of $CD8^+$ T cells. (A, B) Oxidative stress was measured as in Fig. 2. Uninfected (uninf), $n = 6$, same data as Fig. 2B, PbA day 7–9 control, $n = 7$, same data as Fig. 2B, CD8 depletion, $n = 7$, pyrimethamine, $n = 7$; PbXAT, $n = 7$; PbNK, $n = 5$; PyNL, $n = 5$. Numbers beneath photographic images in (A) indicate the range of parasitemia when analysed. $^{**}P < 0.01$, Mann–Whitney U test (control versus the other group). N.D., not detected; NS, not significant. (C) Survival rate (a) and parasitemia (b, c) are shown. Parasitemia values are means ± S.D. All data were calculated from one to three pooled individual experiments (PbA control: open circle, $n = 16$; PbA $CD8^+$ depletion: closed circle, $n = 7$; PbA pyrimethamine: gray circle, $n = 6$; PbXAT: dashed line, $n = 8$; PbNK: dotted line, $n = 10$; PyNL: closed square, $n = 8$).

that oxidative stress is induced in the brain during ECM (Reis et al., 2010; Ong et al., 2013). Reis et al. (2010) and Ong et al. (2013) showed oxidative stress is enhanced in ECM. They measured the level of lipid peroxidation in the brain for a quantitative assessment of oxidative stress. Both hypoxia-reoxygenation caused by sequestration of pRBCs in cerebral microvessels and free haeme released by rupture of schizonts produce reactive oxygen species (ROS), resulting in damage to cerebral tissues (Becker et al., 2004). In response to oxidative stress during ECM, antioxidant protein HO-1 is induced (Fig. 2C). This molecule contributes to attenuating ECM through catalysing haeme into iron, biliverdin and carbon monoxide (Tenhunen et al., 1968; Pamplona et al., 2007). Induction of HO-1 is known to be regulated by Nrf2 (Alam et al., 1999), which implies that oxidative stress during ECM activates Nrf2; therefore, we tested the utility of OKD48 mice as a means of visualising the development of ECM.

Our method offers several advantages. This easy-to-use method takes less time to obtain results and is performed in an objective manner using live animals, which may minimise observer biases.

CM is a serious complication caused by *P. falciparum* malaria in humans. To better control malaria, the pathogenesis of cerebral malaria must be analysed in detail. Our system, which can sequentially monitor the development of ECM in living mice, might serve as a useful platform for studying precise mechanisms involved in CM and aid in the development of pharmaceutical agents to prevent the disease.

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Short Report: A Case of Quadruple Malaria Infection Imported from Mozambique to Japan

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Abstract. A 35-year-old Japanese man had an intermittent fever and mild headache for eight weeks after he returned to Japan from working in Mozambique. He had taken antimalarial prophylaxis (doxycycline) for 25 weeks, and stopped taking this drug two weeks after his return. Microscopic examination of a peripheral blood smear showed a mixed infection with *Plasmodium vivax*, *P. falciparum*, and *P. ovale*. In addition, a nested polymerase chain reaction and subsequent sequencing detected specific DNA sequences of four species of *Plasmodium*, including *P. malariae*. The patient was successfully treated with artemether-lumefantrine and primaquine phosphate. The present case is a rare instance of a mixed infection with four species of *Plasmodium*. Nonimmune persons in malaria-endemic areas may have a risk of mixed infection. All four species must be identified by using sensitive and specific tests, such as a nested polymerase chain reaction, in addition to conventional morphologic identification.

Although many cases of mixed malaria infection have been reported in malaria-endemic countries, coincidental infection with more than one species of *Plasmodium* spp. is rare in non-endemic countries.[1,2] In Japan, only approximately 50–80 cases of malaria are reported each year, and are all imported malaria.[3] In this report, we describe a Japanese man who was given a diagnosis of a mixed infection with four species of *Plasmodium* (*P. vivax*, *P. falciparum*, *P. ovale wallikeri*, and *P. malariae*) by using a nested polymerase chain reaction (PCR) after he returned from Mozambique.

CASE REPORT

A 35-year-old Japanese man had intermittent fever and mild headaches for eight weeks after returning from Mozambique. He returned to Japan at the end of June after working as an instructor in operating construction machinery during for two years (June 2010–June 2012). He had taken antimalarial prophylaxis (doxycycline) for 25 weeks from January through the second week of July in 2012. He had no symptoms suggestive of malaria while in Mozambique, but a slight fever developed on August 25, 2012, in Japan. A high fever developed on August 30 and he was admitted to Tokai University Hospital on September 3.

Physical examination at admission showed a body temperature of 37.9°C, a respiratory rate of 27 beats/minute, a heart rate of 100 beats/minute, a blood pressure of 108/68 mm Hg, anemia in the palpebral conjunctiva, hepatomegaly, and splenomegaly. The hemoglobin level was 11.3 g/dL, the mean corpuscular volume was 88.7 fL, and the platelet count was 54×10^9/L. Biochemical tests showed increased serum levels of aspartate aminotransferase (32 U/L, reference value < 30 U/L), lactate dehydrogenase (379 U/L, reference range = 110–219 U/L) and C-reactive protein (5.57 mg/dL, reference value < 0.3 mg/dL). A coagulation test result for the prothrombin time–international normalized ratio was slightly prolonged (1.18, reference range = 0.80–1.10). An ultrasonographic examination showed hepatomegaly (140 mm thickness along the mammary line), splenomegaly (160 × 75 mm), and reactive swelling of the portal hepatic lymph nodes.

A rapid diagnostic test (OptiMAL-IT; DiaMed, Cressier, Switzerland) for malaria with blood of the patient showed three positive bands for the control, *Plasmodium* spp., and *P. falciparum*. A thin blood smear stained with Giemsa showed *P. vivax*, *P. falciparum*, and *P. ovale* (Figure 1). Trophozoites of *P. vivax* were dominant in the microscopic examination, and the total parasitemia including other species was 1.7%.

The patient was treated with Coartem (artemether 20 mg/lumefantrine 120 mg) in a six-dose regimen for three days (total = 24 tablets), followed by primaquine, 15 mg/day for 14 days. The patient soon became afebrile and subsequently left the hospital on the seventh day of hospitalization. Since that time, *Plasmodium* has not been detected, and his symptoms did not recur.

To identify the exact *Plasmodium* species, *Plasmodium* DNA was extracted from the patient's blood by using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany)[4,5] and was subjected to a nested PCR specific for the 18S ribosomal RNA gene. *Plasmodium* genus–specific primers and four pairs of species-specific primers described in a previous study were used for the nested PCR.[6] Results showed that the patient was infected with four species of *Plasmodium*, including *P. malariae*, which was not identified in the blood smear by microscopy (Figure 2). Amplified fragments were cloned into a vector and sequenced. The fragment amplified with *P. falciparum*-specific nested primers showed 100% identity with the *P. falciparum* 18S ribosomal RNA gene (GenBank accession no. JQ627152.1, expected = 3×10^{-39}). Fragments amplified with *P. vivax*-specific, *P. ovale*-specific, and *P. malariae*-specific nested primers showed a single nucleotide substitution in the *P. vivax* 18S ribosomal RNA gene (GenBank accession no. JQ627158, expected = 1×10^{-38}), the *P. ovale* 18S ribosomal RNA gene (GenBank accession no. AB182490.1, expected = 9×10^{-44}) and the *P. malariae* 18S ribosomal RNA gene (GenBank accession no. M54897.1, expected = 9×10^{-44}), respectively.

Because two types of *P. ovale* strains (classic and variant types) are prevalent in Africa and Asia, and it has been reported that the two types are distinct species,[7] we generated specific nested-primers specific for the *P. ovale* cytochrome b

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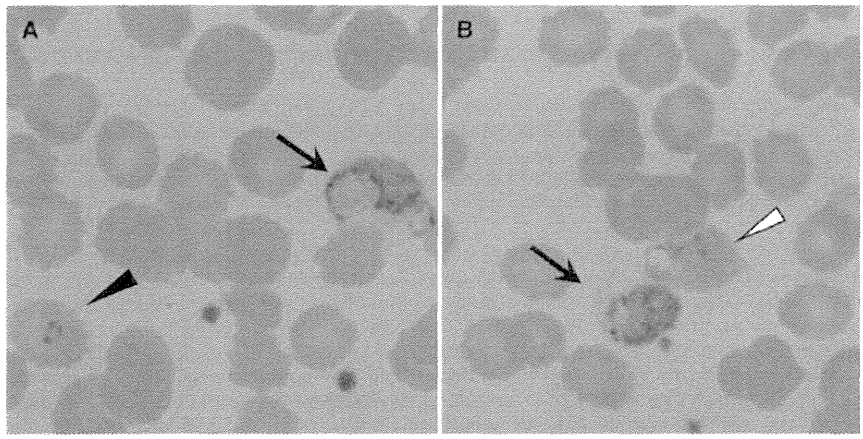

FIGURE 1. Microscopy of a Giemsa-stained peripheral blood smear of the patient. **A**, Ring form of *Plasmodium falciparum* (**arrowhead**) and a late trophozoite of *P. vivax* (**arrow**) were observed. **B**, Trophozoite of *P. vivax* (**arrow**) and trophozoite of *P. ovale* (**white arrowhead**) were observed.

gene (*cytb*) to determine the *P. ovale* species (Figure 3A). Specific bands corresponding to a 257-basepair fragment, were amplified from the sample. No specific bands were amplified from *P. vivax* genomic DNA (MRA-41, provided by the Malaria Research and Reference Reagent Resource Center) under these conditions when used as a negative control. Sequencing of the amplified fragment showed 100% identity with the *P. ovale wallikeri cytb* gene (GenBank accession no. HQ712053, expected = 1×10^{-127}), and single-nucleotide polymorphisms specific for *P. ovale wallikeri* were detected (Figure 3B). These results showed that the patient was infected with four malaria species (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale wallikeri*).

DISCUSSION

Some triple or quadruple mixed malaria infections have been reported among semi-immune residents in malaria-endemic areas of Asia[8,9] and Africa.[10,11] However, triple or quadruple mixed malarial infections have been rare among non-residents of such areas or among nonimmune persons. The present case is the first imported case of quadruple malaria in a nonimmune Japanese patient.

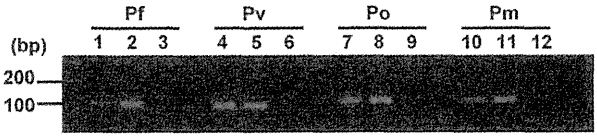

FIGURE 2. Detection of a *Plasmodium* 18S ribosomal RNA gene fragment by using nested polymerase chain reaction analysis. Amplification was performed by using a standard protocol for 35 and 20 cycles for the first and the second amplifications, respectively. Samples were first amplified with *Plasmodium* genus-specific primers and then amplified with *P. falciparum*-specific (lanes 1–3), *P. vivax*-specific (lanes 4–6), *P. ovale*-specific (lanes 7–9), and *P. malariae*-specific (lanes 10–12) oligonucleotide sets, respectively. Specific bands corresponding to *P. falciparum* (101 basepairs [bp], lanes 1 and 2), *P. vivax* (104 bp, lanes 4 and 5), *P. ovale* (115 bp, lanes 7 and 8), and *P. malariae* (115 bp, lanes 10 and 11) were amplified. Patient samples: lanes 1, 4, 7, and 10. Positive controls: lanes 2, 5, 8, and 11. Negative controls: lanes 3, 6, 9, and 12.

In mixed malaria infections, the level of parasitemia is different for each *Plasmodium* spp. In Japan, some mixed infections have been confirmed by combined use of morphologic detection and other alternative methods, including immunoassays and the molecular analyses.[12,13] The findings in this report, as well as in other reports,[14,15] indicate that molecular detection methods, such as nested PCR and real-time PCR, have superior sensitivity and specificity, which enables identification of low levels of infection and differentiation of species.

For antimalarial chemoprophylaxis, mefloquine is recommended in Japan, but it cannot be used in areas with mefloquine-resistant malaria. Doxycycline and atovaquone/proguanil can be used in all malaria-endemic areas, and these drugs are used in Japan. These drugs are effective against erythrocytic forms and also inhibit development of the normal liver stages, but they do not affect hypnozoites. Therefore, development of clinical malaria from hypnozoites of *P. vivax* or *P. ovale* has been reported more than two months after returning among travelers who continued chemoprophylaxis based on standard regimens.[16]

Daily use of doxycycline is recommended during travel to malaria-endemic areas and for four weeks after returning to a area where malaria is not endemic. However, the patient in this study stopped taking doxycycline after two weeks. Similar prophylactic failures for doxycycline against *P. falciparum* or *P. vivax* are associated with use of inadequately low doses.[17,18] Moreover, the prevalence of *P. falciparum* isolates with reduced susceptibility to mefloquine and doxycycline has been reported in Africa.[19,20]

In nonimmune persons taking chemoprophylaxis, efficacy differs for each *Plasmodium* spp. Furthermore, different levels of drug resistance to each strain and the dose of drugs used influence the clinical manifestations in cases with chemoprophylactic failure. *P. falciparum* might dominate over *P. vivax* by inducing a primary infection, as occasionally observed in patients simultaneously infected with the two species. In the present case, however, *P. vivax* predominated over *P. falciparum*. A partial effect of doxycycline was conceivably obtained because the parasitemia with *P. falciparum* and *P. malariae* was low. Detection of *P. malariae* was difficult by

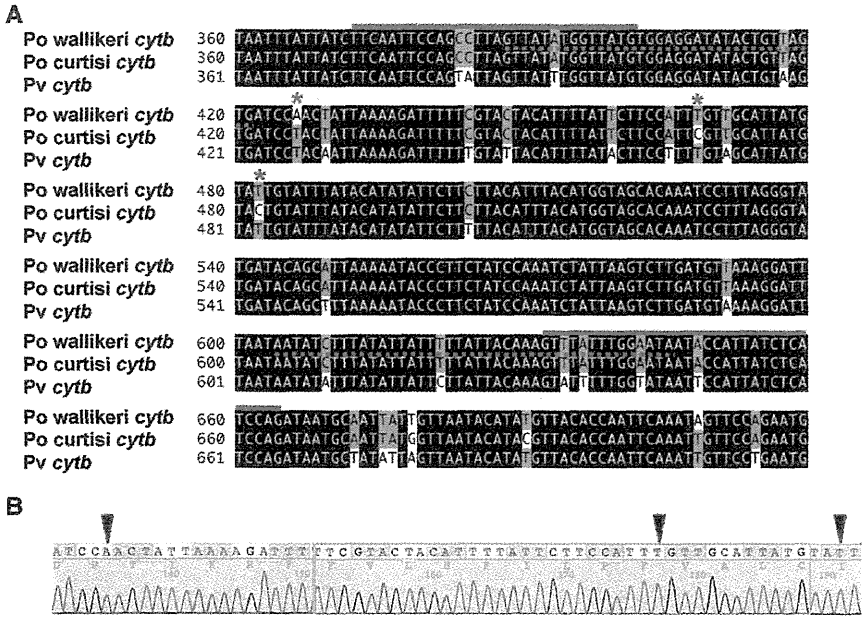

FIGURE 3. Sequence analysis of the *Plasmodium ovale* (Po) *wallikeri cytb* gene. **A**, Nucleotide alignment of the *cytb* genes from *P. ovale wallikeri* (Gen Bank accession no. GU723535), *P. ovale curtisi* (GU723514), and *P. vivax* (Pv) (AY791525). Red lines indicate regions where nested primers are annealed, respectively. Oligonucleotide sequences used to amplify polymorphic regions of *P. ovale* spp. were outer primers PoCytb-1F (5′-TTC AAT TCC AGC CTT AGT TAT ATG GTT ATG-3′) and PoCytb-2R (5′-CTG GAT GAG ATA ATG GTA TTA TTC CAA ATA AAC-3′) and inner primers PoCytb-3F (5′-GTT ATA TGG TTA TGT GGA GGA TAT ACT GTT AGT G-3′) and PoCytb-4R (5′-TAT TCC AAA TAA ACT TTG TAA TAA AAA TAA TAT AAT GAT ATT-3′). A polymerase chain reaction was performed as described above for 40 and 30 cycles for the first and second amplifications, respectively. Three single nucleotide polymorphism sites included this region specific for *P. ovale wallikeri* are indicated by red stars. **B**, Sequence analysis of a fragment amplified by using nested polymerase chain reaction identified *P. ovale wallikeri*–specific nucleotide polymorphisms, as indicated by arrowheads.

microscopy and was confirmed by using the nested PCR. A variant strain of *P. ovale* could not be detected by PCR for the classic strain, and its infection was confirmed by the more specific protocol of PCR used for the present case.

Although the Duffy-negative phenotype $FY*BE^{ES}$, which is not susceptible to infection with *P. vivax*, is dominant in sub-Saharan Africa,[21] Duffy-negative persons infected with *P. vivax* have been reported and suggested as a possible reservoir of *P. vivax* to Duffy-positive persons, such as those seen in Japanese persons.[22] As more nonimmune persons visit malaria-endemic countries in Africa, there are likely to be more cases of imported mixed infections of *Plasmodium* species, including *P. vivax*. Nonimmune persons who stay in malaria-endemic areas for long periods face an increasing risk of mixed infection, and clinical features are modified by chemoprophylaxis. When nonimmune persons show atypical clinical features for malaria or when chemoprophylaxis failure is suspected, all four species of human malaria parasites should be considered as causative agents, and the patients should be examined by using sensitive and specific tests, such as the PCR, in addition to conventional morphologic analyses.

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A DOT-ELISA TEST USING A *GNATHOSTOMA SPINIGERUM* RECOMBINANT MATRIX METALLOPROTEINASE PROTEIN FOR THE SERODIAGNOSIS OF HUMAN GNATHOSTOMIASIS

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Abstract. Gnathostomiasis caused by *Gnathostoma spinigerum*, is a hazardous food-borne helminthic zoonosis, and is endemic especially in developing countries in Asia. Definitive diagnosis, relying upon identification of worms from human tissues or body, is rarely accomplished. Consequently, sensitive supporting tools such as serological tests have been used widely. But these methods are time consuming, need sophisticated equipment and are impractical in some settings. In the present study a dot enzyme linked immunosorbent assay (dot-ELISA), using *G. spinigerum* recombinant matrix metalloproteinase protein as the antigen, was developed and assessed using sera of gnathostomiasis and other parasitosis patients as well as healthy controls. The accuracy, sensitivity, specificity, positive and negative predictive values were 97.4%, 100%, 96.1%, 92.9%, and 100%, respectively. The dot-ELISA appears to be a suitable rapid test for diagnostic purpose as well as epidemiological studies.

Keywords: *Gnathostoma spinigerum*, dot-ELISA, human gnathostomiasis, recombinant matrix metalloproteinase

INTRODUCTION

Gnathostomiasis is an important food-borne helminthic zoonosis caused by spirurid round worms of the genus *Gnathostoma*. The disease is endemic in Asia and the Americas (Waikagul and Diaz Camacho, 2007) and often reported in travelers returning from those areas (Moore *et al*, 2003; Katchanov *et al*, 2011). *Gnathostoma spinigerum* is a major causative species in Asian countries, *ie* Japan, Thailand, Vietnam, etc (Daengsvang, 1981; Nawa, 1991; Xuan le *et al*, 2002; Herman and Chiodini, 2009). Humans become infected by eating raw or undercooked flesh of a wide range of animals including freshwater fish, chicken, frogs which contains *Gnathostoma* advanced third-stage

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larvae (AL3). Ingested larvae migrate aimlessly in the human body and produce various signs and symptoms depending on the organs involved. Involvement of vital organs, *ie*, central nervous system, eye, etc., may lead to severe disease and death (Daengsvang, 1981; Nawa, 1991; Katchanov *et al*, 2011).

Parasitological diagnosis of human gnathostomiasis is usually done by identifying larvae recovered from the human body. Worms can be recovered after drug treatment, surgery, or after they have spontaneously exited the body. However, direct recovery of larvae is rare. Consequently, diagnosis of gnathostomiasis is more commonly reached by using clinical features, history of consuming risky foods, blood eosinophilia and serological test results. To date, a number of serological diagnostic tests, including enzyme-linked immunosorbent assays (ELISA) (Maleewong *et al*, 1988; Diaz Camacho *et al*, 1998) and immunoblotting using *Gnathostoma* AL3 extract (Tapchaisri *et al*, 1991; Wongkham *et al*, 2000; Anantaphruti *et al*, 2005; Laummaunwai *et al*, 2007), have been evaluated and reported. Recently, a recombinant matrix metalloproteinase (rMMP) protein of *G. spinigerum* AL3 has been produced and can be used as the sensitive and specific antigen for serodiagnosis of human gnathostomiasis by immunoblotting (Janwan *et al*, 2013a,b). The rMMP protein can be used as a diagnostic antigen and potentially replace native parasite antigens for development of a gnathostomiasis diagnostic kit.

However, standard ELISA and immunoblotting need sophisticated equipment and are too complicated to be performed routinely under field conditions. A dot enzyme linked immunosorbent assay (dot-ELISA) using rMMP protein as the antigen provides a simple, rapid and practical test. Consequently, the aim of the present study is to develop and test the performance of rMMP based dot-ELISA for serodiagnosis of human gnathostomiasis.

MATERIALS AND METHODS

Human sera

All serum samples were retrieved from the serum bank of the Faculty of Medicine, Khon Kaen University. They were anonymized before use: (i) negative control-healthy adult volunteer group (n = 21), which included samples from subjects visiting Srinagarind Hospital, Faculty of Medicine, Khon Kaen University whose stool examination by formalin ethyl acetate concentration technique (Elkins *et al*, 1986) revealed no parasites at the time of blood collection. Pooled sera from all of the healthy individuals was also used as negative control for individual assays; (ii) gnathostomiasis group consisting of samples taken from parasitologically confirmed gnathostomiasis patients (n = 9) and from patients who presented with clinical signs and symptoms indicative of cutaneous and visceral gnathostomiasis as well as neurognathostomiasis (n = 30) (Daengsvang, 1981; Punyagupta *et al*, 1968; Boongird *et al*, 1977; Intapan *et al*, 2010) and who had produced IgG antibody specifically against the 21/24 kDa *G. spinigerum* larval antigen, as detected by immunoblotting; (iii) serum samples from other parasitosis patients (n = 56). Infections in this last group were identified by parasitological methods except cysticercosis cases, which were diagnosed via computerized tomography scan and were serological positive (Intapan *et al*, 2008) (Table 1). Informed consents were obtained from all adult participants and from parents or legal guardians in the case of minors. The study protocol was

Table 1
Type of serum and dot enzyme linked immunosorbent assay results against the recombinant matrix metalloproteinase protein.

Type of serum	Group[a]	No. positive/no. total (%)
Healthy control	i	0/21 (0%)
Confirmed gnathostomiasis	ii	9/9 (100%)
Suspected gnathostomiasis	ii	30/30 (100%)
Cysticercosis	iii	0/5 (0%)
Taeniasis	iii	0/5 (0%)
Opisthorchiasis viverrini	iii	1/5 (20%)
Fascioliasis	iii	0/5 (0%)
Paragonimiasis	iii	0/4 (0%)
Angiostrongyliasis	iii	0/5 (0%)
Strongyloidiasis	iii	0/6 (0%)
Hookworm infection	iii	0/5 (0%)
Capillariasis	iii	1/6 (16.7%)
Ascariasis	iii	0/4 (0%)
Trichinosis	iii	1/6 (16.7%)

[a] See materials and methods.

approved by the Khon Kaen University Ethics Committee for Human Research (HE561477).

Dot-ELISA

The rMMP protein was constructed from a cDNA encoding the MMP protein of *G. spinigerum* AL3, cloned and expressed as previously described (Janwan *et al*, 2013a) with some modifications using pQE-31 expression vector and *Escherichia coli* XL-1Blue (Qiagen, Germany) as the expression system. The rMMP protein was solubilized using solubilizing solution (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 8.0) and collected. The recombinant protein fused with 6-Histidine (6-His)-tagged residues, and was purified using Ni-NTA His Bind Resin (Novagen, Darmstadt, Germany). The optimum concentration of the rMMP protein, 0.5 μg, was previously determined by checkerboard titration and used as antigen in this study. Ten microliters (0.5 μg) of the antigen diluted in solubilizing solution, were spotted separately onto a nitrocellulose (NC) membrane (GE Healthcare, Piscataway, NJ), air-dried for 15 minutes at room temperature (RT) and incubated at 37°C for 2 hours. The unoccupied sites of the NC membrane were then blocked by immersion of the membrane for 30 minutes in 3% skimmed milk in phosphate buffered saline (PBS), pH 7.5, containing 0.1% Tween-20 (PBST). Next, the NC membrane was washed with 1% skimmed milk in PBST (2 times) and was cut into 8x25 mm strips (2 spots of antigen/strip) and stored at -20°C until use. Each strip was incubated with individual human serum samples (diluted 1:100 in 1% skimmed milk in PBS) absorbed with *E. coli* lysate for 30 minutes at RT. The strips were washed with 1% skimmed milk in PBST (5 times) and incubated with goat anti-human IgG (H+L) HRP conjugate

(Invitrogen, Carlsbad, CA) at a dilution of 1:20,000 (in 1% skimmed milk in PBS) for 1 hour at RT. After a further 5 washes with 1% skimmed milk in PBST, the precipitate dots on strips were rendered visible in 3, 3′-diaminobenzidine-tetrahydrochloride solution. The reaction was stopped after 5 minutes by washing strips with distilled water. The developed color dots were read with the naked eye after the blot had dried and the results were interpreted arbitrarily, according to color intensity, as positive (a color with a clear contrast to the background), weakly positive (there is a color but lower intensity) and negative (no color development was observed).

Reading and data analysis

The results were independently read, on the same day but 30 minutes apart, by two independent observers, to estimate inter-observer variability. The precision of the dot-ELISA was also investigated by performing the test on different days using the same pooled positive and negative sera, the same batch of antigens, and the same conditions. Similar results were shown from all, which revealed that day to day variation was minimal. The level of cross-reaction was estimated independently for individual parasitosis sera. The diagnostic accuracy, sensitivity, specificity, and positive and negative predictive values were calculated as previously described (Galen, 1980).

RESULTS

For clinical interpretation, readings of "positive" and "weakly positive" were both regarded as indicating a positive result (the one patient returning a weakly positive result – Fig 1 - had clinical symptoms indicative of gnathostomiasis). The sera were scored as positive when each of the two observers gave the same interpretation. In fact, both observers agreed on the readings in every case. Dot-ELISA employing the rMMP protein for diagnosis of human gnathostomiasis was evaluated using individual sera from healthy control, gnathostomiasis patients and patients with other parasitic diseases (Fig 1). The results are summarized in Table 1. The calculated values for accuracy, sensitivity, specificity, positive and negative predictive values are 97.4%, 100%, 96.1%, 92.9%, and 100%, respectively. Sera from opisthorchiasis (1/5), capillariasis (1/6), and trichinosis (1/6) patients appeared to cross-react (data not shown) in the dot-ELISA (Table 1).

DISCUSSION

The dot-ELISA is a versatile solid-phase immunoassay for antigen or antibody detection. The method uses small volumes of reagent dotted onto solid surfaces such as nitrocellulose and other membranes which strongly bind proteins. After reaction with antigen-specific antibody and enzyme-conjugated anti-antibody, the addition of a chromogenic substrate produces a colored precipitate dot on the membrane which is read by the naked eye (Pappas, 1988). The dot-ELISA has been used widely in the serodiagnosis of human helminthiases, including fascioliasis (Intapan *et al*, 2003), paragonimiasis (Maleewong *et al*, 1997), cysticercosis (Piña *et al*, 2011), toxocariasis (Bojanich *et al*, 2012), trichinosis (Mahmoud and Moustafa, 2003). For serodiagnosis of human gnathostomiasis, dot-ELISA using adult-worm extracts of *G. spinigerum*, *G. hispidum* and *G. doloresi* has been reported (Ishiwata *et al*, 2003; Sakamoto *et al*, 2004). Here, the dot-ELISA using rMMP protein of *G. spinigerum* as antigen was developed. The results demonstrated

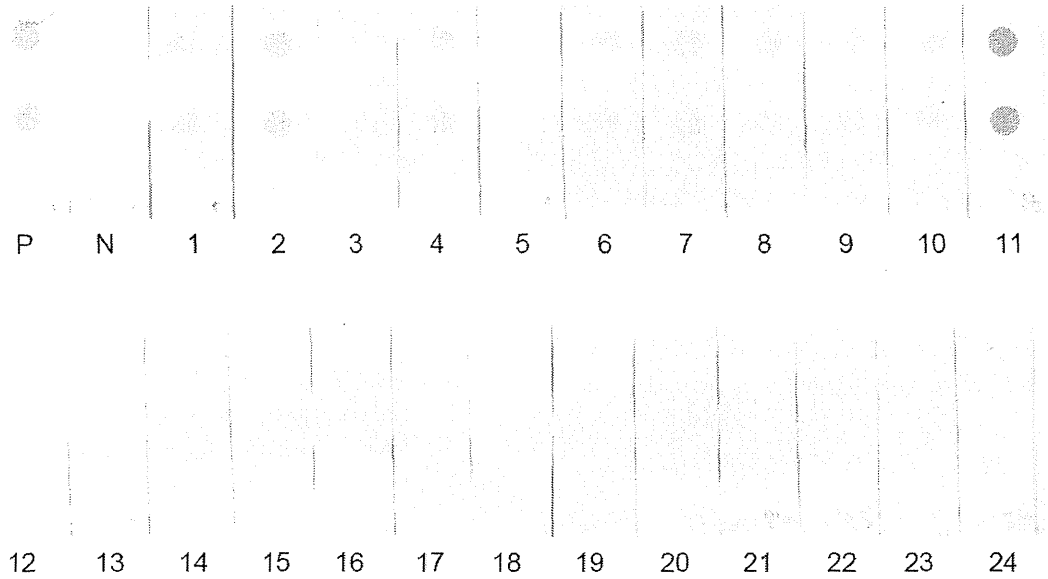

Fig 1–Representative dot enzyme linked immunosorbent assay using recombinant matrix metalloproteinase protein as the antigen probed with 1:100 diluted human sera. Pooled human gnathostomiasis patients (P), pooled negative controls (N), parasitologically confirmed cutaneous gnathostomiasis (1-3), ocular gnathostomiasis (4-6), clinically suspected gnathostomiasis (7-11), healthy control (12, 13), trichinosis (14), paragonimiasis (15), hookworm infection (16). capillariasis (17), fascioliasis (18), ascariasis (19), cysticercosis (20), opisthorchiasis viverrini (21), taeniasis (22), strongyloidiasis (23), angiostrongyliasis (24). Lanes P, 1-4 and 6-11 were scored by both observers as positive. Lane 5 was scored as weakly positive and lanes N and 12-24 were scored as negative.

high accuracy, sensitivity and specificity. The recombinant protein can be reliably mass-produced and the antigen strips are stable for at least three months at -20°C (data not shown).

Some cross-reactions with opisthorchiasis (1 of 5), capillariasis (1 of 6), and trichinosis (1 of 6) sera were found. It is possible that these patients might have had previous subclinical infection with *G. spinigerum*. Findings of such a cross reaction does not pose a problem in differential diagnosis because these parasitic infections usually present with clinical features distinguishable from those of gnathostomiasis. However, this test needs to be evaluated with more samples. Results will need to be interpreted cautiously if it is applied in an area endemic for opisthorchiasis, capillariasis and trichinosis.

The dot-ELISA method is specific, sensitive, rapid, reagent-conservative, cost effective, easy to perform and to interpret, portable and does not require sophisticated equipment that is often unavailable in developing countries. Moreover, the dot-ELISA could be adapted to employ microtiter plates for large-scale serological surveys or dipsticks for small numbers of sera in a local hospital.

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イムノクロマトキットを用いた幼虫移行症の迅速血清診断

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[抄録] 幼虫移行症として重要なトキソカラ症とマンソン孤虫症の抗体検出を目的とした簡便で迅速なイムノクロマト法を用いた迅速検査キットを開発したので紹介する。

キーワード；幼虫移行症、トキソカラ症、マンソン孤虫症、イムノクロマトキット、迅速血清検査

Rapid serodiagnostic tools for larva migrans using immunochromatographic kits

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Abstract: Simple and rapid serodiagnostic tools for toxocariasis and sparganosis in humans using immunochromatographic kits has been introduced.

Key Words; larva migrans, toxocariasis, sparganosis, immunochromatographic kit, rapid serodiagnosis

[はじめに]

ヒトのトキソカラ症はイヌ回虫(*Toxocara canis*)やネコ回虫(*Toxocara cati*)の幼虫寄生によって惹起される幼虫移行症として世界的に患者発生が見られる[1,2]。一方、マンソン裂頭条虫(*Spirometra erinaceieuropaei*)の幼虫(プレロセルコイド)寄生によるマンソン孤虫症は東南アジア～東アジア地域で多くの患者発生が見られる[3,4]。これらの幼虫移行症ではIgG抗体検出による検査が診断に有効であり、トキソカラ症の血清診断にはイヌ回虫幼虫の分泌・排泄物excretory-secretory substances[5,6]や遺伝子組換え抗原[7,8]が用いられている。一方、マンソン孤虫症の血清診断には、プレロセルコイドのシステインプロテイナーゼ[9,10]などが用いられている。著者らは、最近、特異性の高い遺伝子組換えイヌ回虫幼虫抗原を用いたイムノクロマトキット(iToxocara kit)[11]とマンソン孤虫プレロセルコイドから生化学的手法で精製したシステインプロテアーゼを用いたキット(iSpa kit、図1)を開発したので[12]、簡便、かつ迅速検査法として紹介する。

[材料と方法]

イヌ回虫幼虫由来の遺伝子組換え抗原とマンソン裂頭条虫プレロセルコイド由来のシステインプロテアーゼはそれぞれ既法に基づいて精製し、抗原として用いた[7-9]。イムノクロマトキットの評価には、血清学的、ならびに臨床所見からトキソカラ症と診断された患者(ブラジル人と日本人)血清(54検体)、マンソン孤虫症は寄生虫学的に確定診断された患者(日本

人とタイ人)血清(13 検体)、陰性対照として健常人(ブラジル人、日本人)血清(57 検体)を用いて最適化した。図 1 にはマンソン孤虫症のイムノクロマトキットを例示した。キットにはイムノクロマト device1 枚、血清希釈用 sample buffer と発色レベル判定用の color board が含まれている。

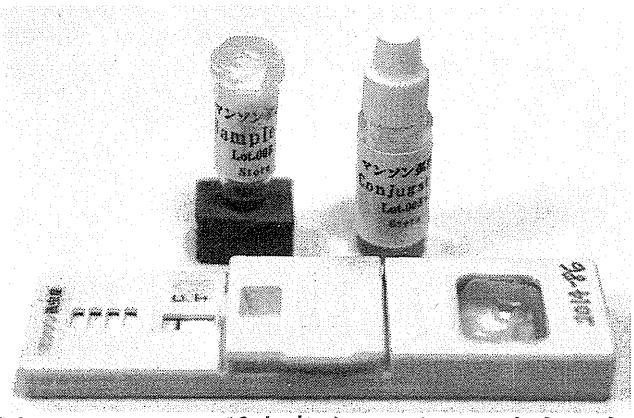

図 1. マンソン孤虫症イムノクロマトキット (iSpa kit).

検査手順は以下のとおりである(図 2)。

1) 被検血清 5 μL をマイクロチューブに分注し、sample buffer 20 μL を加えて 5 倍に希釈する(a)。
2) 希釈された血清 5μL を希釈血清の部分に吸着させる(b)。
3) strip のカバーを開け、二次抗体液を 1 滴垂らす(c)。カバーを閉じる。
4) 展開液の入ったバッグを指で押す(d)。
5) 室温で 20 分間放置する。
6) 結果判定は Test line (T)に青色バンドが出現するか否かで、判定する。

図 2. 検査手順.

[結果と考察]

トキソカラ症キットに関する評価試験では、ブラジル人トキソカラ症患では 42 例中 37 例(88.1%)、また、日本人 12 例中 10 例(83.3%)で陽性であった。一方、ブラジル人、日本人の計 57 例の健常者血清ではいずれも陰性であった。イムノクロマトキットによるバンドの強度と ELISA の吸光度とはよく相関し、相関関係を表す Spearman 係数は 0.799 と高い値を示した。最近、マレーシアで実施した評価試験では、有病率 27.3% (39/143)における感度は 84.8%、特異性は 90.7%であった(Lim PKC, *et al.*, in submission)。

一方、マンソン孤虫症キットについては、マンソン孤虫症確定例（日本人 10 例、タイ人 3 例)、健常者血清(日本人 50 例、タイ人 9 例)、さらにマンソン孤虫症以外の寄生虫症確定症例 74 例、計 146 検体について検討したところ、有病率 8.9% (13 /146)では、マンソン孤虫症 13 例は全例陽性であり、感度は 100%、特異性は 97.0%であった[12]。

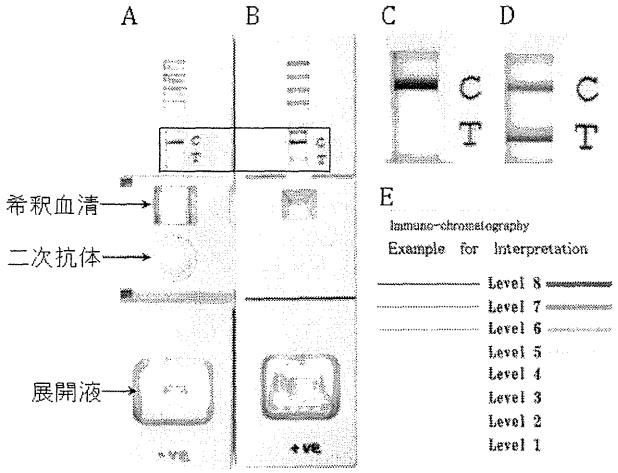

図 3. イムノクロマトキットと結果判定. イムノクロマト device のカバーが開いた状態(A)と閉じた状態(B). T ラインに青色バンドが出なければ陰性(C), 出れば陽性(D). 発色強度は color board を参考に肉眼的に判定する(文献 12 を改変).

今回、開発したトキソカラ症とマンソン孤虫症のイムノクロマトキットは、感度、特異性ともに高く、検査結果の信頼性は高いと考えられる。ただ、抗体価が相対的に高い場合、イムノクロマトキットで非特異的な陽性反応が見られることがあるが、この場合には臨床症状や食歴など総合的な所見から判断する必要がある。

現在、遺伝子組換え抗原を用いた顎口虫症イムノクロマト検査キットの開発も行っており、2015 年には完成の予定である。

[おわりに]

ここで紹介した幼虫移行症のイムノクロマトキットは、汎用されている ELISA などに比べ、より簡便で迅速に行える検査法である。これらのキットは、現在、国立感染症研究所寄生動物部で保管しており、ご要望があれば、無償で提供している。幼虫移行症検査法の標準化に向け、ご活用いただきたい。

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[文献]

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