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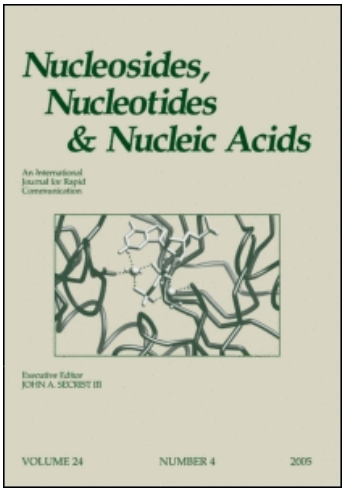

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DNA Diagnosis of Ovale Malaria and Malariae Malaria Using Microtiter Plate-Hybridization

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NUCLEOSIDES & NUCLEOTIDES, 13(6&7), 1363-1374 (1994)

DNA DIAGNOSIS OF OVALE MALARIA AND MALARIAE MALARIA USING MICROTITER PLATE-HYBRIDIZATION §

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ABSTRACT: We have developed a colorimetric assay, "microtiter plate-hybridization", for the detection of human malaria parasite, *Plasmodium ovale* and *Plasmodium malariae*, in which the target DNA sequence of the 18S ribosomal RNA gene is amplified by polymerase chain reaction and hybridized with the species-specific probes immobilized on a microtiter well.

INTRODUCTION

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]

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§ This paper is dedicated to Dr. Morio Ikehara, Emeritus Professor of Osaka University, on the celebration of the 70th birthday.

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There are four plasmodial species, *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, that cause human malaria. Several researchers have reported various DNA probe-based or PCR-based diagnostic methods for the detection of *P. falciparum*.[3-16] Recently, sequences of the gene coding for the 18S small subunit ribosomal RNA (18S rRNA) of malaria parasites have been reported[17-19] and used as the target for specific detection of malaria parasites.[10,16,20,21] These 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 be of great advantage.

We have developed 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. This assay system allowed us to detect and identify the two human malaria parasites, *P. falciparum* and *P. vivax* in blood samples, using the DNA sequence of 18S rRNA gene as the target.[22] Here we report the construction of *P. ovale*-specific and *P. malariae*-specific microtiter plate-hybridization using the 18S rRNA gene sequence and the results of *P. ovale*-specific microtiter plate-hybridization on clinical blood samples from ovale malaria patients. Our DNA diagnostic method can detect the four species of human malaria parasites, showing that it has the potential for replace microscopy and will contribute to malaria control.

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MATERIALS AND METHODS

Clinical blood samples

Blood samples were obtained from ovale malaria patients in the Hospital of the Institute of Medical Science, University of Tokyo. They had visited ovale malaria-endemic areas and returned to Japan: Patient A, a 30-years-old male, who had been infected in Equatorial Guinea; Patient B, a 32-years-old male, who had stayed in Ghana; Patient C, a 26-years-old female, who had visited Mari, Ghana, and Côte d'Ivoire; Patient D, a 22-years-old male, who had stayed in Tanzania and Kenya. In these cases, *P. ovale* parasites were detected by thin blood-smear microscopy with Giemsa staining. *P. ovale* DNA was extracted from the blood obtained from Patient A and Patient B. The DNA from Patient B was amplified by PCR, and used for sequence analysis. Blood samples obtained from Patient C and Patient D were subjected to a microtiter plate-hybridization assay.

Blood samples were obtained from a malariae malaria patient who was a 29-years-old male and had been infected in Cameroon, and used for examination and evaluation of the microtiter plate-hybridization. *P. vivax* DNA was also prepared from a vivax malaria

patient and *P. falciparum* DNA was prepared from *in vitro* culture. These DNA were used as control templates in this study.

For a microtiter plate-hybridization assay, 10 µl of whole blood was suspended in 150 µl of phosphate-buffered saline containing 10 units/ml nystatin and 25 µg/ml gentamicin (PBS-NG).[15] Simultaneously, a thin blood smear was also prepared and stained with Giemsa for microscopic examination. Parasites were counted until 500 leukocytes have been counted, and parasite density (number of parasite per 1 µl blood) was calculated.

Preparation of *P. ovale* DNA

Whole blood (20 ml) was obtained with a heparinized syringe from Patient A and Patient B. The blood was suspended in 180 ml of phosphate-buffered saline (PBS) and centrifuged at 1,000 x *g* for 10 min. The precipitate was resuspended in 30 ml of PBS containing 0.15% saponin and incubated at 37°C for 20 min. One hundred and eighty milliliters of PBS was added to the mixture and centrifuged at 1,000 x *g* for 15 min at room temperature. After centrifugation, the precipitate was washed with PBS (1,000 x *g* for 15 min) three times. The pellet was suspended in PBS and centrifuged at 7,000 x *g* for 5 min. The pellet obtained was again suspended in 1.5 ml of TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) containing 0.1 M NaCl, 0.5% sodium dodecyl sulfate (SDS), and proteinase K (200 µg/ml). The mixture was incubated overnight at 37°C with gentle shaking. A one-third volume of saturated sodium chloride was added to the mixture, and the suspension was shaken for 15 sec and centrifuged at 7,000 x *g* for 15 min. DNA was isolated from the supernatant by phenol-chloroform extraction.[23] *P. vivax* DNA was also extracted from the blood by the same method described above. For preparation of *P. falciparum* DNA, 1 ml of a culture was suspended in 10 ml of PBS, centrifuged at 1,000 x *g* for 10 min, and treated with the same procedures described above.

PCR primers

Two sets of oligonucleotide primers, PF-1 (5'-GAACGAAAGTTAAGGGAGT-3') and PF-2 (5'-ACTGAAGGAAGCAATCTAA-3'), and MPH-1 (5'-TCAGATACCGTCG TAATCTT-3') and MPH-2 (5'-CCAAAGACTTTGATTTCTCAT-3'), which are specific to the highly conserved regions of the 18S rRNA genes of *P. falciparum*,[17] *P. vivax*,[18] and *P. malariae*[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 PF-2 and 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 fragments for PF-2 and MPH-1 were reacted with ε-caproylamidobiotin-*N*-hydroxysuccinimide ester (Bethesda Research Laboratories,

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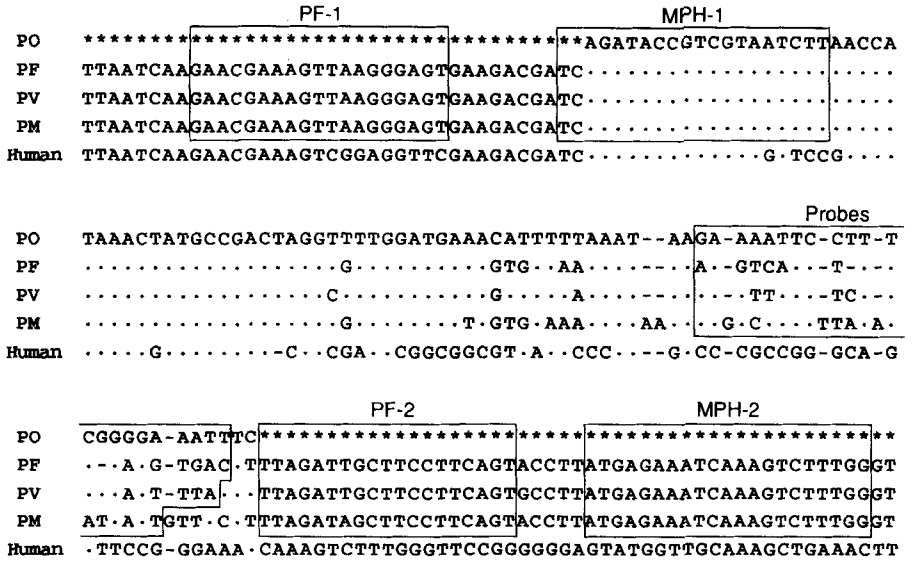

FIG. 1. Partial sequences of the 18S rRNA genes in the four species of human malaria parasites. PO = *P. ovale*, PF = *P. falciparum*, PV = *P. vivax*, PM = *P. malariae*. Sequences are aligned with reference to *P. ovale* sequence. Nucleotide homology is indicated by points. A dash shows that no corresponding nucleotide exists. An asterisk shows that the corresponding nucleotide has not been determined. The regions used for primers or probes are enclosed by solid lines. The sequence of human 18S rRNA gene is also presented.[25]

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Gaithersburg, Maryland, USA). The labeled fragments were purified by gel filtration and reversed phase HPLC.

Determination of partial DNA sequence of the 18S rRNA gene of *P. ovale*

P. ovale DNA extracted from the blood of Patient B was subjected to PCR using PF-1 and PF-2 as primers. The resulting PCR products were reamplified using PF-1 and 5'-biotinylated PF-2 as primers. Single-stranded DNA was prepared from the PCR products using streptavidin-coated magnetic beads (Dynabeads, Dynal AS, Olso, Norway). The biotinylated strand was sequenced by the dideoxy-nucleotide chain termination method (Sequenase Version 2.0, United States Biochemical, Cleveland, Ohio, USA) using PF-1.

***P. ovale*-specific and *P. malariae*-specific probes**

A species-specific probe (5'-AATTTCCCCGAAAGGAATTTTC-3') was designed from the sequence of the 18S rRNA gene of *P. ovale*, as shown in Fig. 1. *P. malariae*-specific probe (5'-ACTCATATATAAGAATGTCTC-3') was also designed from the published sequence.[19] Tandem repetitive probes were prepared and immobilized on microtiter wells (MS-3508F, Sumitomo Bakelite, Tokyo, Japan) by the method of Kawai et al.[24]

Cell lysis and DNA amplification of parasites

To hemolyze all erythrocytes, saponin (final concentration, 0.02%) was added to the blood suspension (10 µl of whole blood in 150 µl of PBS-NG), which was then maintained for 5 min at room temperature (25°C). The mixture was centrifuged at 7,000 x *g* for 10 min to remove components that might inhibit the PCR.[11, 13, 15] After the pellet was washed with 200 µl of PBS, 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, and 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 MPH-2 and 5'-biotinylated MPH-1, 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, and 1 unit of Tth DNA polymerase (Toyobo, Osaka, Japan)) was added to the above solution and the 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

Fig. 2 shows the principle of the 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*-nitrophenyl phosphate 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. *P. ovale*-specific, *P. falciparum*-specific, and *P. vivax*-specific microtiter plate-hybridization assays were performed on the clinical blood samples from Patient C and Patient D. A complete assay required 6 hours from sample preparation to diagnosis.

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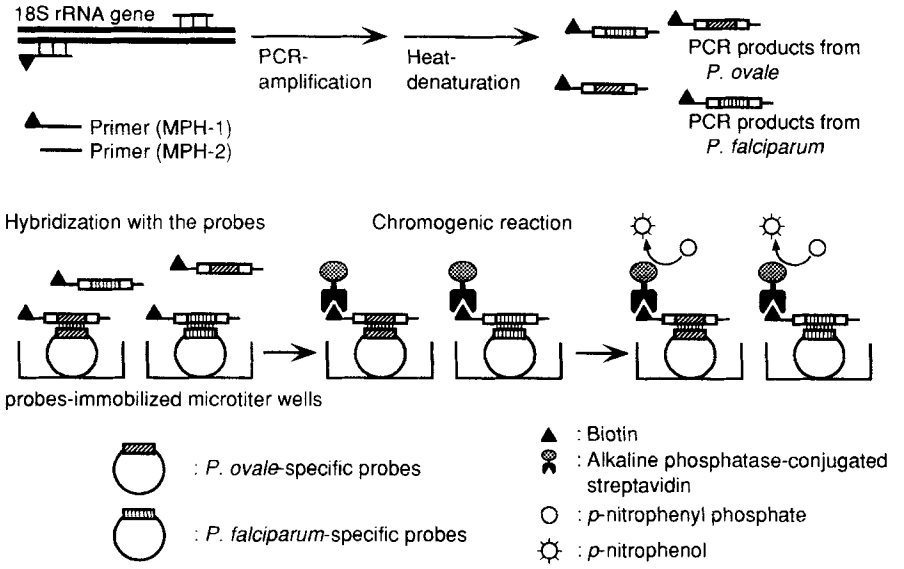

FIG. 2. Principle of microtiter plate-hybridization.

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Specificity of the species-specific microtiter-plate hybridization

Experiments were conducted to evaluate the specificity of the species-specific microtiter-plate hybridization. *P. ovale* DNA extracted from the blood of Patient A was amplified by the PCR using MPH-2 and 5'-biotinylated MPH-1 as primers. *P. vivax* DNA and *P. falciparum* DNA were also amplified using the same primers. The PCR products were purified by HPLC, and 30 ng of each DNA was added to a probes-immobilized microtiter well. The blood sample obtained from the malariae malaria patient was lysed and subjected to the PCR-amplification as described above. The PCR products of *P. malariae* parasites were reamplified in the same conditions, and 10 μl of the resulting mixture was used for the microtiter plate-hybridization.

RESULTS

Sequence of the 18S rRNA gene of *P. ovale*

The 90 base pairs (bp) of the 18S rRNA gene sequence of the PCR product of *P. ovale* have been determined (DNA Data bank of Japan; accession no. D17580) and are presented in Fig. 1. The comparison of the DNA sequences reveals a high degree of conservation and a significant difference within the four species of human malaria parasites.

We constructed a *P. ovale*-specific probe from the species-specific region in the determined sequence. We also constructed a *P. malariae*-specific probe from the published sequence.[19]

Specific detection of *P. ovale* DNA by microtiter plate-hybridization

We examined specificity of *P. falciparum*-specific,[22] *P. vivax*-specific,[22] *P. ovale*-specific, and *P. malariae*-specific microtiter plate-hybridization using amplified DNA of each species of parasites (Table 1). Each species-specific probe gave a positive result with the corresponding PCR products. No cross reaction on the four kinds of species-specific microtiter plate-hybridization was observed. *P. malariae*-specific probes gave a low level of absorbance with amplified *P. malariae* DNA.

Clinical blood samples examined by the microtiter plate-hybridization

We examined 3 blood samples (day 1, day 4, and day 28, after admission) from Patient C, and 7 samples (day 1 to day 7) from Patient D. In the case of Patient C, chloroquine phosphate (600 mg base in a single dose) was administrated to the patient on day 1, after the first blood sample had been obtained. The results of *P. ovale*-specific microtiter plate-hybridization were 0.374 (day 1), 0.209 (day 4), and 0.006 (day 28). Parasites were detected on a thin blood smear of day 1 (350 parasites/μl), but not detected on day 4, while *P. ovale*-specific microtiter plate-hybridization gave a positive result on day 4. On day 28, both *P. ovale*-specific microtiter plate-hybridization and thin-smear microscopy gave negative results.

Fig. 3 shows the clinical course of Patient D. In this case, chloroquine phosphate was administrated on day 1 (total 900 mg base; 600 mg as first dose, and 300 mg 12 hours later), day 2 (300 mg base in a single dose), and day 3 (300 mg base in a single dose). Primaquine diphosphate (15 mg base in a single dose per day) was given from day 4 to day 17 (total 14 days). The results of *P. ovale*-specific microtiter plate-hybridization showed a correlation with the clinical course. However, there were two discrepancies between the results of *P. ovale*-specific microtiter plate-hybridization and the parasite densities. From day 1 to day 2, parasite density reduced from 2,240 to 1,060 parasites/μl, while the absorbance showed an extreme reduction (from 0.735 to 0.230). On the other hand, absorbance increased from day 2 (0.230) to day 3 (0.341), although parasite density decreased from 1,060 to 59 parasites/μl. The blood sample of day 5 gave an absorbance of 0.117, which was higher than other negative results. This sample was, however, judged as negative, because the absorbance of the chromogenic reagent at this time was 0.148, which was higher than that of day 5.

In the two cases, none of the samples gave a positive result by *P. falciparum*-specific or *P. vivax*-specific microtiter plate-hybridization. We did not perform *P. malariae*-specific microtiter-plate hybridization with the samples, because *P. malariae*-specific microtiter plates had not been prepared at that time.

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TABLE 1. Species-specificity of microtiter plate-hybridization.

PCR products	Absorbance at 405 nm of species-specific microtiter plate-hybridization			
	PF	PV	PO	PM
PF	0.64	0.00	0.01	0.01
PV	0.00	1.14	0.01	0.01
PO	0.00	0.00	0.55	0.01
PM	0.01	0.02	0.02	0.14

PF = *P. falciparum*, PV = *P. vivax*, PO = *P. ovale*, PM = *P. malariae*.

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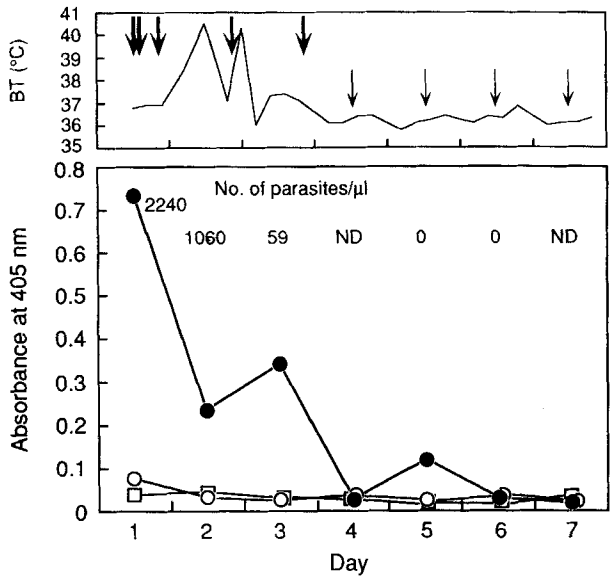

FIG. 3. The clinical course of an ovale malaria patient. Body temperatures (BT) and drug administrations are presented in the upper panel. A thick arrow shows an administration of one tablet of chloroquine phosphate (300 mg base). A thin arrow shows an administration of primaquine diphosphate (15 mg base). Primaquine was given for total 14 days. Results of species-specific microtiter plate-hybridization and calculated parasite densities are shown in the lower panel. Closed circles = *P. ovale*-specific microtiter plate-hybridization. Open circles = *P. falciparum*-specific microtiter plate-hybridization. Open squares = *P. vivax*-specific microtiter plate-hybridization. ND = not done.

DISCUSSION

We determined 90 bp of the 18S rRNA gene of *P. ovale*. It is likely that the full sequence includes the same sequences of PF-1 and PF-2, because we used the PCR-product amplified with these primers for the sequence analysis. *P. ovale* DNA was also amplified with MPH-1 and MPH-2, and the resulting product gave a band of 137 bp by agarose gel electrophoresis (data not shown). This size of the band corresponds to that of the expected products if the full sequence contains the same sequences of MPH-1 and MPH-2. Therefore, we think that the sequences of MPH-1 and MPH-2 are also included in the full sequence of the 18S rRNA gene of *P. ovale*. Further sequence analysis will reveal these points.

P. ovale-specific microtiter plate-hybridization gave a species-specific result with *P. ovale* DNA, and no cross reaction was observed. The sequence of the target DNA was obtained from the blood of Patient B, from which the *P. ovale*-specific probe was constructed. The *P. ovale*-specific probe hybridized with the amplified DNA derived from Patient A, Patient C, and Patient D. Therefore, we think that the *P. ovale*-specific probe has been established.

The *P. malariae*-specific probe was constructed from the published sequence. Species-specificity of the probe was shown in Table 1, which is good enough for the diagnosis of malariae malaria. We tested the probe with blood samples obtained from a malariae malaria patient, whose amplified DNA gave a positive, but low level of absorbance. It was not likely that amplified products contained only a small number of the target sequences, because the blood sample used for PCR-amplification contained enough number of *P. malariae* parasites (33,580 parasites/10 μl blood). We think the low absorbance might be due to a difference of the target sequence between the parasites in the blood sample and the published sequence. Amplified target DNA, whose sequence might be different from the probe sequence, might have hybridized weakly with the *P. malariae*-specific probe, causing the low absorbance. We will make a sequence analysis of amplified DNA from *P. malariae* parasites, although we rarely have a case of malariae malaria in Japan. Further experiments will reveal above points.

In the case of Patient C, absorbance as a result of *P. ovale*-specific microtiter plate-hybridization decreased, corresponding to the reduction of parasite density and improvement of the clinical course. The sample of day 4 was positive by *P. ovale*-specific microtiter plate-hybridization, but negative by thin-smear microscopy, suggesting the usefulness of the microtiter plate-hybridization. However, an extensive follow-up should have been made on this patient, because she was pregnant and so had not been given primaquine treatment.

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In the case of Patient D, a certain degree of correspondence was shown between absorbances of *P. ovale*-specific microtiter plate-hybridization and parasite densities. However, there were some discrepancies between DNA diagnosis and microscopy. We think that there are two possibilities for the explanation of the discrepancies. First, clinical blood samples from an ovale malaria patient contain *P. ovale* parasites in all stages of its erythrocytic life cycles, including schizonts and microgametocytes that have multiple nuclei.[8] Therefore, a parasite density does not always mean a genome density, causing variation of number of the target sequences in a blood sample. Previous researchers have focused their interests on detecting *P. falciparum*, the only species whose infected blood mostly contains ring forms with one chromatin. Thus, they could use parasite density as a parallel with target density. In our experiments, there is not correspondence between parasite density and target density, for the detection of *P. ovale*, *P. vivax*, and *P. malariae*. The second explanation is that microscopic examination might not allow us to know the level of degeneration of parasite DNA in response to a drug administration. Parasite DNA on day 2 might have been degenerated by chloroquine treatment, causing the decrease of the target sequences, although microscopic images of parasites were still intact. On day 3, number of parasites also decreased on the blood film, while number of the target sequences might not have been changed. If this explanation is adequate, our microtiter plate-hybridization might show the response of parasites to a drug treatment more accurately than microscopic examination.

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In this study, we could not determine the range of detection of *P. ovale* or *P. malariae* parasites in human blood by the species-specific microtiter plate-hybridization. Another experiment using other blood samples revealed that *P. ovale*-specific microtiter plate-hybridization could detect at least 100 parasites in 10 μl of blood (data not shown). We think that *P. ovale*-specific microtiter plate-hybridization has similar range of detection to *P. falciparum*-specific and *P. vivax*-specific microtiter plate-hybridization, in which there was correlation between absorbances and parasite densities within 65 to 13,000 parasites in 10 μl of blood.[22] A blood sample containing larger number of parasites may give a saturated absorbance. The *P. malariae*-specific probe seemed to have lower sensitivity than the *P. ovale*-specific probe, by an experiment using blood samples obtained from only one case of malariae malaria. We will determine the actual detection range of both *P. ovale*-specific and *P. malariae*-specific microtiter plate-hybridization, although we rarely have a case of ovale malaria or malariae malaria in Japan.

In clinical conditions, *P. ovale*-specific microtiter plate-hybridization is useful for evaluation of drug treatment. We will evaluate both *P. ovale*-specific and *P. malariae*-specific microtiter plate-hybridization under field conditions in endemic areas. Our microtiter plate-hybridization, which is simple, rapid, and adapted to field applications,[22]

would be of great advantage. Moreover, the system allowed us to detect and identify the four species of human malaria parasites, showing that it has the potential for replace microscopy and will contribute to malaria control.

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