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The Prevalence of Falciparum Malaria in the Solomon Islands Investigated by a Filter Paper Disk-PCR Method

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Abstract

We investigated the prevalence of falciparum malaria in the Solomon Islands with the aid of the polymerase chain reaction (PCR), examining 405 samples collected on filter paper disks. Giemsa-stained thin and thick blood films were also made from each subject and examined for malaria in two different centers. For PCR, we targeted two different DNA segments of the *Plasmodium falciparum* parasite: the dihydrofolate reductase-thymidylate synthetase junction (DHFR-TS junction, of 410 bp), and a fragment of the gene that encodes the *Plasmodium falciparum* gametocyte cytoplasmic protein (Pfg 27/25 gene, of 598 bp). Results of the PCR were compared with those of the Giemsa stain method. By PCR, sensitivity was 80.2% for the 410-bp and 91.9% for the 598-bp segments and the specificity was 87.5% and 79.8%, respectively. However, by combining the results of these two PCRs, sensitivity was improved to 98%. The detection limit for malaria parasites by the optical microscopy method varies from 50 to 5000 parasites/μl, so most patients with low parasitemia are not detected by the conventional method. According to our preliminary study, the detection limit with the PCR method was 4 parasites/μl. Results of field studies showed a definite discrepancy between the Giemsa stain and PCR techniques, indicating that examination of Giemsa-stained blood films alone is not adequate for accurate estimation of the prevalence of malaria in epidemiological studies.

Key words: Malaria, *Plasmodium falciparum*, filter paper disk-PCR, Solomon Islands

Introduction

The Solomon Islands, together with Papua New Guinea and Vanuatu, are hyperendemic for malaria (Wataya *et al.*, 1993). In such hyperendemic areas, malaria is one of the principal causes of child mortality. On the other hand, people who survive repeated infections become relatively resistant to the disease, showing either very slight symptoms or, sometimes, none at all. These patients usually show very low parasitemia and are difficult to detect by the classical Giemsa stain method, but they are important as a reservoir of the disease. The detection limit of the optical method for diagnosing malaria varies from 50 to 5,000 parasites/μl of blood, depending upon the ability of the microscopist and his perseverance.

Attempts to diagnose malaria using labeled specific DNA probes have been tried (Bruce-Chwatt, 1987; Fucharoen *et al.*, 1988) and shown to have improved sensitivity, but the detection limit was still about 40–100 parasites/μl blood (Barker *et al.*, 1986; Waters and McCutchan, 1989).

Recently, the use of the polymerase chain reaction (PCR) has been reported for the detection of

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many infectious agents, including malaria parasites (Jaureguiberry *et al.*, 1990; Barker Jr. *et al.*, 1992; Kain *et al.*, 1992; Sethabutr *et al.*, 1992). Theoretically, a single malaria parasite should be detectable by PCR. In this paper, we report the prevalence of malaria in the Solomon Islands, determined by examining 405 samples of blood from inhabitants aged from 1 to 89 years. The blood was collected on filter paper disks and *Plasmodium falciparum* infection was detected by a PCR method. The results were compared with those of the conventional Giemsa stain method.

Material and Methods

Blood sampling

For collecting the blood, we used a modification of the method of Kain and Lanar (1991). Five microliters of blood were obtained by finger-prick as a drop on a filter paper disk (Whatman 3MM) 6mm in diameter. The disk was then air-dried and transported at room temperature to the laboratory for isolation of the DNA.

Isolation of the DNA from the filter paper disk

The blood on the filter paper was hemolysed with 0.5% saponin in phosphate-buffered saline (PBS) and the filter was washed twice with PBS. The filter was then vortexed for 1 min in 200 μl of 5% aqueous Chelex-100 (BioRad, Tokyo, Japan) to adsorb substances such as iron and other metals that might inhibit the PCR. The tube containing the sample was heated in a hot block at 94°C for 8 min and immediately quenched in ice for 2 min. The aqueous phase containing the malaria parasite DNA was separated and used for the PCR reaction.

PCR method

For amplification of the DNA, a Program Temperature Control System (PC-700, Astec, Tokyo, Japan) was used. Ten microliters of the solution prepared from the sample was added to an amplification mixture containing 1.5 mmol $MgCl_2$, 200 μmol dNTPs, 1.25 U of Taq DNA polymerase (TaKaRa, Kyoto, Japan), and the upper and lower primers (50 pmol each) corresponding to the targeted unique sequence of the *Plasmodium falciparum* DNA (total volume per sample subjected to PCR: 50 μl). Two different fragments of the *Plasmodium falciparum* DNA were amplified by PCR. One fragment, of 410 bp, was the junction portion of the dihydrofolate reductase-thymidylate synthetase gene (DHFR-TS) (Bzik *et al.*, 1987; Wataya *et al.*, 1993) and the other fragment, of 598 bp, was part of the gene that encodes the *Plasmodium falciparum* gametocyte cytoplasmic protein, Pfg 27/25 (Alano *et al.*, 1991). A set of 25mer primers was used for amplification of the 410-bp fragment (5' TGT TAG CGA TGT ATA TAC TAG TAA C 3' and 5' TTC GAT CAC TTT GTT TAT TTC CAT T 3'), and for amplification of the 598-bp fragment we used 19mer and 20mer primers (5' GGA TAG TGC CAA GCC CTT G 3' and 5' GGT TCA TCA GGT GTT ACG GG 3'). The PCR consisted of 35 repetitive cycles including 30 s at 94°C for denaturation, 1 min at 58°C for annealing, and 2 min at 72°C for extension. A positive control (blood containing *Plasmodium falciparum*, 10^5 parasites/μl) and a negative control (uninfected blood) were amplified concomitantly with each set of samples.

Hybridization of the PCR products

The PCR-amplified 410-bp DNA was electrophoresed in 1.5% agarose and transferred to a cellulose membrane by Southern blotting. For hybridization, we used a 36mer oligoprobe (5' TTA TGA AAA TGA TGA TGA TGA TGA AGA AGA AGA TGA 3') labeled with ^{32}P-ATP. This oligoprobe had a sequence corresponding to an internal segment of the 410-bp fragment.

Results

Sensitivity of the PCR method for detection of Plasmodium falciparum

Firstly, a preliminary study was performed to determine the detection limit of the PCR using these two pairs of primers. A culture of erythrocytes infected with *Plasmodium falciparum* was serially diluted (1:10) five times with uninfected blood. Five microliters of each dilution was spotted onto a filter disk and processed in the same manner as the sample. The detection limit of *Plasmodium falciparum* by PCR was 4 parasites/μl blood, corresponding to a parasitemia of about 0.0001% (Fig. 1).

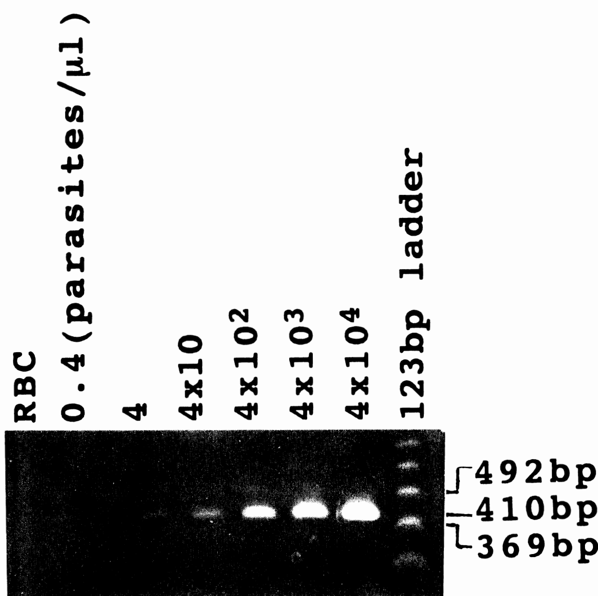

Fig. 1 Detection limit of *Plasmodium falciparum* by the 410-bp PCR method.
P.f. infected red blood cells were serially diluted (1:10) with uninfected blood to determine the detection limit of the PCR (410 bp). The detection limit was 4 parasites/μl of blood (corresponding to a parasitemia of 0.0001%).

PCR detection of Plasmodium falciparum *infection in residents of the Solomon Islands*

In our two visits to the Solomon Islands (in January and August of 1994), we were able to collect 405 samples from inhabitants aged from 1 to 89 years in 3 villages, about 50 to 70 km from Honiara City, the capital of the Solomon Islands. At least two sets of thin and thick blood films were also prepared from each subject for Giemsa staining and comparison with the results obtained by PCR. One set of films was immediately examined by a local microscopist, and another set was brought to our laboratory in the Jichi Medical School for subsequent confirmation and comparison of the results. Up to 100 fields were examined on each Giemsa-stained thick blood film before it was declared negative.

Of the 405 persons examined in our laboratory, 197 were positive and 208 were negative by Giemsa staining; 158 of the positive samples and 182 of the negatives coincided with the results of the 410-bp PCR (sensitivity 80.2% and specificity 87.5%) and 181 positive samples and 166 negatives agreed with the results of the 598-bp PCR (sensitivity 91.9%, specificity 79.8%). However, by summation of both PCR results, the sensitivity was elevated to 98%, despite a slight decrease in specificity to 77.9% (Table 1). Hybridization of the 410-bp PCR product increased the sensitivity from 80.2% to 87.5%. However, a decrease in specificity was observed, from 87.5% to 61.5% (data not shown).

Because of the difficulties in determining the exact number of malaria parasites in the sample processed by PCR, we defined the result obtained with the positive control (10^5 parasites/μl) as (4+). The PCR results were classified into groups from (–) to (4+) according to the intensity of the band. Results from the Giemsa-stained blood films were also graded from (–) to (4+), according to the number of malaria parasites/100 white blood cells (Fig. 2). A significant correlation was found between Giemsa and 410-bp PCR methods (correlation coefficient r=0.80). *Plasmodium vivax* infection was diagnosed only by the Giemsa stain method, since PCR for detection of vivax malaria was not performed. As shown in Table 2, *Plasmodium falciparum* formed

Table 1 Sensitivity and specificity of the 410-bp and 598-bp PCR singly or in combination for the diagnosis of malaria compared with the Giemsa staining method

PCR (410 bp)				PCR (598 bp)				PCR (410 and 598 bp)			
Giemsa	+	–	Total	Giemsa	+	–	Total	Giemsa	+	–	Total
+	158	39	197	+	181	16	197	+	193	4	197
–	26	182	208	–	42	166	208	–	46	162	208
Total	184	221	405	Total	223	182	405	Total	239	166	405
sensitivity=80.2% specificity=87.5%				sensitivity=91.9% specificity=79.8%				sensitivity=98.0% specificity=77.9%			

Results of Giemsa staining were determined by examining 100 fields of a thick blood film at 1000X magnification. PCR results were determined by visualization of the amplified DNA band after electrophoresis and staining with ethidium bromide.

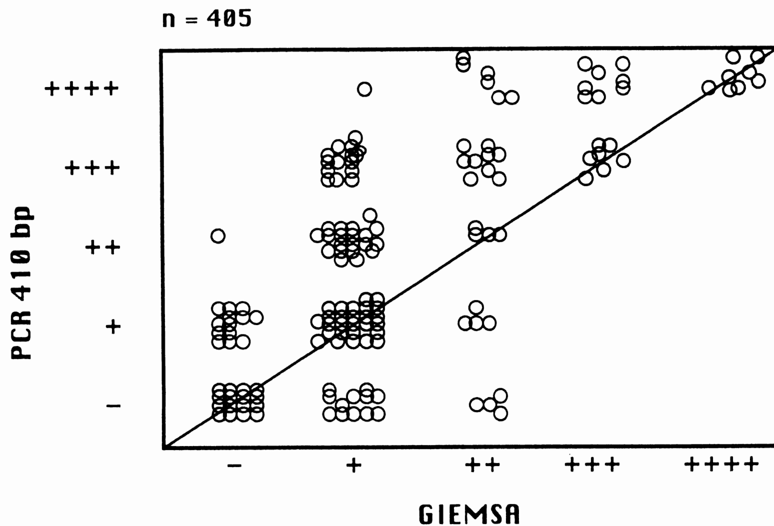

Fig. 2 Correlation of the results obtained by PCR with those from Giemsa-stained blood films (correlation coefficient r=0.8).
PCR products were electrophoresed and stained with ethidium bromide. Results were graded from (–) to (4+) according to the intensity of the band. We considered as (4+) samples with intensity equivalent to or stronger than the positive control (10^5 parasites/μl). Results obtained by the Giemsa staining method were also graded from (–) to (4+) according to the number of malaria parasites/100 white blood cells (4+ means 4 or more parasites/100 white blood cells).

the overwhelming majority of the malarial infections. In all three villages, the prevalence of falciparum malaria was higher in January than in August. In one of the villages (Talaura), the prevalence of falciparum malaria was almost 80% in January. Conversely, *Plasmodium vivax* showed a tendency to be more common in August than in January. We also observed some patients with a double infection of both *P. vivax* and *P. falciparum.*

Table 2 Prevalence of malaria in three villages in the Solomon Islands

Village	Month	Number of cases (%)				
		P.falciparum	*P.vivax*	P.f.+P.v.	Negative	Total
Talaura	Jan	63 (78.8%)	1 (1.3%)	3 (3.8%)	13 (16.3%)	80 (100%)
	Aug	30 (54.5%)	1 (1.8%)	3 (5.5%)	21 (38.2%)	55 (100%)
Mbambala	Jan	38 (48.7%)	5 (6.4%)	7 (9.0%)	28 (35.9%)	78 (100%)
	Aug	32 (48.5%)	9 (13.6%)	2 (3.0%)	23 (34.8%)	66 (100%)
Kolona	Jan	43 (58.1%)	4 (5.4%)	5 (6.8%)	22 (29.7%)	74 (100%)
	Aug	17 (32.7%)	10 (19.2%)	0 (0%)	25 (48.1%)	52 (100%)
	Total	223	30	20	132	405

Blood was sampled in January and August of 1994. An absolute preponderance of falciparum malaria was observed in all three villages, with a higher prevalence in January.

Comparison of the results by PCR and Giemsa staining

The results obtained in both investigation centers agreed for 128 negative samples and 123 positive samples (Fig. 3). However, 111 subjects shown to be positive by PCR were considered as negative by the local microscopist. Of this group, *Plasmodium falciparum* infection was confirmed in 70 persons by re-examination of the blood films in our laboratory. The remaining 41 persons in this group were positive only by PCR. In 34 samples diagnosed as positive for falciparum malaria by the local microscopist, *Plasmodium falciparum* was not demonstrated by PCR and these were considered to be misidentifications by the microscopist, since 12 samples of this group contained *P. vivax* and not *P. falciparum*, and in the remaining 22 samples parasites were not detected even by re-examination of the films. In four cases negative by PCR, the parasite was detected by our parasitologists on Giemsa-stained films.

Correlation of infection intensity with age of the patient

It is well known that children are more susceptible to malaria infection than adults, and may suffer severe or complicated disease. However, in this study, no significant correlation was found between the age and results of the PCR (correlation coefficient r=–0.14). This controversial finding should be due to the main population age that was sampled (Fig. 4). Most of the samples were from children younger than 15 years old.

Discussion

More than 200 million patients a year are estimated to be infected with malaria in the world, with approximately 2 million deaths annually, especially involving infants. To design more adequate malaria control programs, it is crucial to know the precise incidence and epidemiology of this disease. Traditionally, malaria infection has been diagnosed by optical microscopy, because of its low cost and suitability under field conditions. However, in areas where malaria is a common disease, most of the

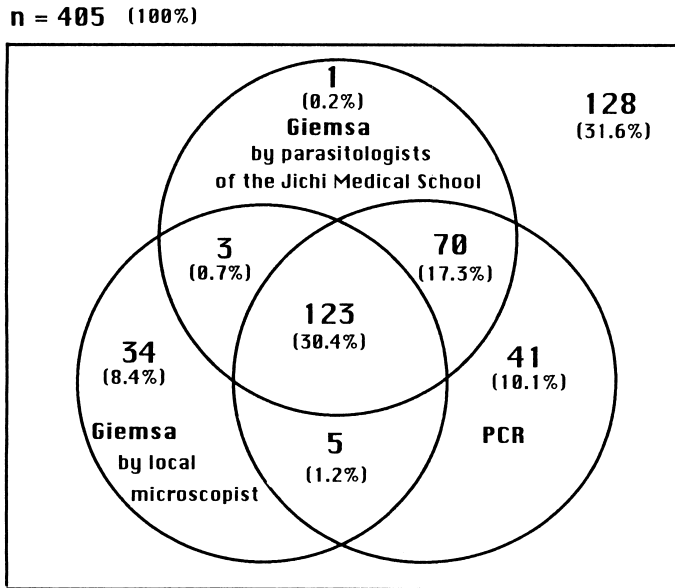

Fig. 3 Comparison of the results obtained by the examination of Giemsa-stained thick blood films in the field by a local microscopist with those obtained by parasitologists in the Jichi Medical School and the PCR method.
Isolated, in the upper right of the square, are the "true" negative (n=128, 31.6%). In the upper-middle circle, are the positives for P.f. in blood films examined at Jichi Medical School by Giemsa method (n=197, 48.6%). In the right circle are the positives by PCR (n=239, 59.0%) and in the left circle, are the positives found by the local microscopist (n=165, 40.7%).

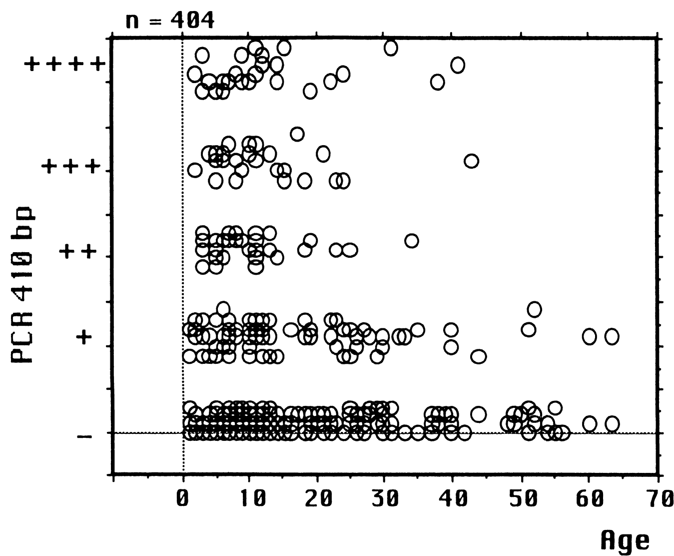

Fig. 4 Correlation of *Plasmodium falciparum* parasitemia with the age of the patient.
No significant correlation was found between age and results obtained by 410-bp PCR method (correlation coefficient r=−0.14). A man of 89 years was excluded in this graph.

population sustain a very low parasitemia, due to a relative immunity or because of the use of drugs in sub-curative doses. Patients with low parasitemia are detected only with difficulty by the ordinary method, since its detection limit is considered to be around 50 to 5,000 parasites/μl (Tirasophon *et al.*, 1991). On the other hand, these apparently healthy patients are the principal population responsible for maintaining the disease in certain areas. These "carriers" usually are not treated because they lack symptoms, or because their symptoms are negligible. So they serve as a long-term source of infection, spreading and perpetuating the disease in the area. Development of the PCR has permitted us to diagnose several microorganisms in minimal numbers, including malaria parasites in the blood. We chose the filter paper disk method for sampling blood for the following reasons: (1) only a small amount of blood is required, (2) ease of sampling, (3) no need for refrigeration to preserve the sample, (4) easy transportation, and (5) simplicity of isolation of the DNA (using this method, the phenol/chloroform step in the isolation of the DNA can be omitted). Different segments of the malarial DNA are reported to be unique to *Plasmodium falciparum* and these have been used as targets for PCR detection (Alano *et al.*, 1991; Barker *et al.*, 1992; Sethabutr *et al.*, 1992; Wooden *et al.*, 1993). However, concerning the sensitivity and specificity of the method, it is difficult to determine exactly the best point for amplification, especially for protozoan parasites such as malaria. Variants of the same strain have already been reported, indicating that malaria parasites may frequently change their antigenicity and thus increase their ability to survive in the host organism (Kain and Lanar, 1991; Cheng *et al.*, 1993; Kain *et al.*, 1992; Janse, 1993; Prescott *et al.*, 1994). We targeted two different sites for PCR amplification: the DHFR-TS junction segment (Bzik *et al.*, 1987; Snewin *et al.*, 1989; Wataya *et al.*, 1993) and the Pfg27/25 gene (Alano *et al.*, 1991), in order to increase the chance of detection even if some variation had occurred. The probability of variations occurring simultaneously in these two targeted segments is lower than the probability of variations ocurring individually in each segment. Therefore, parasites with DHFR-TS variants that would not be recognized by PCR, could be detected by amplification of the Pfg27/25 gene, or *vice versa*. Wataya *et al.* (1993) reported the use of a double-PCR technique for detection of falciparum malaria in the Solomon Islands, in which the detection limit was approximately 5 times more sensitive than that of the single PCR. However, the double PCR method requires about 10 h from preparation of the sample to obtaining the result; in contrast, amplification of 2 distinct DNA segments can be carried out simultaneously, shortening the time to 4 hours. Although we have no direct evidence that variations had occurred in the DHFR-TS junction segment or the Pfg27/25 gene, this possibility was suggested by the occurrence of samples that were positive in only one of the two PCRs performed.

Combination of the two separate PCR results (with sensitivities of 80.2% with 410 bp and 91.9% with 598 bp) increased the sensitivity to 98%. The 41 samples that were negative by the Giemsa method but positive by PCR amplification may have included patients with parasitemia below the detection limit of the microscopic method. This group also may have included some false positive results. In a few cases, only one of the two PCRs was positive. A possible explanation for this discrepancy, as mentioned above, is the presence of a variant of the parasite, with an alteration in the targeted gene, or parasitemia near the detection limit of the PCR. The 70 patients diagnosed as negative by the local microscopist but positive by our parasitologists were considered to be the result of a failure in diagnosis by the first microscopist, since PCR and re-examination of the blood films detected parasites. Thirty-four samples diagnosed as positive for *Plasmodium falciparum* by the local microscopist were not confirmed by PCR or by re-examination of the films. This group included 12 misdiagnosed *Plasmodium vivax* infections. With the remaining 22 samples in this group, it is difficult to be certain of the "true" diagnosis, but, as both PCR and re-examination of the films failed to detect *Plasmodium falciparum*, it seems likely that the first, "positive", diagnosis was wrong.

Giemsa staining has long been used for diagnosis of malaria because it is the most accessible method under field conditions. However, our data show that the result obtained by examination of Giemsa-stained blood films alone may not always reflect the true

prevalence of this disease. Some discrepancies were observed between the results obtained by the PCR and those from the Giemsa-stained blood films. These differences do not necessarily reflect examiner error; they may often have been due to the relatively high limit of detection of the optical microscopy method and the limit to the number of slides that can be accurately examined by a microscopist in one day. Although it remains to be seen whether the PCR is practicable in the field for detection of malaria, we conclude that another, sensitive method of detection, in addition to Giemsa staining, is required for an adequate epidemiological study of malaria. Another problem in diagnosing malaria, is the existence of patients with multiple infections, commonly observed in malaria-endemic areas (Brown *et al.*, 1992; Snounou *et al.*, 1993). DNA and ribosomal RNA of the four human malaria parasites have been sequenced (Waters and McCutchan, 1989; Kain *et al.*, 1992; Snounou *et al.*, 1993), allowing us to perform genetic diagnosis of these infections. More recently, Kimura *et al.* (1994) and Arai *et al.* (1994) have developed a microtiter plate hybridization method for DNA diagnosis of malaria, with good prospects for a field study.

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