Detection and Differentiation of Malaria Parasites in DNA Extracted from Blood Samples by the Polymerase Chain Reaction (PCR)

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ABSTRACT. The purpose of this work was to construct a screening method to detect the small subunit rRNA of malaria parasites, especially *Plasmodium falciparum* and *P. vivax*, in blood samples. The PCR method, using the nested multiplex primer set that has been effective for the detection and differentiation of both *P. falciparum* and *P. vivax*, was applied to DNA extracted from patients of the Solomon Islands and Papua New Guinea with abnormal hemoglobin, Hb J-Tongariki or Hb I-Toulouse. Since it was, however, not enough to certify the respective *Plasmodia* in patients infected by multiple species, confimation by the PCR method using a specific primer set required to detect the respective *Plasmodia* finally might be necessary.

Key words : *Plasmodium falciparum* — *Plasmodium vivax* — polymerase chain reaction (PCR) — Hb J-Tongariki — Hb I-Toulouse

Malaria is the most widespread parasitic disease in humans and, even today, there are an estimated 1.5 to 2.7 million deaths, mostly of children, annually. Four species of *Plasmodia* (*P. falciparum, P. vivax, P. ovale* and *P. malariae*) are responsible for causing human malaria. *P. falciparum* is the most dangerous and, if untreated, can lead to death. *P. vivax* has been observed worldwide while observation of *P. ovale* and *P. malariae* has been rare. The clinical condition of the patient infected with these species is not as severe as that of one infected with *P. falciparum*.[1)]

Although conventional methods for detecting *Plasmodia*, such as microscopy or antigen-based methods had and have been used, the polymerase chain reaction (PCR) is now commonly acknowledged as a superior sensitive method.[2)] Recently, Zaman S, *et al*[3)] reported that the nested multiplex primer set was the most optimal primer set for the detection of malaria DNA extracted from blood samples with the advantage

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of simultaneously detecting and differentiating between *P. falciparum* and *P. vivax*. In the present study, we applied these primer sets to DNA extracted from patients with and without malaria to detect *P. falciparum* and *P. vivax* separately and investigated the usefulness of simultaneous screening of these species.

MATERIALS AND METHODS

Peripheral blood samples of inhabitants of villages in the Solomon Islands and Papua New Guinea were collected into the tubes including an EDTA. After diagnosis microscopically with and without malaria, they were separated into two components of red cells and plasma by centrifugation. The red cells (about 0.5 mL) were frozen and transported to the Department of Biochemistry, Kawasaki Medical School, Okayama, Japan. The blood samples used in this study were as follows: the result of microscopic determination, one (sample No. 246) of four carriers of Hb J-Tongariki [α115(GH3)Ala->Asp] infected with *P. falciparum*, and 8 (sample Nos. 104, 107, 114, 137, 139, 142, 405, and 1053) of 16 carriers of Hb I-Toulouse [β66(E10)Lys->Glu] (unstable Hb forming a ferri-Hb = metHb) infected with *P. falciparum* and one (sample No. 126) with *P. vivax*[4,5] (Table 1). In the carriers of a compound heterozygote for Hb J-Tongariki and Hb I-Toulouse, no malaria parasites were observed microscopically. The carriers of Hb J-Tongariki were also carriers of α-thalassemia-1 (genotype: $-\alpha^{3.7}/-\alpha^{3.7}$) or α-thalassemia-2 (genotype: $-\alpha^{3.7}/\alpha\alpha$) (Table 1). A sample (sample No. 101) microscopically negative for malaria parasites was used as a control.

DNA was extracted from red cells by the Mini Prep Kit method (Biorad Laboratories Inc., USA). The DNA concentration (μg/mL) was estimated from absorbance of the DNA solution at 260 nm.

The PCR was performed in two steps, a first and a second or nested PCRs. The first PCR was carried out by using the common primer set for the small subunit rRNA (ssrRNA) of *P. falciparum* and *P. vivax* according to the procedure described by Snounou *et al.*[6] The sequence of the primers used here was as follows: Mal-1F as sense primer: 5'-TTAAAATTGTTGC AGTTAAAACG-3' and Mal-1R as antisense primer: 5'-CCTGTTGTTGCC TTAAACTTC-3'. PCR was performed on a total volume of 50 μL of mixture containing about 0.1 μg of extracted DNA, 200 μmol of dNTPs, 50 pmol of each primer, 1 x polymerase reaction buffer, 2.5 unit of polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA) and an adequate volume of sterilized water. The mixture was set on a thermal cycler (i-cycler, Biorad Laboratories Inc., USA) and the reaction was begun with denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30s, 58°C for 1 min, and 72°C for 1 min with final incubation at 72°C for 5 min. The second or nested PCR using 1 μL of the first PCR product was performed by using the primer set for each *P. falciparum* and *P. vivax* sample or a mixture of these primer sets (multiplex primer). The primer set for the detection of *P. falciparum* was composed of 5'-TTAAACTGGTTTCGGAAAACCAAATATATT-3' as a sense primer (Mal-2F) and 5'-ACACAATGAACTCAATCATGACTACCCGTC-3' as an

antisense primer (Mal-2R). That of *P. vivax* consisted of 5'-CGCTTCTAGC TTAATCCACATAACTGATAC-3' as a sense primer (Mal-VF) and 5'-ACT TCCAAGCCGAAGCAAAGAAAGTCCTTA-3' as an antisense primer (Mal-VR). The conditions for the second PCR were the same as those for the first one. The appearance of 206 bp of PCR product detected by ethidium bromide staining following 2% Nusieve agarose (Biowhittaker Molecular Application, Rockland, ME, USA) gel electrophoresis in a TEA buffer solution (pH 8.3) indicated the presence of *P. falciparum*, while that of 121 bp indicated the presence of *P. vivax*.[7,8] The DNA sequence of these products, 206 bp and 121 bp, removed from gel by Spin Column (Quantum Prep Freeze 'N Squeeze Spin Columns, BioRad Laboratories Inc., Hercules, CA, USA) was determined by the BigDye termination cycle sequencing method (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits, PE Biosystems, Foster City, CA, USA).[9]

RESULTS

The PCR product obtained by the first PCR was about 1 kb in size, and was considered to correspond to the ssrRNA of *P. falciparum* and *P. vivax*.[7,8] The second PCR products of the patients with and without malaria obtained with the multiplex primer revealed the presence of bands corresponding to 206 bp and 121 bp in size (described as 205 bp and 120 bp in the references[3,4]). In the patients examined here, those having been decided microscopically to be carriers of *P. falciparum* with exception of two (sample Nos. 114 and 405) exhibited the 206 bp band (Fig 1 and Table 1), showing the same results with both microscopic and PCR methods. However, patients 222, 048, 406, and 101 (control), who had negative results in the microscopy method, had a positive result for *P. falciparum*. Although *P. vivax* had been detected in only one patient, patient 126, the presence of a 121 bp DNA fragment was observed in many patients, 18 of the total 23, suggests the *P. vivax* may be observed widely in the world. Two carriers (sample No. 408 and 1005) of Hb I-Toulouse were negative, showing identification with both methods, the PCR and microscopy. These results are listed in Table 1.

Additionally, there were some patients (Table 1) who had two bands, 121 bp and 206 bp in size, although the intensity of one of these two electrophoretic bands was weaker than that of other when they were compared with each other (Fig 1). The thin band was further investigated by using the respective specific primer set for *P. falciparum* and *P. vivax* (Fig 2). Consequently, eight patients, 048, 104, 107, 137, 139, 142, 406, and 101 as the control, were considered to be doubly infected with *P. falciparum* and *P. vivax*.

The nucleotide sequences of the two bands of 121 bp and 206 bp removed from the electrophoresed agarose gel were further investigated and showed indentification with a part of the ssrRNAs of *P. falciparum* and *P. vivax*[7,8] (Fig 3), respectively.

TABLE 1. Detection and differentiation of malaria parasites of Solomon Islander and Papua New Guinean patients with Hb J-Tongariki or Hb I-Toulouse by microscopy and the PCR.

Sample No.	Microscopy	PCR	Notes
222		Pf	Hb J-Tongariki+α-Tahl-1
246	Pf	Pf	Hb J-Tongariki+α-Tahl-2
1026		Pv	Hb J-Tongariki+α-Tahl-1
1036		Pv	Hb J-Tongariki+α-Tahl-2
048		Pf+Pv	Hb I-Toulouse, unstable, Ferri-Hb
104	Pf	Pf+Pv	Hb I-Toulouse, unstable, ferri-Hb
107	Pf	Pf+Pv	Hb I-Toulouse, unstable, ferri-Hb
114	Pf	Pv	Hb I-Toulouse, unstable, ferri-Hb
116		Pv	Hb I-Toulouse, unstable, ferri-Hb
126	Pv	Pv	Hb I-Toulouse, unstable, ferri-Hb
135		Pv	Hb I-Toulouse, unstable, ferri-Hb
137	Pf	Pf+Pv	Hb I-Toulouse, unstable, ferri-Hb
139	Pf	Pf+Pv	Hb I-Toulouse, unstable, ferri-Hb
142	Pf	Pf+Pv	Hb I-Toulouse, unstable, ferri-Hb
403		Pv	Hb I-Toulouse, unstable, ferri-Hb
405	Pf	Pv	Hb I-Toulouse, unstable, ferri-Hb
406		Pf+Pv	Hb I-Toulouse, unstable, ferri-Hb
408			Hb I-Toulouse, unstable, ferri-Hb
1005			Hb I-Toulouse, unstable, ferri-Hb
1053	Pf	Pf	Hb I-Toulouse, unstable, ferri-Hb
233		Pv	Hb I-Toulouse/Hb J-Tongariki
256		Pv	Hb I-Toulouse/Hb J-Tongariki
101		Pf+Pv	Subject without Hb variant

Hb J-Tongariki [α114(GH3)Ala→Asp] is a Hb variant without any instability of abnormal function, but Hb I-Toulouse [β66(E10)Lys→Glu] is a Hb with instability for the formation of ferric Hb or methemoglobin. α-Thal means α-thalassemia. The genotypes of α-Thal-2 and α-Thal-1 are $-\alpha^{3.7}/\alpha\alpha$ and $-\alpha^{3.7}/-\alpha^{3.7}$, respectively. Pf and Pv mean *Plasmodium falciparum* and *P. vivax*, respectively.

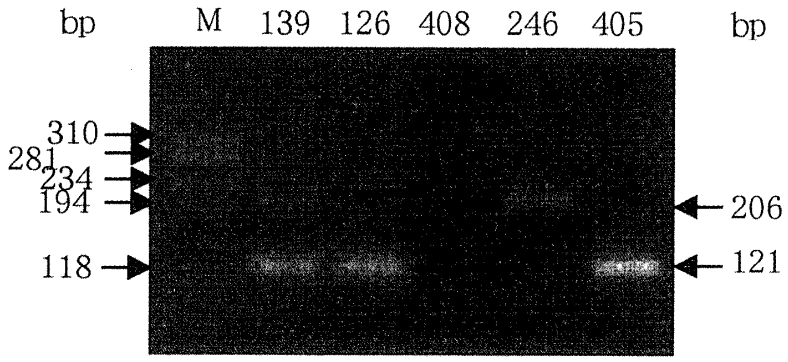

Fig 1. Electrophoresis of the products amplified using multiplex PCR primer set. The band of 206 bp in size is from ssrRNA of *P. falciparum* and that of 121 bp from ssrRNA of *P. vivax*. Sample No. 139 was observed to have a faint band at the region of 206. M : Molecular marker.

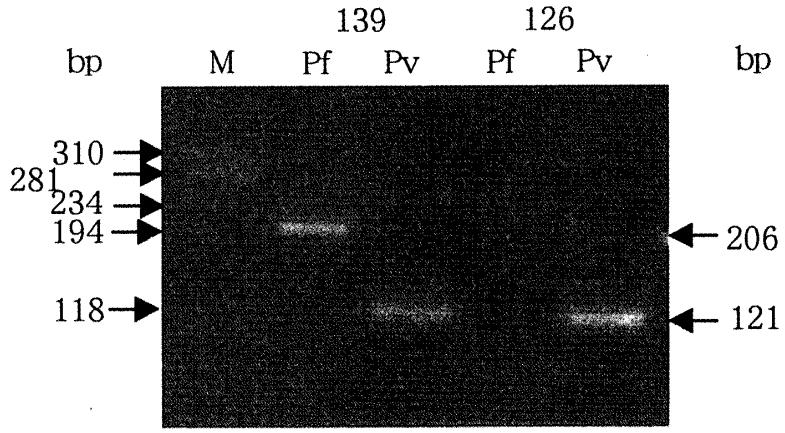

Fig 2. Electrophoresis of the products obtained from samples No. 139 and No. 126 using a specific primer set to detect *P. falciparum* (Pf) and *P. vivax* (Pv) differentially. Sample No. 139 was a carrier of both *P. falciparum* and *P. vivax*, but No. 126 was a carrier of only *P. vivax*.

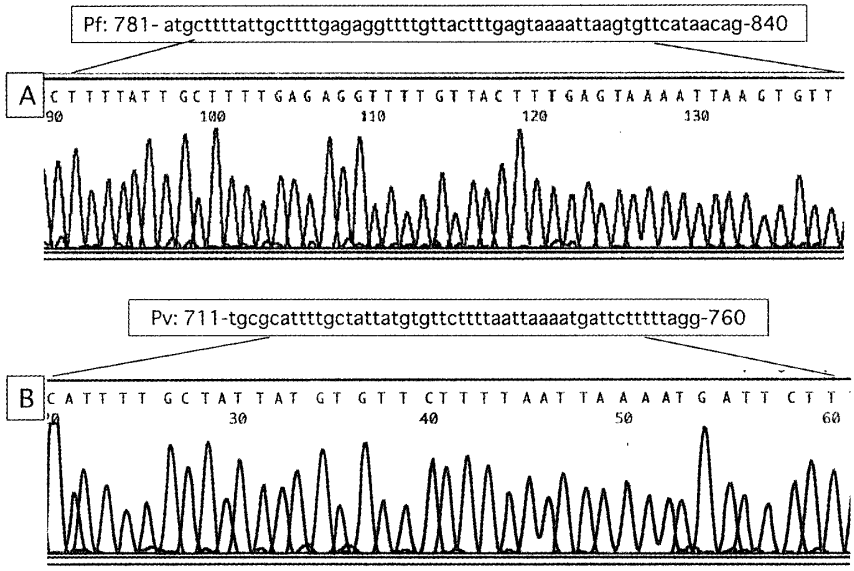

Fig 3. Nucleotide sequencing of the 206 bp and 121 bp PCR products observed on an electrophoresis gel. The sequences of these products were identified with those of ssrRNA of *P. falciparum* (Pf: A) and *P. vivax* (Pv: B) reported previously. The sequences described in the figure above are those cited from references.[7,8]

DISCUSSION

Until recently, examinations for malaria parasites had been performed by microscopic or antigen-based methods, but, during the last decade, the PCR method has developed as a superior sensitive method.[2,3] Recently, many primer sets have been tested for the detection and differentiation of *P. falciparum* and *P. vivax*.[3]

Ziaman, S., *et al*[3] investigated the screening of many primer sets for the purpose of finding the most sensitive PCR primer pair for the detection and differentiation of *P. falciparum* and *P. vivax* in blood samples collected from malaria patients. Consequently, they suggested using the nested multiplex primer set (Mal-2F + Mal-2R + Mal-VF + Mal-VR) as the optimal

primer set for this purpose, after used Mal-1F and Mal-1R (they used other marks) as the first PCR primer set. This first PCR primer set is common to both Plasmodia. It was considered that a mixed primer set of these four primers could detect and differentiate between *P. falciparum* and *P. vivax* with sensitivities of more than 95%.[3]

The PCR product from some patients (Table 1) revealed the presence of two bands of 121 bp and 206 bp, the intensity of which was not same. These bands corresponded to the sequences of a part of the ssrRNA of *P. falciparum* and *P. vivax*.[7,8] To confirm the respective species, it is necessary to use the specific primer set to differentially detect *Plasmodium*.

We investigated the mixture of these primers in 23 (including one control) Solomon Islander and Papua New Guinean patients,[4,5] nine of whom had been diagnosed as the carriers of *P. falciparum* and one carrier of *P. vivax* by microscopy. Four patients besides these 9 carriers of *P. falciparum* were newly detected and, additionally, 18 carriers of *P. vivax* were found, although only one patient had been diagnosed as a *vivax* by microscopy, suggesting that the PCR method is a more sensitive technique than the microscopic method. From these results, many inhabitants of, not only the Solomon Islands and Papua New Guinea, but also in area of malaria prevalence could be infected with both *P. falciparum* and *P. vivax*, but at least with *P. vivax*. It is very important to determine whether the patient is infected with *P. falciparum* causing a severe clinical manifestation and/or *P. vivax* which is observed widely in the world, but which does not cause a severe condition.

In this study, we examined patients of the Solomon Islands and Papua New Guinea who were heterozygotes or compound heterozygotes for Hb J-Tongariki and unstable Hb I-Toulouse, and carriers of Hb J-Tongariki associated with α-thalassemia. Generally, we have believed that the carriers of some abnormal Hb, such as Hb S,[10] Hb E,[11] and Hb C very recently reported,[12] and thalassemia syndromes[13] have some resistance to malaria, namely, they do experience any severe clinical manifestations. Although the clinical manifestations of the patients studied here could not be determined, it was found that most of them were carriers of at least one or two malaria parasite species. *P. vivax* especially seems to be prevalent in these areas.

The presence of malaria parasites in plasma collected from patients was not examined in this study. If this had been done, we might have been able to predict the picture of clinical conditions of these patients.

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