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NOTES

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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 Vietnamese provinces (Song Be, Lam Dong, Duc 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 μl of the blood samples was collected for diagnosis by the MPH method by using a tip ejector pipette, mixed with 150 μl 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 ×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 ×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
were then kept at −20°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-μl 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. A405 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 μl of HBS (HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid]-buffered saline; 140 mM NaCl, 9 mM KCl, 1 mM MgCl₂, and 10 mM HEPES [pH 7.0]). Blood clots (100 μl) were also mixed with 100 μl of HBS. Then, 100 μl 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. 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, Duc 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

<table>
<thead>
<tr>
<th>Isolate by MPH method (no. of isolates)</th>
<th>No. of isolates by AO method</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Pl (68)</td>
</tr>
<tr>
<td>Pl (60)</td>
<td>60</td>
</tr>
<tr>
<td>Pv (31)</td>
<td>25</td>
</tr>
<tr>
<td>Pl + PV (14)</td>
<td>25</td>
</tr>
<tr>
<td>Pm (2)</td>
<td>0</td>
</tr>
<tr>
<td>Negative (1)</td>
<td>0</td>
</tr>
</tbody>
</table>

*P. falciparum; Pv, P. vivax; Pm, P. malariae.

Values in parentheses are total number of isolates.

Parasite species identified by MPH further confirmed by reexamination by AO and Giemsa staining.

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. malarialae*, 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.

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REFERENCES


