A TRIAL FOR A DNA DIAGNOSIS OF PLASMODIUM VIVAX MALARIA RECENTLY REEMERGING IN THE REPUBLIC OF KOREA USING MICROTITER PLATE HYBRIDIZATION ASSAY

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Abstract. The polymerase chain reaction-based microtiter plate hybridization (PCR-MPH) assay was utilized for a DNA diagnosis of Plasmodium vivax malaria, which has recently reemerged in the Republic of Korea. The subjects were 18 parasite-proven patients and 5 healthy controls. Follow-up blood samples were collected from 4 patients after a standard course of treatment. Polymerase chain reaction and electrophoresis of all the patients’ blood showed a prominent band at the 138 base pair area, but not in the controls or after treating the patients. Hybridization of the PCR products with known species-specific probes of the 18S rRNA of various malaria species revealed strong positive reactions against the Plasmodium vivax-specific probe (absorbance 1.30–1.90 at 405 nm) in all of the patients. The absorbance was positively correlated with the degree of blood parasitemia, but with a borderline significance. Sequencing of the probe region of the Korean P. vivax revealed no significant variations from the typical P. vivax. The results show that the PCR-MPH is a highly useful technique for the DNA diagnosis of Korean vivax malaria.

INTRODUCTION

Plasmodium vivax, the only known species of malaria indigenous to the Republic of Korea, was highly prevalent until the 1970s when it disappeared. In 1993, however, a soldier working in the northern part of Kyonggi-do Province, who had never been abroad, developed fever and was diagnosed with P. vivax malaria. Since then, exponentially increasing numbers of malaria patients have been occurring each year: 25 cases in 1994, 107 in 1995, 356 in 1996, 1,724 in 1997, 3,932 in 1998, and 3,621 in 1999. Thus, P. vivax malaria has become an important reemerging disease in the Republic of Korea. Other than this type of malaria, tropical and quartan malaria have been diagnosed among Korean travelers who had been to tropical and subtropical countries.

The diagnosis of malaria is usually based upon the identification of parasites from Giemsa or Wright-stained thick or thin blood films. The method is simple and does not require sophisticated equipment. However, it is time-consuming and at times species diagnosis is difficult when the patient is mixed-infected, modified by a drug treatment, or reveals a low grade parasitemia. As an alternative to the morphological diagnosis, a dipstick test detecting a blood stage antigen known as histidine-rich protein II was introduced in Plasmodium falciparum infection, but it is not applicable to other species of malaria. The serological diagnosis has also been tried to detect antibodies, but it cannot discriminate past and present infections.

To overcome these difficulties, several researchers have developed DNA probe-based, RNA-probe based or polymerase chain reaction (PCR)-based procedures in order to detect malarial genes. Among them, the PCR-based microtiter plate hybridization (MPH) was reported to be a simple but highly useful technique for a DNA diagnosis of different species of malaria. However, more trials are needed to verify the usefulness and feasibility of the MPH technique. After the reemergence of P. vivax malaria in the Republic of Korea, the diagnosis of patients has usually been based on the examination of blood films, and DNA diagnosis has never been tried. Thus, in the present study, we evaluated the usefulness of the MPH technique in the genetic diagnosis of Korean P. vivax malaria.

MATERIALS AND METHODS

Patients. Eighteen P. vivax patients proven by parasites on blood films and 5 healthy controls were subjected to study. The patients consisted of 16 soldiers (males, 20–23 years old), who were admitted to a military hospital located in a northern part of suburban Seoul, and 2 civilians aged 5 years (female) and 19 years (male), who were admitted to the Seoul National University Hospital and Seoul Gangye Paik Hospital, respectively. After collecting the blood samples, all of the patients were treated with chloroquine for 3 days (10 mg/kg plus 5 mg/kg on the first day and 5 mg/kg per day for the following 2 days) and then primaquine for 14 days (15 mg daily). Follow-up blood samples were collected from 4 patients at Days 3–27 after the last dose of primaquine; other patients were not cooperative in follow-up studies.

Parasite density. The degree of parasitemia was determined by counting the total number of malaria-infected red blood cells (RBC) per 500 high power fields (x 1,000) in thin blood films. The percent parasitemia was calculated by dividing it by the total number of RBCs, and was expressed as the parasite density per μL blood.

Target sequences of malaria parasites. It is well known that the plasmodial genes encoding 18S rRNA contain species-specific regions: P. vivax, P. falciparum, Plasmodium malariae, Plasmodium ovale, and Plasmodium ovale-specific regions: P. vivax, P. falciparum, Plasmodium malariae, Plasmodium ovale, and Plasmodium ovale-specific probe (absorbance 1.30–1.90 at 405 nm) in all of the patients. The absorbance was positively correlated with the degree of blood parasitemia, but with a borderline significance. Sequencing of the probe region of the Korean P. vivax revealed no significant variations from the typical P. vivax. The results show that the PCR-MPH is a highly useful technique for the DNA diagnosis of Korean vivax malaria.
Species-specific probes (P. vivax, 5'-TAAACTGGAA-GAGAAATTC-3', P. falciparum, 5'-GTCCTTGAAAGTGACTT-3', P. malariae, 5'-ACTATATAAAGTATGTTC-3', P. ovale, 5'-GAAAATTCGAAAGTTTCCAAAG-GAATTTC-3') were prepared in the plasmid pUC-Si2 as described previously.11 The plasmid pUC-Si2 was used as a cloning vector for the probe.11 The procedure set forth by Kimura and others13 was used to immobilize the probes on plate wells.

Treatment of blood samples. Ten microliters of heparinized blood were suspended in 150 μL phosphate-buffered saline (PBS) containing 10 U/mL of nystatin and 25 μg/mL of gentamycin. The samples were kept at 4°C until use. They were hemolyzed by adding 18 μL of 0.2% saponin/PBS (final concentration about 0.02%) and incubated at room temperature for 5 min. The mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The pellet was re-suspended with 200 μL PBS, vortexed, and centrifuged again under the same conditions. The destruction and proteolysis of the parasites were carried out by adding to the pellet 40 μL of the lysis solution,17 together with one drop of mineral oil followed by incubation at 60°C for 20 min. The mixture was incubated at 95°C for 10 min to inactivate the proteinase K, and then cooled to 50°C.

PCR amplification. The sample was spun down and mixed with 10 μL of the PCR reagent mixture containing 10 mM Tris-HCl (pH 8.9), 1.5 mM MgCl₂, 500 μg of bovine serum albumin per mL, 0.1% Triton X-100, 5 μg each of primer MPH-1 and primer MPH-2 per mL, 1 mM deoxy-nucleoside triphosphates, and 100 U of Thermus thermophilus (Tth) polymerase (Toyobo, Osaka, Japan). The mixture was subjected to 30 cycles of PCR in a thermal cycler (Combi Thermal Reactor TR-2; Hybaid, Teddington, Middlesex, UK). The conditions for PCR amplification were as follows: denaturation at 91°C for 30 sec, annealing at 52°C for 60 sec, and extension at 72°C for 60 sec. After amplification, the tubes were gradually cooled to room temperature, and the amplified DNA was denatured by heating at 95°C for 10 min followed by rapid cooling on ice for 10 min.

Hybridization and colorization. Microtiter plate wells coated with probes specific for P. vivax, P. falciparum, P. malariae, P. ovale, and P. ovale-variant were filled with 100 μL/well of 5x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After 5 μL each of the PCR products were placed in the wells and incubated at 60°C for 1 hr, the solution was removed, and the wells were washed three times with 200 μL/well of 2x SSC. Then 100 μL of alkaline phosphatase-labeled streptavidin (Bethesda Research Lab., Bethesda, MD), diluted to 1:1,000 with solution 1 (0.1 M Tris-HCl pH 7.5, 0.1 M NaCl, 2 mM MgCl₂, and 0.05% Triton X-100), was added to each well.

The wells were washed three times with 300 μL of solution 1, and 100 μL of PNPP solution (1 M diethanolamine pH 9.8, 0.5 mM MgCl₂, and 10 mM para-nitrophenyl phosphate) was added to each well as the substrate. Then the mixture was incubated at 25°C for 30 min. The color development was measured using an enzyme immunoassay reader (MPR-A4, TOSOH, Tokyo, Japan) at 405 nm. A positive value was identified where the optical density (OD) was higher than the double of the healthy controls (0.083 ± 0.009, n = 66).18

Agarose gel electrophoresis. The PCR products were analyzed by agarose electrophoresis on 1.2% agarose gel (Seakem LE agarose, FMC Bioproducts, Rockland, ME) in 1x TBE running buffer (0.89 M Tris, 0.89M boric acid, and 0.02 M EDTA). Five microliters of the PCR products were added to the lanes of the gel, and the gels were run at 100 V for 30 min. The gels were stained with ethidium bromide (5 μL/ml) for 5 min, and soaked in distilled water. Photographs of the gels were taken using a UV transilluminator. The size of the product was estimated by comparison with 123 and 246 base pair (bp) ladder DNA size markers.

Partial sequencing of 18S rRNA of Korean P. vivax DNA fragments of the 138 bp area (the target region of MPH) were amplified using the primers MPH-1 and MPH-2, and purified by a microconcentrator (Centricon, Amicon, Japan). The purified DNA solution was processed to a cycle sequencing using the DNA terminator cycle sequencing kit (PE Applied Biosystems, CA). Its nucleotide sequence was determined by an automatic DNA sequencer (Autosequencer 373S, PE Applied Biosystems, CA).

Statistics. The correlation coefficient (and equation) between the parasite density and MPH absorbance at 405 nm was obtained on a personal computer using SigmaPlot version 5.0 for Windows (SPSS Inc., Chicago, IL). The Student’s t-test was applied to compare the observed data with the theoretical ones, and a value of P < 0.1 was considered significant.

RESULTS

Agarose gel electrophoretic patterns of PCR products. The PCR products of the blood were amplified using the primer pair MPH-1 and MPH-2, and subjected to agarose gel electrophoresis. This revealed a prominent band at the 138 bp area in all of the parasite-proven patients (Figure 1). The band indicated the presence of malarial genes regardless of the species. On the other hand, no significant band was observed in the PCR products of the blood of the healthy controls or the post-treatment follow-up patients (Figure 1).

Results of MPH. All the parasite-proven patients revealed strong positive reactions against only the P. vivax-specific probe (Table 1). The absorbance of the reaction in OD was 1.30–1.90 (positive criterion = 0.18) at 405 nm. However, none reacted positively against the probes specific for other species of malaria. In contrast, the post-treatment patients and healthy controls revealed negative reactions against the P. vivax-specific probe (OD: 0.07–0.11) as well as to other species-specific probes (OD: 0.07–0.09).

Parasite density in the blood and its correlation with the absorbance in MPH. The parasite density in the blood of 18 parasite-proven patients was between 94 and 7,222 per μL blood (0.002% to 0.14% in parasitemia). The parasite density in the blood was positively correlated with the absorbance in the MPH following the equation Y = 0.05X + 1.40, where X is the parasite density in the logarithmic scale and Y is the OD in the MPH (Figure 2). However, the degree of correlation was extremely low (r = 0.408), and only a low-grade statistical significance was observed (0.05 < P <
FIGURE 1. Agarose gel electrophoresis patterns of blood PCR products of malaria patients. SM = DNA size marker. Lanes 1–18 = bloods of patients microscopically diagnosed as *Plasmodium vivax* infected. HC = blood of a healthy control. Lanes 19–22 = bloods of post-treatment follow-up cases. Lanes *P.f.* to *P.m.* = positive controls with DNA of *Plasmodium falciparum*, *P. vivax*, *Plasmodium ovale*, *Plasmodium ovale*-variant, and *Plasmodium malariae*, respectively. Lane H₂O = negative control with distilled water. All of the 18 parasite-proven patients show a single band of about 138 base-pair size, indicating the presence of malarial parasites. The blood of a healthy control, 4 post-treatment follow-up cases, and distilled water revealed negative reactions.

FIGURE 2. The correlation between the parasite density in blood films and the optical density (OD) in the microtiter-plate hybridization (MPH) of 18 *Plasmodium vivax* patients (positive criterion 0.18). Closed circle (●) represents one patient. The linear regression equation is $Y = 0.05X + 1.40$, where $X$ is the parasite density in the logarithmic scale and $Y$ is the OD value in the MPH ($r = 0.408$, $0.05 < P < 0.10$).

TABLE 1
Results of the polymerase chain reaction-based microtiter plate hybridization (MPH) using the blood of malaria patients

<table>
<thead>
<tr>
<th></th>
<th>No. of cases examined</th>
<th><em>Plasmodium vivax</em></th>
<th><em>Plasmodium falciparum</em></th>
<th><em>Plasmodium malariae</em></th>
<th><em>Plasmodium ovale</em></th>
<th><em>Plasmodium ovale</em>-variant</th>
</tr>
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<tr>
<td>Febrile patients†</td>
<td>18</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Post-treatment follow-up cases‡</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* see Figure 2 for the absorbance of positive cases.
† diagnosed as *P. vivax* infection on blood films.
‡ at Days 3–27 post-treatment with primaquine.

DISCUSSION

In order to cope with the disadvantages of the morphological and serological techniques for diagnosing malaria, various DNA assays have been introduced. Simple, double, and nested PCR amplifications of malarial DNA were reported as sensitive and specific diagnostic tools. However, these techniques required complicated and time-consuming manipulations to extract the DNA and confirm the amplified products. As an alternative measure, the PCR-based MPH assay was tested for its practical utility and reported as a useful tool for the differential diagnosis of various species of malaria.

In the present study, we confirmed the usefulness and feasibility of the PCR-based MPH technique for the genetic diagnosis of the reemerging Korean *P. vivax* malaria. Using
FIGURE 3. Partial nucleotide sequences of genes encoding 18S rRNA of *Plasmodium vivax* in the blood of three patients (Nos. 2, 6, and 17 in Figure 1) in comparison with those already known in A-type (blood stage) and C-type (mosquito stage) of *P. vivax*. The regions used for primers MPH-1, MPH-2, and the probe are enclosed by a solid line. The nucleotide identity is indicated by dots; a lack of corresponding nucleotides is indicated by dashes. Ambiguous bases are abbreviated as follows: R: A or G; Y: C or T; W: A or T; M: A or C; and K: G or T.

This technique it was possible to provide a specific diagnosis of human malaria. The technique was further suggested to be useful in evaluating the efficacy of the treatment, although the post-treatment testing could be done only in 4 of 18 patients. Sequencing of the probe region of 18S rRNA of *P. vivax* revealed no significant variation from that of the typical *P. vivax*.12,13

The basic goal of the present study was to confirm whether the MPH technique is applicable to the reemerging Korean *P. vivax* malaria, since there could be variations in the sequence of the probe region of the malarial genes. An example of the variations in the probe region of 18S rRNA among malaria parasites was reported for *P. ovale*, which was found in Vietnam.14

Genetic variations of Korean *P. vivax* have been strongly suggested because of its peculiar and atypical clinical manifestations (e.g., exceptionally long incubation periods,3,12,22 atypical fever cycles,3,23 and higher frequency of relapses).3,21–24 When human experimental infections were performed with the North Korean strain of *P. vivax*, the incubation period was about one month in 25% of the patients and ranged between 5 and 13 months in the remaining 75% of the patients. The recent reemerging malaria is very similar in its incubation period.3

The typical 48-hr fever cycle was observed in only 40% of the Korean tertian malaria patients.21 In addition, the Korean *P. vivax* malaria showed higher frequencies of thrombocytopenia, leucopenia, elevation of transaminases, and low blood parasitemia compared with other geographical strains.21,22 Therefore, quite a few of the reemerging Korean malaria cases have been initially misdiagnosed as other febrile diseases and confirmed later as malaria.22,27 Although the probe region of the 18S rRNA of the Korean *P. vivax* showed no variations from the typical *P. vivax*, genetic variations at other regions should be investigated further.

The agarose gel electrophoretic patterns of the PCR products of the patients’ blood showed successful amplification of the target area of 138 bp size in all of the parasite-proven patients. In contrast, there were no recognizable amplified products in any of the healthy controls and post-treatment follow-up patients. The disappearance of the band at the 138 bp area in the post-treatment patients suggests that the MPH technique could differentiate acute febrile cases of *P. vivax* from those in the convalescent stage. To verify this implication, however, studies dealing with sufficient numbers of post-treatment follow-up cases are required.

The MPH assay had been shown to be superior to the simple PCR assay because it could provide information on the severity of the infection.13 The present study revealed a positive correlation between the parasite density in the blood and OD in the MPH assay. However, the degree of correlation and the statistical significance were both too low to draw definite conclusions.

Unfortunately, in the present study the lowest parasite density detectable by the MPH assay could not be determined due to the inadequate size of the sample population. It can be inferred, however, that in both the *P. vivax* and *P. falciparum* infections the MPH technique can detect as few as 1.3 parasites per μL blood.13

The cost of the MPH test is estimated roughly at US$3.00 per test, which is a little more expensive than examining a blood smear, but cheaper than treating a suspected malaria patient showing negative blood smears. In summary, the PCR-based MPH assay is a highly feasible tool for a DNA diagnosis of the Korean strain of *P. vivax* malaria.

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