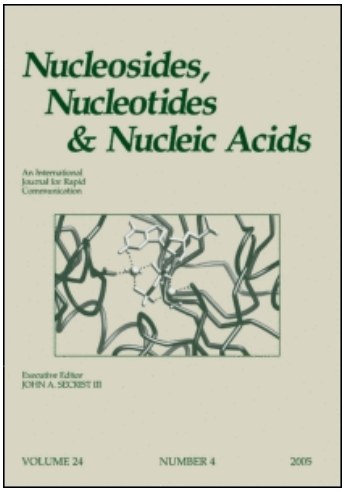

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A Colorimetric DNA Diagnostic Method for Falciparum Malaria and Vivax Malaria: A Field Trial in the Solomon Islands

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A COLORIMETRIC DNA DIAGNOSTIC METHOD FOR FALCIPARUM MALARIA AND VIVAX MALARIA: A FIELD TRIAL IN THE SOLOMON ISLANDS

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Abstract: We have developed a colorimetric assay, "microtiter plate-hybridization", for the detection of malaria parasites *Plasmodium falciparum* and *P. vivax* in human blood, in which the target DNA sequences (18S small subunit ribosomal RNA gene) amplified by polymerase chain reaction (PCR) are hybridized with the species-specific probes immobilized on a microtiter well. This assay system was tested in Guadalcanal, Solomon Islands, where malaria is highly endemic. We obtained blood samples by finger puncture from 130 asymptomatic donors. Among the 130 samples, 30 (23 %) were *P. falciparum* positive, 28 (22 %) were *P. vivax* positive, and 8 (6 %) were mixed infections. The results of our DNA diagnostic method showed good correlation with those of acridine orange microscopy.

The conventional method for malaria diagnosis is microscopic examination of stained blood films. However, this procedure is labor-intensive and requires a highly trained microscopist for accurate identification when few parasites are present. A promising alternative to microscopy is RNA/DNA probe technology, in particular, the use of *in vitro* amplification using the polymerase chain reaction (PCR) [1,2].

Several researchers have reported various DNA probe-based or PCR-based diagnostic methods for the detection of *Plasmodium falciparum* [3-17]. Recently, sequences of the gene coding for the 18S small subunit ribosomal RNA (SSUrRNA) of malaria

§ This paper is dedicated to Dr. Yoshihisa Mizuno, Emeritus Professor of Hokkaido University, on the celebration of the 75th birthday.

parasites have been reported [18-20] and used as the target for specific detection of malaria parasites [10,17,21,22]. However, these reported methods require complicated manipulations, such as DNA extraction from the blood sample, dot blot hybridization, or handling of radioisotopes, which make them impractical under clinical or field conditions. A diagnostic assay, based on the PCR, which is simple, specific, rapid, and adapted to field applications, would therefore be of great advantage.

We report here a solid-phase approach, "microtiter plate-hybridization", in which the PCR-amplified products are captured by species-specific probes immobilized on a microtiter well, and visualized by a subsequent colorimetric reaction [23]. This assay system allowed us to detect and identify the two species of human malaria parasites, *P. falciparum* and *P. vivax* in blood samples, using the DNA sequence of the SSUrRNA gene as the target. We also report the results of *P. falciparum specific and P. vivax* specific microtiter plate-hybridization assays in Guadalcanal, Solomon Islands, where malaria is highly endemic. Our DNA diagnostic method has the potential to replace microscopy and will be a useful tool in malaria control programs.

MATERIALS AND METHODS

Parasites. *P. falciparum* strains FCR-3 (ATCC 30932), Honduras-1 (ATCC 30950), and FCO-1 (isolated in Sumatra by Dr. A. Ishii) were grown *in vitro* according to the method of Trager and Jensen [24]. *P. falciparum* parasites grown *in vitro* were diluted with heparinized whole blood from an uninfected donor. The blood samples were derived from asynchronous cultures, which contained the organism in all stages of its erythrocytic life cycle, including schizonts. Therefore, they had more DNA per infected erythrocyte than field or clinical samples, which usually contain only ring-infected or gametocyte-infected erythrocytes [8]. Because the calculated nucleus density was 30 % higher than the parasitemia in a thin blood smear from the culture, parasitemias were multiplied by 1.3 to rectify the difference in the nucleus density between asynchronous culture and infected erythrocytes. Blood samples (10 µl each) containing 10 to 10,000 parasites were prepared and suspended in 150 µl of phosphate-buffered saline containing 10 units/ml nystatin and 25 µg/ml gentamicin (PBS-NG), as previously reported [16].

Blood samples from vivax malaria patients were kindly provided by Dr. M. Kimura, Department of Infectious Diseases and Applied Immunology, Institute of Medical Science, University of Tokyo. The blood was diluted with heparinized whole blood from an uninfected donor for trial use in testing human blood infected with *P. vivax* .

DNA preparation. DNA was extracted from *P. falciparum* culture and blood from vivax malaria patients [23]. The culture or blood was suspended in phosphate-buffered

saline (PBS) and centrifuged. The precipitate was suspended in PBS containing 0.15 % saponin to hemolyze the red blood cells (RBCs). The mixture was centrifuged and the precipitate was washed with PBS to remove hemoglobin, then treated with sodium dodecyl sulfate and proteinase K. DNA was prepared from the lysed material by phenol/chloroform extraction and ethanol precipitation. Prepared DNA was used for positive controls throughout the study.

Field samples. Field samples were collected at Rogu village (n = 14), Babala village (n = 12), Tenaru Secondary School (n = 102), and the Solomon Islands Medical Training and Research Institute (MTRI, n = 2), in Guadalcanal, Solomon Islands, in January and February, 1993. Blood samples (10 µl each) were obtained by finger puncture and suspended in 150 µl of PBS-NG without heparinization. Microtiter plate-hybridization assays were performed at MTRI in the Solomon Islands. Simultaneously, one thick and two thin blood smears were prepared from each donor. Thick blood smears were stained with Giemsa and examined by microscopists of the Solomon Islands, who studied the slides for about 5 min each by the rapid method recommended by WHO [25]. One set of thin blood smears were stained with acridine orange (AO) and examined for 10 min per slide (x200 and x400 magnification), using an interference filter, at Nagoya University (Nagoya, Japan) [26,27]. The microtiter plate-hybridization, thick-smear microscopy, and AO microscopy were performed by double blind experiment. The other set of thin blood smears were stained with Giemsa.

PCR primers and probes. Oligonucleotide primers (MPH-1, 5'-CAGATACCG-TCGTAATCTTA-3', and MPH-2, 5'-CCAAAGACTTTGATTTCTCAT-3'), which are specific to the highly conserved regions of the SSUrRNA genes of *P. falciparum* [18] and *P. vivax* [19] were designed from the published sequences as shown in FIG. 1. The oligonucleotides were synthesized on a DNA synthesizer (381A, Applied Biosystems, Foster City, California, USA). For labeling the 5' end of MPH-1 with biotin, an alkylamine linker (Aminolink II, Applied Biosystems) was incorporated in the final step of the synthesis.

After standard deprotection and gel filtration (Sephadex G-50, Pharmacia LKB Biotechnology, Uppsala, Sweden), the fragment for MPH-1 was reacted with ε-caproylamidobiotin-*N*-hydroxysuccinimide ester (Bethesda Research Laboratories, Gaithersburg, Maryland, USA). The labeled fragments were purified by gel filtration and reversed phase HPLC.

P. falciparum-specific probe (5'-GTCACCTCGAAAGATGACTT-3') and *P. vivax*-specific probe (5'-TAAACTCCGAAGAGAAAATTC-3') were designed from the

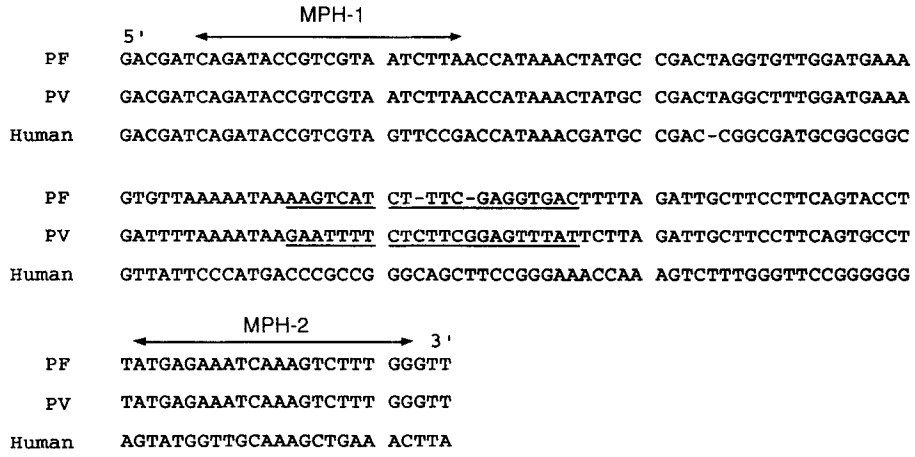

FIG. 1. Target sequences of the SSUrRNA genes of *P. falciparum* and *P. vivax*. PF = *P. falciparum*, PV = *P. vivax*. Species-specific regions used for the probes are underlined. MPH-1 and MPH-2 show the regions of the primer set used for the PCR amplification. The sequence of human SSUrRNA gene is also aligned.

sequences of the SSUrRNA genes (FIG. 1). Tandem repetitive probes were prepared and immobilized on microtiter wells (MS-3508F, Sumitomo Bakelite, Tokyo, Japan) by the method of Kawai *et al.* [28].

Cell lysis and DNA amplification of parasites. To hemolyze all RBCs, saponin (final concentration, 0.02 %) was added to the blood suspension, which was then maintained for 5 min at room temperature (25 °C). The mixture was then centrifuged at 7,000 x g for 10 min to remove components that might inhibit the PCR [11, 13, 16]. After the pellet was washed with 200 µl of PBS without antibiotics, it was suspended in 40 µl of the lysis solution (110 mM Tris-HCl pH 8.9 / 1.5 mM $MgCl_2$ / 80 mM KCl / 500 µg/ml bovine serum albumin (BSA) / 0.1 % sodium cholate / 0.1 % Triton X-100 / 200 µg/ml proteinase K / 0.45 % Tween 20 / 0.45 % Nonidet P-40) and the suspension was incubated at 60 °C for 20 min and subsequently at 95 °C for 10 min. Ten microliters of the reaction mixture (0.8 µM each primer / 1 mM each dATP, dGTP, dCTP, and dTTP / 10 mM Tris-HCl pH 8.9 / 80 mM KCl / 1.5 mM $MgCl_2$ / 0.1 % Triton X-100 / 0.1 % sodium cholate / 500 µg/ml BSA / 1 unit of Tth DNA polymerase (Toyobo, Osaka, Japan)) was added to the above solution and the reaction mixture was subjected to 30 cycles of PCR amplification on a Combi Thermal Reactor TR-2 (Hybaid, Teddington, Middlesex, UK). The conditions

of the PCR amplification were as follows: denaturation at 94 °C for 30 sec, annealing at 50 °C for 60 sec, and extension at 72 °C for 60 sec.

Procedure for microtiter plate-hybridization. One hundred microliters of hybridization solution: 5 x SSC (standard saline citrate, 0.15 M NaCl / 0.015 M sodium citrate) containing 200 μg/ml herring sperm DNA, was dispensed into microtiter wells coated with the probes. Ten microliters of the heat-denatured PCR product was mixed into the hybridization solution and incubated at 60 °C for 1 hour. The hybridization solution was removed from the well, which was then rinsed three times with 200 μl of 2 x SSC. One hundred microliters of alkaline phosphatase-conjugated streptavidin solution (Bethesda Research Laboratories), diluted 1/1,000 with incubation solution (0.1 M NaCl / 0.1 M Tris-HCl pH 7.5 / 2 mM $MgCl_2$ / 0.05% Triton X-100), was added to the well and the well was incubated for 15 min at 25 °C. After incubation, the well was rinsed three times with 200 μl of incubation solution, then 100 μl of 1 M diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$ and 10 mM *p*-nitrophenylphosphate as substrate was added, and the colorimetric reaction proceeded at 25 °C for 30 min. Absorbance of each well was read at 405 nm using a microtiter-plate reader (MPR-A4, Tosoh, Tokyo, Japan). Data were corrected by subtraction of the background corresponding to the chromogenic reagent itself.

RESULTS

Detection sensitivity of microtiter plate-hybridization. We performed experiments using blood samples containing cultured *P. falciparum* parasites. The PCR-amplified products were analyzed by agarose gel electrophoresis and fragments which corresponded to the expected size of 135 base pairs were observed (data not shown). As shown in FIG. 2, there was correlation between absorbances at 405 nm of the microtiter plate-hybridization assay and parasite densities. Thirteen parasites in 10 μl of blood, which corresponds to 0.000026 % parasitemia were sufficient for detection by *P. falciparum* specific microtiter plate-hybridization assay. We used phenol/chloroform-extracted DNA of *P. falciparum* and *P. vivax* as positive controls. No cross reaction on the two kinds of species-specific microtiter plate-hybridization was observed. There was no significant difference in sensitivity among the three strains of *P. falciparum* parasites, FCO-1, FCR-3, and Honduras-1 (data not shown). The experiments using a series of samples containing *P. vivax* parasites revealed similar sensitivity of *P. vivax*-specific microtiter plate-hybridization, to that of *P. falciparum*-specific microtiter plate-hybridization (data not shown). In each experiment, two samples, one containing blood from an uninfected donor and the other containing all the components other than blood- or parasite-DNA, were used as negative

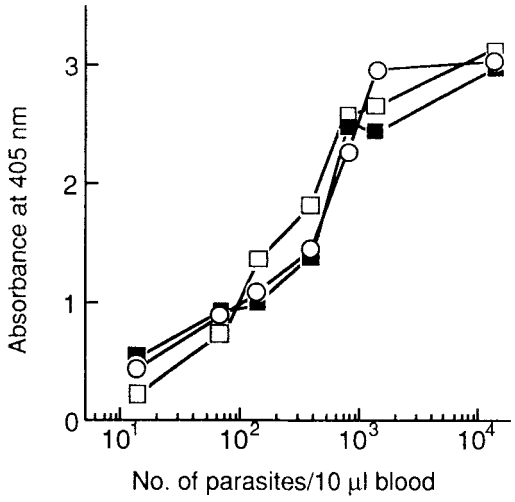

FIG. 2. Correlation between parasite densities and results of *P. falciparum*-specific microtiter plate-hybridization using cultured parasites. Mean values of duplicate samples are plotted. Three experiments were conducted independently.

controls. These negative controls (n = 66 each) gave no positive-signals by the assay. The values of the optical density of negative control were 0.083 ± 0.009 (mean ± SD, n = 66). Cutoff values were determined from twice optical dencity for negative control.

Field samples. Experiments were conducted to evaluate the usefulness of *P. falciparum* specific and *P. vivax* specific microplates in Guadalcanal, Solomon Islands. The results of the microtiter plate-hybridization assay, AO stained thin-smear microscopy, and thick-smear microscopy by the local microscopists are summarized in TABLE 1. As shown in TABLE 1, the results of microtiter plate-hybridization showed good correlation with those of AO microscopy. There were 10 samples which gave inconsistent results by microtiter plate-hybridization and AO microscopy for *P. falciparum*-infections and *P. vivax*-infections (FIG. 3A and FIG. 3B). There was considerable disagreement between the results obtained by thick-smear microscopy and those obtained by microtiter plate-hybridization or AO microscopy. There were 78 cases (60 %) whose results by thick-smear microscopy were coincidental with those of microtiter plate-hybridization, and 70 cases (54 %) whose results obtained by thick-smear microscopy were coincidental with those obtained by AO microscopy. *P. malariae* parasites were observed in 7 cases (5 %) by AO microscopy, and 6 of them were mixed infections in which *P. falciparum* or *P. vivax*

TABLE 1. Summary of comparisons among three diagnostic methods in the Solomon Islands.

Results of thick-smear microscopy			Results of microtiter plate-hybridization				Results of acridine orange microscopy				
			Negative	PF	PV	PF/PV	Negative	PF (+/PM) [c]	PV (+/PM) [c]	PF/PV	PM alone
Negative	63	(20) [a]	44	7	11	1	43	7 (0)	10 (3)	2	1
PF	29	(5) [b]	5	19	2	3	6	16 (1)	2 (0)	5	0
PV	37	(12) [b]	15	3	15	4	15	2 (2)	14 (0)	6	0
PF/PV	1		0	1	0	0	0	1 (0)	0 (0)	0	0
Total	130		64	30	28	8	64	26 (3)	26 (3)	13	1

PF = *P. falciparum*, PV = *P. vivax*, PM = *P. malariae*, PF/PV = mixed infection of *P. falciparum* and *P. vivax*

a : False negative (positive by acridine orange microscopy or microtiter plate-hybridization)

b : False positive (negative by both acridine orange microscopy and microtiter plate-hybridization)

c : Mixed infection with *P. malariae*

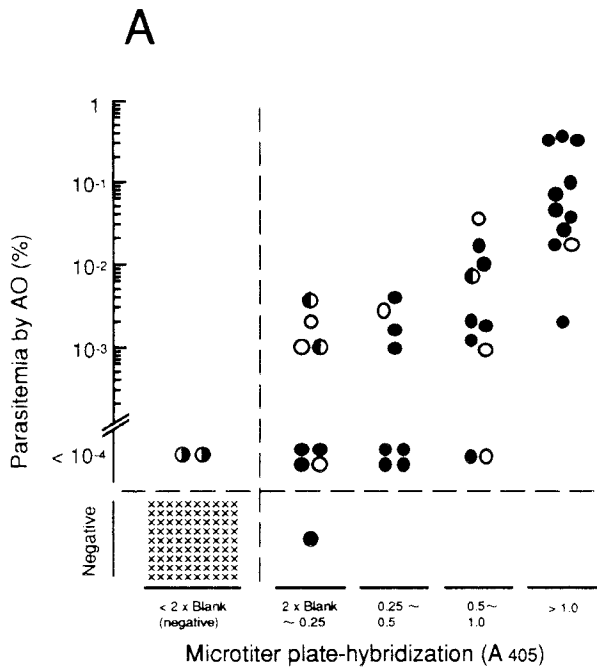

FIG. 3. Results of *P. falciparum* specific (A) and *P. vivax* specific (B) microtiter plate-hybridization assays in the Solomon Islands.
AO = acridine orange microscopy. Crosed circles show the cases which were positive only for either *P. vivax* or *P. falciparum* by either AO microscopy or microtiter plate-hybridization. Open circles show the cases which gave positive results by both *P. falciparum*-specific and *P. vivax*-specific microtiter plate-hybridization. Left-semiclosed circles show the cases which were both *P. vivax* and *P. falciparum* positive by AO microscopy, but positive only for *P. falciparum* by microtiter plate-hybridization. Right-semiclosed circles show the cases which were both *P. vivax* and *P. falciparum* positive by AO microscopy, but positive only for *P. vivax* by microtiter plate-hybridization. Small cross marks show the cases which were negative for *P. falciparum* or *P. vivax* by both AO microscopy and microtiter plate-hybridization. 2 x Blank : cutoff values

parasites were also detected. Neither the *P. falciparum* specific probe nor the *P. vivax* specific probe showed cross reactivity with *P. malariae* parasites [23]. The local microscopists could not identify *P. malariae* parasites in the 7 thick smears. Single infection of *P. malariae* by AO microscopy in TABLE 1 was classfied as negative by our methods and AO microscopy in FIG. 3, because *P. malariae*-specific microtiter plates [23] had not been prepared at that time.

The correlation between parasitemias of the field samples and their corresponding results of the microtiter plate-hybridization assay in Guadalcanal are shown in FIG. 3A

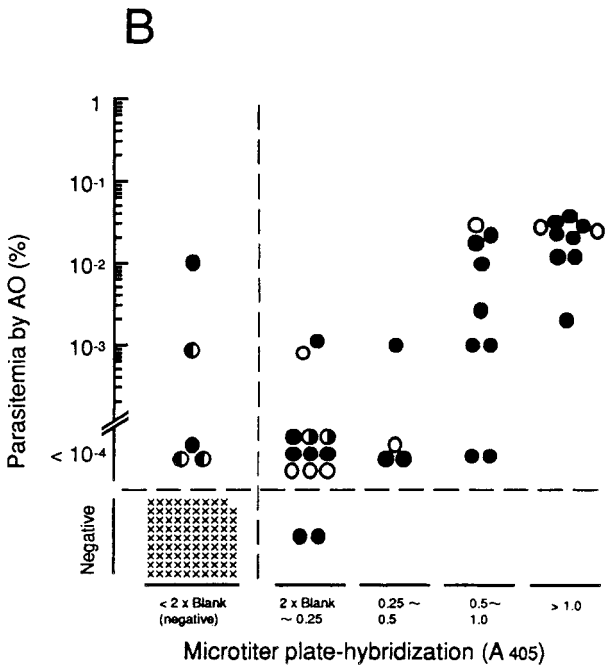

FIG. 3. Continued

and FIG. 3B. Samples of high parasite densities gave high absorbances by both *P. falciparum*-specific and *P. vivax*-specific microtiter plate-hybridization. The details of the results in FIG. 3A and FIG. 3B are mentioned and discussed in the next section.

DISCUSSION

We have developed a new DNA diagnostic system which provides a rapid, sensitive, and specific means of detecting the amplified sequences without the need for prior DNA extraction, filtration, preparation of radiolabeled probes, gel electrophoresis, or blotting techniques. In this method, the target sequence is amplified using primers that are specific to human malaria parasites, after which, the amplified products are detected with a species-specific probe. The two-step selection of the target sequence contributes to the high specificity. The complete assay of microtiter plate-hybridization can be performed within 6 hours.

The sensitivity is limited by the efficiency of the amplification reaction; 13 parasites in 10 μl of blood can be detected reproducibly under the conditions described in the paper. The sensitivity corresponds to that of thin-smear microscopy with the examination

of approximately 4,000 oil immersion fields, and is higher than that of routine thick-smear microscopy. Significant differences were not found in the sensitivity of the *P. falciparum* specific microtiter plate-hybridization among the three strains of *P. falciparum* , FCR-3 (Gambia), FCO-1 (Indonesia), and Honduras-1 (Honduras). *P. falciparum* parasites in Guadalcanal were also detected by our method. Therefore, the microtiter plate-hybridization can be used worldwide for the detection of *P. falciparum* parasites in human blood. Experiments using cultured parasites and field blood samples showed correlation between parasite densities and the results of species-specific microtiter plate-hybridization assay, indicating that this method can be used not only to detect the presence of malaria parasites, but also to obtain semiquantitative information about parasite density in the blood.

The microtiter plate-hybridization method allowed us to detect and identify *P. falciparum* and *P. vivax* . We have also constructed *P. ovale* specific and *P. malariae* specific microtiter plate-hybridizations using the same primers for clinical blood samples [23]. Therefore, our microtiter plate-hybridization method can be used for species-specific detection of the four species of human malaria parasites. Recently, Snounou et al. reported a PCR-based diagnostic method, in which a species-specific sequence of the SSUrRNA gene allowed identification of the four species [17]. The system requires phenol/chloroform extraction of DNA from the blood, and 4 separate PCR-amplifications must be carried out with every sample to detect each species. Our microtiter plate-hybridization method requires only a single PCR-amplification with a single set of primers.

Among the 130 samples tested, 5 were *P. vivax* positive by AO microscopy, but negative by *P. vivax* specific microtiter plate-hybridization as shown in FIG. 3B. It was not likely that the *P. vivax*-specific probe failed to detect a small number of *P. vivax* parasites, because 2 of the 5 cases had considerably high parasitemia (0.01 % and 0.0008 %). Moreover, 3 of the 5 cases were *P. falciparum* positive by both microtiter plate-hybridization and AO microscopy as shown in FIG. 3A, suggesting that PCR-amplification had been successful. It follows that if *P. vivax* parasites were contained in the samples, positive results must have been obtained. We think that the parasites identified as *P. vivax* in these 5 cases might be the new malarial species, *P. vivax*-like parasite, which was reported by Qari et al. [29]. The *P. vivax*-like parasite, which may not be distinguishable from *P. vivax* by microscopy, was found in Papua New Guinea [29], near the Solomon Islands. Therefore, it is possible that the *P. vivax*-like parasite also exists in the Solomon Islands and the sequence of the SSUrRNA gene of this species differs from that of *P. vivax* . Species-identification analysis is now in progress using DNA extracted from the Giemsa-stained blood films [30].

In two cases, microtiter plate-hybridization gave positive results only with the *P. vivax*-specific probe, while parasites of *P. vivax* and only gametocytes of *P. falciparum*

were detected by AO microscopy, as shown in FIG. 3A and FIG. 3B. These discrepancies were possibly due to the fragility of gametocyte-infected RBCs due to the induction of gametogenesis [31]. Gametocyte-infected RBCs were probably disrupted during sample storage and parasite DNA was dissolved in PBS during the washing process, causing a decrease in the efficiency of PCR-amplification. Conversely, three cases were positive by microtiter plate-hybridization, but negative by AO microscopy. Reexamination of Giemsa-stained thin smears detected malaria parasites (parastemia: 0.00005~0.00007%). We think these discrepancies were due to the higher sensitivity of the microtiter plate-hybridization than that of AO microscopy.

Although it had been reported that there are *P. malariae* infections in Malaita, Solomon Islands [32], we tested only *P. falciparum*-specific and *P. vivax*-specific microplates in Guadalcanal, because *P. malariae*-specific microtiter plates had not been prepared at that time. In this study, AO microscopy revealed that a considerable number of *P. malariae* infections exist in Guadalcanal. It is a problem that the coincident rate of thick-smear microscopy with microtiter plate-hybridization was much lower than that of AO microscopy with microtiter plate-hybridization. The local microscopists did not care for dehemoglobinization of RBCs, that caused unclear background on the slides and made it difficult to distinguish parasites from ghosts of RBCs or other particles.

Evaluation of the microtiter plate-hybridization has been done with clinical blood samples. We examined 404 blood samples from 183 donors who was suspected of malaria at the Institute of Medical Science, University of Tokyo. Among the 183 cases, 32 (17 %) were *P. falciparum*-positive, 25 (14 %) were *P. vivax*-positive, 12 (7 %) were *P. ovale*-positive, and 2 (1 %) were *P. malariae*-positive by species-specific microtiter plate-hybridization (details will be published elsewhere). These results were completely consistent with those of thin-smear microscopy. In clinical cases, absorbance as a result of species-specific microtiter plate-hybridization decreased, corresponding to the reduction of parasite density and improvement of the clinical course [23].

The microtiter plate-hybridization is simple and sensitive detection system which does not require either prior DNA extraction from the blood or radiolabeled isotopes. It should also be emphasized that our DNA diagnosis does not depend upon the skill of individual examiners, making it a useful tool in malaria control programs.

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