

## NOTES

### Sequence Variation in the 18S rRNA gene, a Target for PCR-Based Malaria Diagnosis, in *Plasmodium ovale* from Southern Vietnam

FUMIHIKO KAWAMOTO,<sup>1\*</sup> HIROFUMI MIYAKE,<sup>2</sup> OSAMU KANEKO,<sup>3</sup> MASATSUGU KIMURA,<sup>4</sup>  
NGUYEN THI DUNG,<sup>5</sup> NGUYEN THE DUNG,<sup>5</sup> QING LIU,<sup>1</sup> MIAN ZHOU,<sup>1</sup> LE DUC DAO,<sup>6</sup>  
SHINTARO KAWAI,<sup>7</sup> SHIN ISOMURA,<sup>1</sup> AND YUSUKE WATAYA<sup>2</sup>

Department of Medical Zoology, Nagoya University School of Medicine, Nagoya,<sup>1</sup> Faculty of Pharmaceutical Sciences, Okayama University, Okayama,<sup>2</sup> Department of Medical Zoology<sup>3</sup> and Laboratory of Biophysics,<sup>4</sup> Osaka City University Medical School, Osaka, and Wakunaga Pharmaceutical Co., Takatagun, Hiroshima,<sup>7</sup> Japan, and Center for Tropical Diseases, Ho Chi Minh City,<sup>5</sup> and Institute of Malariology, Parasitology and Entomology, Hanoi,<sup>6</sup> Vietnam

Received 22 April 1996/Returned for modification 14 May 1996/Accepted 29 May 1996

**Field surveys of malaria were performed in southern Vietnam by using an acridine orange staining method for rapid diagnosis and a PCR-based, microtiter plate hybridization method for accurate diagnosis. A total of three patients of *Plasmodium ovale* infection were detected, but PCR-amplified DNA of the *P. ovale* isolates from two of the patients did not hybridize with the *P. ovale*-specific probe. Analysis of the target sequence in the 18S rRNA gene indicated that in the DNA of isolates from both patients three nucleotides in the probe region from the typical *P. ovale* sequence were different, with deletions of two nucleotides and the substitution of one nucleotide. These results may suggest that in addition to molecular biological methods, careful microscopic examination of stained thin blood films is still required in studies of the prevalence of different malaria species.**

*Plasmodium ovale* infections have commonly been found in tropical Africa, the Middle East, Iranian Jaya, and Papua New Guinea but only sporadically in Southeast Asia (3, 5). For instance, in Thailand three new cases of *P. ovale* infections were recently detected by PCR (11, 12) about 25 years after the last published report of a *P. ovale* infection in that country (4). In Vietnam, four *P. ovale* infections were detected in American men in southern parts of the country in 1969 (5), but no cases have officially been recorded in Vietnam. Because accurate species diagnosis based on ordinary microscopic examination may be difficult, we hypothesized that *P. ovale* infections may have been overlooked in Vietnam.

The presence of *P. ovale* infections was investigated in field surveys conducted in four southern Vietnam provinces (Song Be, Lam Dong, Dac Lac, and Khanh Hoa) between July 1994 and December 1995 by an acridine orange (AO) staining method (7, 8) for rapid diagnosis in the field and a PCR-based, microtiter plate hybridization (MPH) method (1, 2, 10) for accurate diagnosis in central laboratories. By the MPH method, a segment of the small-subunit rRNA (18S rRNA) gene is amplified by PCR and is then hybridized with species-specific probes designed from the A genes of four human malaria parasites. The 18S rRNA gene is commonly used to make phylogenetic inferences and diagnoses, since its mutation rate has been shown to be very slow (6, 14). Here we report on three patients in different provinces in southern Vietnam with

*P. ovale* infections and describe sequence variations in the 18S rRNA genes of two *P. ovale* isolates which did not hybridize with the *P. ovale*-specific probe.

Thin smears were prepared with blood collected by finger prick from outpatients presenting in malaria clinics; the smears were fixed with methanol and stained with AO as described previously (7, 8). At the same time, 10  $\mu$ l of the blood samples was collected for diagnosis by the MPH method by using a tip ejector pipette, mixed with 150  $\mu$ l of sterile phosphate-buffered saline (pH 7.4), and kept on ice for 3 to 4 days before examination in Ho Chi Minh City. All thin smears were examined in the field at  $\times 400$  magnification for 3 to 5 min under ordinary light microscopes in combination with a halogen light source equipped with an interference filter (Toyo Optics, Fuchu, Japan). By the AO method, all developmental stages of malaria parasites and their characteristic shapes can easily be identified at  $\times 400$  magnification without immersion oil, since they fluoresce against the dark background (7, 8). In addition, observation of the morphologies of infected erythrocytes, i.e., enlargement or fimbriation, is also possible by removing the interference filter and examining the erythrocytes under a normal light so that most of the criteria for species identification which have been applied to conventional staining techniques, except for the detection of morphological traits associated with malaria pigments, can also be used by this method (8).

For patients positive by the AO method, an additional two to four thin and thick blood smears were prepared for further confirmatory microscopic examination and DNA isolation and sequencing (see below). If the patient agreed, 1.5 ml of venous blood was also collected. Sera and clotted blood, which were separated by centrifugation, were kept on ice in the field, and

\* Corresponding author. Mailing address: Department of Medical Zoology, Nagoya University School of Medicine, Nagoya 466, Japan. Phone: 81-(52)-744-2113. Fax: 81-(52)-744-2114. Electronic mail address: hiko@med.nagoya-u.ac.jp.

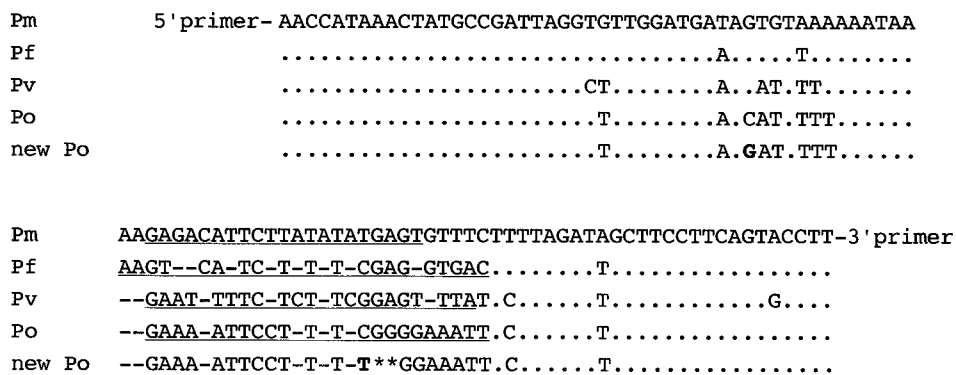


FIG. 1. Partial sequences of the A genes of four human malaria parasites and a new mutated form of *P. ovale*. Pm, *P. malariae*; Pf, *P. falciparum*; Pv, *P. vivax*; Po, *P. ovale*. Sequences are aligned with reference to the *P. malariae* sequence. Nucleotide identity is indicated by dots, and the lack of the corresponding nucleotide is indicated by dashes. Underlined sequences indicate the probe regions of each species used for diagnosis by the MPH method. Boldface letters indicate mutated nucleotides in the new *P. ovale*, and asterisks indicate nucleotides deleted from a typical sequence.

were then kept at  $-20^{\circ}\text{C}$  in laboratory facilities until they were used.

The MPH method was performed as described previously (1, 2, 10). Briefly, the *Plasmodium*-specific 18S rRNA gene was amplified from 10- $\mu\text{l}$  blood samples by using a pair of universal primers, biotinylated MPH-1 (5'-biotin-CAGATACCGTCGT AATCTTA-3') and MPH-2 (5'-CCAAAGACTTTGATTTCT CAT-3'). The 138- to 150-bp PCR product was then hybridized with species-specific probes for the four human malaria parasites (Fig. 1), which were previously immobilized in microtiter plate wells and detected with alkaline phosphatase-conjugated streptavidin.  $A_{405}$  measurements were made with a microplate reader (MPR-A4; Tosho Co., Tokyo, Japan).

Parasite DNA was also isolated from thick blood smears or, when available, clotted blood samples from all AO-positive patients. The procedures were adapted from a previously described technique (9). Briefly, thick smears (air dried and methanol fixed) were scraped with a clean razor blade and mixed with 100  $\mu\text{l}$  of HBS (HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-buffered saline; 140 mM NaCl, 10 mM KCl, 1 mM  $\text{MgCl}_2$ , and 10 mM HEPES [pH 7.0]). Blood clots (100  $\mu\text{l}$ ) were also mixed with 100  $\mu\text{l}$  of HBS. Then, 100  $\mu\text{l}$  of 30% Chelex-100 in water was added to these samples, and the mixture was boiled for 10 min, vortexed briefly, and centrifuged. The supernatant separated from Chelex-100 was used as a DNA template for PCR.

The amplified DNA products were sequenced by a TA cloning system (Invitrogen, San Diego, Calif.) and a Dye Terminator kit with a 373A Sequencer (Applied Biosystems, Foster City, Calif.) by following the manufacturer's instructions. In addition, parasite DNA isolated from thick smears and clotted blood was amplified by PCR with the same MPH primers, but without biotin, and the PCR products were directly sequenced. The amplified DNAs were electrophoresed in a 2.5% agarose gel, excised, and purified with a Cleanmix kit (Talent, Trieste, Italy). Then, the purified PCR products were sequenced with the Dye Terminator kit. The nucleotide sequences obtained were compared with reported sequences.

During field surveys in July and August 1994, 249 malaria infections were diagnosed by the AO method in 780 outpatients (either symptomatic or asymptomatic) in the provinces of Song Be and Lam Dong. Of the samples from these malaria-positive patients, 108 were further blindly examined by the MPH method (Table 1). When discordant species diagnoses were obtained (18 samples), AO- and Giemsa-stained thin

smears were further examined. For all but one patient, the MPH-based diagnosis was confirmed. The only exception was a 13-year-old girl from Phu Rieng, Song Be Province. For this patient, the first AO-based diagnosis in the field was a mixed infection with *P. vivax* and *P. malariae*, but the DNA product (ca. 135 bp) amplified by the MPH method did not hybridize with any species-specific probe. Further examination of AO- and Giemsa-stained thin smears revealed the presence of trophozoites and young gametocytes resembling *P. ovale* and *P. malariae* (total parasitemia, 0.6%).

Analysis of the target sequences in 11 clones obtained by the TA cloning system and also by direct sequencing revealed that, except for four nucleotides, they were similar to that of the A gene of *P. ovale* (Fig. 1). In the region targeted by the species-specific probe, two nucleotides (G-G) were deleted, and a nucleotide substitution (from C to T) was also found. No clone was found to have *P. malariae* sequences, suggesting that the parasites identified as *P. malariae* isolates might be atypical *P. ovale* affected by artemisinin, because the patient had been given that drug 12 h before examination.

Two other cases of *P. ovale* infection were found in field surveys performed from December 1994 to December 1995, when more than 500 samples from AO-positive patients were examined. One case of infection was found in a 4-year-old girl from Bong Kran, Dac Lac Province, in July 1995, and only *P. ovale* was detected by both the AO (a single trophozoite) and the MPH diagnostic methods. No deletion or mutation was found in the 18S rRNA sequence of this isolate.

TABLE 1. Comparison of diagnostic results between the AO and MPH methods<sup>a</sup>

Isolate by MPH method (no. of isolates)	No. of isolates by AO method			
	Pf (68) <sup>b</sup>	Pv (31)	Pf+Pv (8)	Pv + Pm (1)
Pf (60)	60	0	0	0
Pv (31)	3 <sup>c</sup>	25	3 <sup>c</sup>	0
Pf + Pv (14)	5 <sup>c</sup>	4 <sup>c</sup>	5	0
Pm (2)	0	2 <sup>c</sup>	0	0
Negative (1)	0	0	0	1 <sup>d</sup>

<sup>a</sup> Pf, *P. falciparum*; Pv, *P. vivax*; Pm, *P. malariae*.

<sup>b</sup> Values in parentheses are total number of isolates.

<sup>c</sup> Parasite species identified by MPH further confirmed by reexamination by AO and Giemsa staining.

<sup>d</sup> Later identified as an *P. ovale* isolate with a sequence variation.

The final patient with a *P. ovale* infection was found in Bao Loc, Lam Dong Province, in December 1995. At the Bao Loc Hospital, thin and thick smears were collected from 94 malaria-positive patients during September to December 1995, and transported to Japan. By the AO diagnostic method, one of the patients (an 80-year-old woman) was found to be infected with three species (*P. ovale*, *P. malariae*, and *P. vivax*; total parasitemia, 0.022%). Parasite DNA was isolated from a thick smear and was applied to the MPH method for diagnosis. However, the patient was negative for *P. ovale* by the MPH method, as was the case for the first *P. ovale*-infected patient (the 13-year-old girl). By analyzing the target sequence in the 18S rRNA gene, the same sequence variation found in the gene from the isolate from the first patient was found in the *P. ovale* isolate from the 80-year-old woman.

Our findings confirm the presence of *P. ovale* in southern Vietnam (5) and suggest that infections with this species may be missed by the 18S rRNA-based PCR diagnostic method in the presence of deletions and mutations in the target sequences. Thus, we conclude that a careful microscopic examination of either AO- or Giemsa-stained thin smears is still required, in addition to molecular biological methods, in studies of the prevalence of different malaria species. The same sequence variation was also recently observed in *P. ovale* isolates from two Japanese patients infected in Madagascar and Kenya, respectively (13). Analysis of the full sequences (about 2 kbp) of the 18S rRNA gene of these two types of *P. ovale* isolates is in progress at Okayama University to determine whether these parasites represent a new variant of *P. ovale*.

We recommend the preferential use of blood clots for parasite DNA extraction, but stress that air-dried thick smears fixed with methanol are adequate for storage and transportation under field conditions and may be used to isolate parasite DNA when venous blood samples are difficult to obtain, as in Vietnam.

We are grateful to T. K. Anh, N. V. Kim, P. Tuyet-Anh, N. D. Sy, and H. V. Thien and to the Departments of Immunology and Entomology, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, for their help. We also thank the many Vietnamese collaborators who participated in the project.

This study was supported by Grants-in-Aid for International Scientific Research: Field Survey from the Ministry of Education, Science, and Culture, Japan (grants 06041053 and 07041159).

#### REFERENCES

1. Arai, M., K. Kunisada, S. Kawai, M. Kimura, and Y. Wataya. 1994. DNA diagnosis of *ovale* malaria and *malariae* malaria using microplate-hybridization. *Nucleosides Nucleotides* **13**:1363-1374.
2. Arai, M., K. Kunisada, H.-S. Kim, H. Miyake, C. Mizukoshi, T. Kakutani, A. Yamane, S. Nakagami, S. Kawai, H. Nakano, F. Kawamoto, and Y. Wataya. 1996. A colorimetric DNA diagnostic method for *falciparum* and *vivax* malaria: a field trial in the Solomon Islands. *Nucleosides Nucleotides* **15**:719-731.
3. Bangs, M. J., Purnomo, and R. L. Anthony. 1992. *Plasmodium ovale* in the highlands of Irian Jaya, Indonesia. *Ann. Trop. Med. Parasitol.* **86**:307-308.
4. Cadigan, F. C., and R. S. Desowitz. 1969. Two cases of *Plasmodium ovale* malaria from central Thailand. *Trans. R. Soc. Trop. Med. Hyg.* **63**:681-682.
5. Gleason, N. N., G. U. Fisher, R. Blumhardt, A. E. Roth, and G. W. Gaffney. 1970. *Plasmodium ovale* malaria acquired in Viet-Nam. *Bull. W.H.O.* **42**:399-403.
6. Gunderson, J. H., M. L. Sogin, G. Wollett, M. Hollindale, V. F. De La Cruz, A. P. Waters, and T. F. McCutchan. 1987. Structurally distinct, stage-specific ribosomes occur in *Plasmodium*. *Science* **238**:933-937.
7. Kawamoto, F. 1991. Rapid diagnosis of malaria by fluorescence microscopy using light microscope and interference filter. *Lancet* **337**:200-202.
8. Kawamoto, F., and P. F. Billingsley. 1992. Rapid diagnosis of malaria by fluorescence microscopy. *Parasitol. Today* **8**:69-71.
9. Kimura, M., O. Kaneko, A. Inoue, A. Ishii, and K. Tanabe. 1995. Amplification by polymerase chain reaction of *Plasmodium falciparum* DNA from Giemsa-stained thin blood smears. *Mol. Biochem. Parasitol.* **70**:193-197.
10. Kimura, M., H. Miyake, H.-S. Kim, M. Tanabe, M. Arai, S. Kawai, A. Yamane, and Y. Wataya. 1995. Species-specific PCR detection of malaria parasites by microtiter plate hybridization: clinical study with malaria patients. *J. Clin. Microbiol.* **33**:2342-2346.
11. Snounou, G., S. Viriyakosol, W. Jarra, S. Thaithong, and K. N. Brown. 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol. Biochem. Parasitol.* **58**:283-292.
12. Snounou, G., S. Viriyakosol, X. P. Zhu, W. Jarra, L. Pinheiro, V. E. do Rosario, S. Thaithong, and K. N. Brown. 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol. Biochem. Parasitol.* **61**:315-320.
13. Wataya, Y., et al. Unpublished data.
14. Waters, A. P. 1994. The ribosomal RNA genes of *Plasmodium*. *Adv. Parasitol.* **34**:33-79.