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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

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Genus- and species-specific sequences are present within the small subunit ribosomal RNA genes of the four human malaria parasites. Oligonucleotide primer pairs specific to each species were designed for specific amplification by the Polymerase Chain Reaction (PCR), to detect each malaria species. DNA equivalent to 5 μl of blood was sufficient for the detection of each of the species. Blood samples obtained from 196 patients attending a malaria clinic in Trad province (Thailand) were analyzed. Detection and identification of the parasites, solely by electrophoretic analysis of the PCR products, has proven to be more sensitive and accurate than by routine diagnostic microscopy. A high proportion of mixed species infections were brought to light by the PCR assay. Implications for medical treatment and epidemiological studies are discussed.

Key words: *Plasmodium falciparum*; *Plasmodium vivax*; *Plasmodium malariae*; *Plasmodium ovale*; Polymerase chain reaction; Diagnosis; Mixed infection

Introduction

Detailed knowledge of the incidence and the transmission dynamics of the four malaria species is central to the design of effective malaria control measures. Accurate diagnosis is also clearly essential for successful treatment. In both epidemiological data collection and medical diagnosis, microscopy is universally used. Its practicality makes it particularly suited for rapid diagnosis under field conditions. The time required from blood collection to diagnosis is relatively short when large numbers of parasites are present (1000 or more per mm^3 of blood). Microscopic examination of the blood smear for 15 min or more are required for detection when very low parasitemias are encountered, a situation frequently found with immune populations or when drugs are taken at sub-curative levels. Identification of the species poses few problems to the experienced microscopist, except when one species is numerically dominated by another in a mixed infection. Thus, the major limitation of microscopy resides in the length of time required for blood smear examination.

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Abbreviations: PCR, polymerase chain reaction; ssrRNA, small subunit ribosomal RNA.

This is acutely felt when large numbers of samples need to be accurately examined. This is the case not only in many epidemiological investigations, but also in clinical, therapeutic and immunization trials where the malarial status of a number of subjects must be determined over a prolonged period of time.

In recent years, many researchers have addressed these problems by exploiting molecular biological techniques [1–4]. Generally, nucleic acid sequences specific to a particular species are used. These sequences form targets for hybridization by labeled DNA probes, or act as targets for PCR amplification. In view of the virulence and mortality associated with *Plasmodium falciparum*, most of the research undertaken was concentrated on the detection of this species. High sensitivities for *P. falciparum* detection, down to one parasite, have been claimed by combining the 2 approaches [5,6]. In this paper we present an accurate and practical laboratory based PCR analysis method for use in conjunction with epidemiological and clinical investigations. We have extended previously described methods, by the detection of the 3 other species of malaria infecting man, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. In addition, the hybridization step for amplification product detection is replaced by a simple electrophoretic analysis.

It has been recently discovered that some portions of the sequences coding for the small subunit ribosomal RNA (ssrRNA) are specific to the parasite species from which they are derived [7–10]. This was put to elegant use by designing oligonucleotide probes which specifically and sensitively detect parasites by hybridization to RNA purified from small volumes of blood [9,11]. We have exploited the species-specific sequences in the ssrDNA genes to design oligonucleotide primers suitable for use in the PCR amplification technique [12]. Oligonucleotide pairs specific for each of the four parasite species were synthesized and tested on blood samples obtained from Thai patients. The PCR assay has been shown to be more sensitive than routine diagnostic microscopy. It further allows unequivocal identification of all the human parasite species present in a given sample.

Materials and Methods

Sample collection. Blood samples were collected from consenting patients attending the Malaria Clinic at the village of Borai, Trad Province Thailand. This village is situated close to the border between Thailand and Cambodia. Many of those attending the clinic are migrant gemstone miners. They routinely spend between 1–3 weeks prospecting in the Cambodian jungle, where presumably most of their infections originate. On their return many attend the Malaria Clinic either because they have malaria symptoms (headache, fever or chills) or just to ensure that they are parasite free. The blood samples used were collected for a study initiated by S. Thaithong (WHO/FIELDMAL ID900195). In some cases the samples were obtained in a follow-up examination of patients treated for malaria in the previous 1–3 weeks. 99 and 97 samples were collected on 2 separate occasions, 24th June 1991 and 24th February 1992, respectively. Approximately 1 ml of whole blood was obtained by venepuncture into heparinized tubes by qualified staff. Thin and thick blood smears were made before aliquoting the blood. These samples were stored on ice and brought back on the same day to the laboratory at Chulalongkorn University in Bangkok. Samples from the first collection were stored at −70°C prior to DNA purification. Following the second collection, the blood was stored at 4°C overnight, the plasma was then removed before storage at −70°C.

Microscopy. On arrival of the patients, thick blood smears were made from finger-prick samples, stained with Giemsa and examined by expert microscopists at the Malaria Clinic. Up to 200 microscope fields were examined to establish the diagnosis, to which results from the PCR assay were compared. Duplicate smears were made from the venous blood and brought back to Chulalongkorn Univer-

sity. These were examined by one of us (S.T.) to confirm the PCR assay results. Exact parasite enumeration was not performed. Patients whose thick smears required more than 10 min of examination before a parasite was detected, are referred to as having very low parasitemias.

DNA template preparation. DNA was purified from the samples as follows. The blood was allowed to thaw on ice before being mixed with cold phosphate-buffered saline (PBS; 1.3 ml final volume). The parasites and the unlysed erythrocytes were recovered by centrifugation (5 min, 6000 × *g*). The supernate was discarded and the pellet resuspended in 1 ml cold PBS, saponin was then added to a final concentration of 0.05%. Immediately after lysis was observed, the parasites, as well as white blood cells, were recovered by centrifugation as above. The pellet was immediately resuspended in 25 μl of lysis buffer (40 mM Tris pH 8.0 (with HCl)/ 80 mM EDTA pH 8.0/ 2% SDS and 2 mg ml^{-1} Pronase E). Sterile distilled water was added to a final volume of 100 μl, the mixture was vortexed and then incubated for 4 h at 37°C. A further 300 μl of water was added to the mixture before phenol extraction of the DNA. An equal volume of phenol equlibrated with 0.1 M pH 7.0 Tris (with HCl) was added and the two phases were mixed by vortexing. The aqueous phase was recovered by centrifugation (10 min, 13 000 × g) and re-extracted as above with phenol/chloroform (phenol equilibrated with 0.1 M Tris pH 7.0 (with HCl)/chloroform/isoamyl alcohol at a 50:48:2 ratio). DNA was precipitated by overnight incubation at −70°C following the addition of Na acetate pH 5.0 (final concentration 0.3 M), and 2 volumes of cold absolute ethanol. The DNA was pelleted by centrifugation (10 min, 13 000 × g), washed in 70% ethanol, air dried, and then resuspended in TE buffer (10 mM Tris pH 8.0 (with HCl), 0.1 mM EDTA pH 8.0). The volume of TE used is such that 1 μl of the suspension is equivalent to approx. 5 μl of whole blood.

DNAs used as positive controls for the amplification reactions were purified as above from the following: *P. falciparum* infected blood was obtained from in vitro cultured parasites routinely maintained at the National Institute for Medical Research (London). Samples of blood from chimpanzees individually infected with *P. vivax*, *P. malariae* and *P. ovale* were obtained from W. Collins (Center for Disease Control, Atlanta, USA).

Oligonucleotide synthesis. The published sequences of the ssrRNA genes of the human Plasmodia [7–10] were used to design the oligonucleotide primers used in this study. The sequences and positions of these primers are given in Fig. 1. Synthesis of the oligonucleotides was performed on a 380B DNA Synthesizer (Applied Biosystems, Foster City, USA).

PCR amplification. All PCR reactions were carried out in a total volume of 50 μl. In all cases amplification was performed in 50 mM KCl/10 mM Tris pH 8.3 (with HCl)/ 0.1 mg ml^{-1} gelatin/ 125 μM of each deoxyribonucleotide triphosphate/ 1.0 unit of AmpliTaq Polymerase (Perkin Elmer Cetus, USA). 1 μl of the purified template DNA was used per reaction. A separate reaction was carried out with every sample for the detection of each species. Only the oligonucleotide primers corresponding to that species were used in the reaction mixture. For the rPF1/rPF2, rPM1/rPM2 and rPO1/rPLU6 primer pairs, $MgCl_2$ at a final concentration of 3 mM was used, whereas 1 mM $MgCl_2$ was used for the rPV1/rPV2 primer pair. The oligonucleotide primers were used at 175 nM final concentration (each) for rPF1, rPF2, rPV1 and rPV2, and at 250 nM final concentration for rPM1, rPM2, rPO1 and rPLU6. The PCR assays were performed using a heating block (PTC-100, MJ Research Inc., USA). The amplification program was as follows. Step 1, 95°C for 5 min; step 2, annealing at 55°C (rPF1/rPF2 and rPV1/rPV2) or 52°C (rPM1/rPM2 and rPO1/rPLU6) for 2 min; step 3, extension at 72°C for 2 min; step 4, denaturation at 94°C for 1 min; repeat steps 2–4 39 times, then step 2, and finally step 3 for 5 min. On termination of the

amplification reaction, the temperature was reduced to 20°C.

PCR product analysis. 10 μl of 5× loading buffer (50 mM Tris pH 8.0 (with HCl)/ 75 mM EDTA pH 8.0/ 0.5% SDS/ 10% Ficoll/ 30% Sucrose/ 0.2% Orange G) were added to each amplification reaction. Product was detected by electrophoresis of 10 μl from the reaction on agarose (BRL, USA) or agarose/Nusieve agarose (FMC Bioproducts, USA) gels. The resolution range of the Nusieve agarose/ agarose gels makes them suitable for accurate molecular size determination of the expected PCR products. Agarose gels, were made and run in TAE buffer (40 mM Tris/ 20 mM Na-acetate/ 2 mM EDTA, with the pH adjusted to 7.7 with glacial acetic acid). Nusieve agarose/ agarose (3:1) were made and run in TBE buffer (100 mM Tris/ 100 mM boric acid/ 5 mM EDTA). The amplification products obtained from field samples were electrophoresed on agarose gels, rather than on Nusieve agarose/ agarose gels in order to reduce costs. The DNA was visualized on an ultraviolet transilluminator following ethidium bromide staining.

Results

Specificity of the assay. The variable sequences that are present on the ssrRNA genes of *Plasmodia* (shaded areas in Fig. 1) were exploited to design species-specific oligonucleotide primers. The sequences of the primer pairs used to detect each species by PCR amplification are given in Fig. 1.

Control genomic DNAs from the four human malaria species as well as from humans were used to confirm the specificity of each oligonucleotide pair, and to optimize the PCR assay conditions. Under optimum buffer and PCR cycling parameters, a DNA fragment is amplified only when the genomic DNA of the corresponding species is present (Fig. 2). The size of the fragment corresponds to that predicted from the published sequences, namely 918 bp for *P. falciparum*, 714 bp for *P. vivax* and 513 for *P. malariae*. The full sequence of the *P. ovale* ssrRNA gene is not available. The 3′-end oligonucleotide was made to correspond to the only published *P. ovale* sequence, the 5′-end primer was complementary to a genus-specific sequence of plasmodial ssrRNA genes.

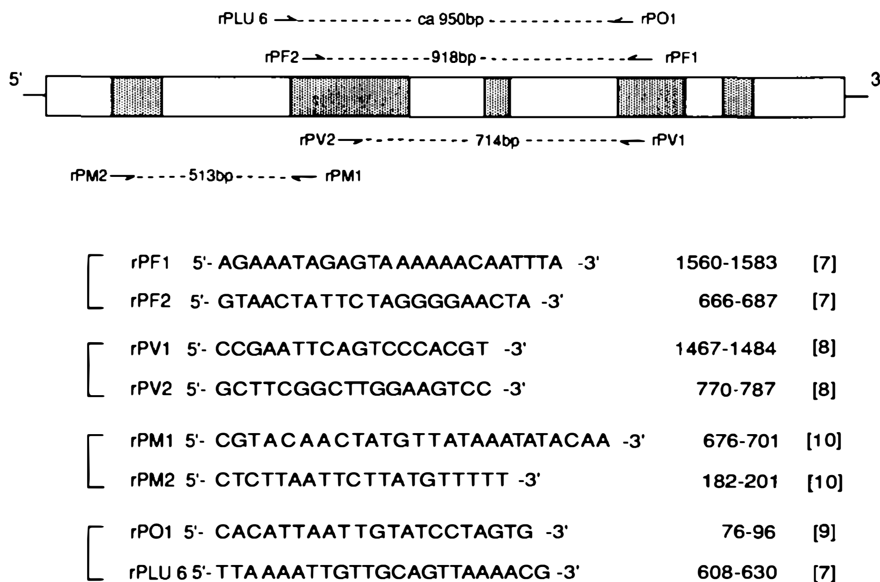

Fig. 1. Schematic representation of the genes coding for the ssrRNA of Plasmodia. Blank areas represent sequences conserved in all the species, whereas stippled areas represent variable sequences unique to each species. The position of the oligonucleotides primers used for PCR amplification is represented by an arrow, and the fragment amplified by a broken line. The sequence of each primer as well as their exact location, as obtained from the published works indicated, is also given.

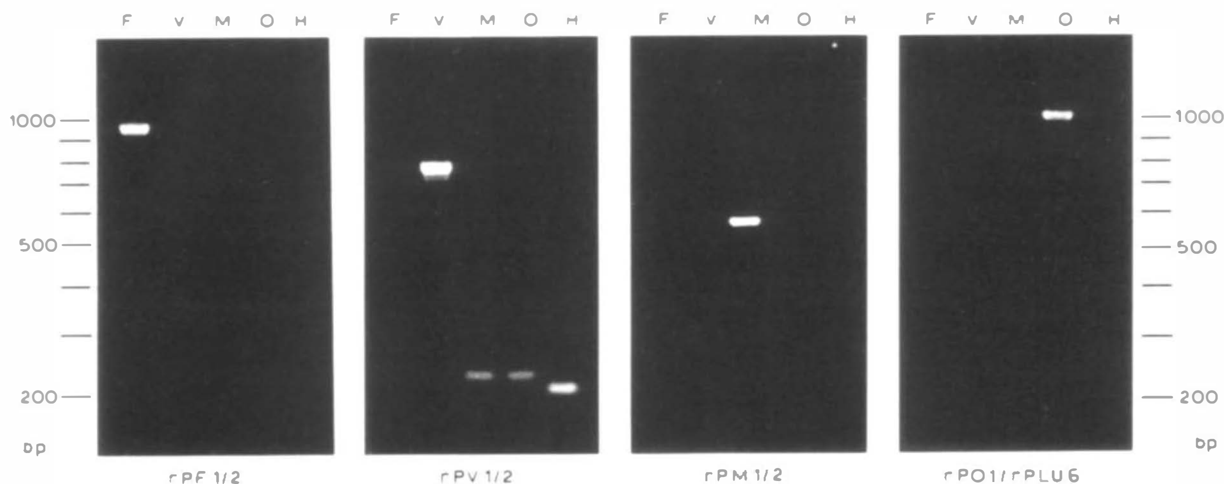

Fig. 2. Specificity of the amplification assays. The electrophoretic mobility of the products of PCR amplification using the indicated oligonucleotide primer pairs (bottom of each panel). The total genomic DNA templates used are indicated above each lane: F, *P. falciparum* from in vitro cultured T9-94 clone; V, *P. vivax*; M, *P. malariae*; O, *P. ovale*; and H, human control. V, M and O obtained from infected chimpanzee blood. In each case approx. 0.5 μg of DNA was used in the PCR assays which were performed as in Materials and Methods. Electrophoresis was in a 3% Nusieve agarose/agarose (3:1) gel run in TBE buffer. The DNA size marker is a 100-bp ladder.

The 960-bp fragment obtained confirms that the structure of the *P. ovale* gene is similar to that of the other 3 species. Using the *P. vivax* specififc oligonucleotides (rPV1/rPV2) a fragment of approx. 200 bp is seen following PCR amplification of *P. malariae* and *P. ovale* control genomic DNAs, a fragment of smaller size is observed with human DNA. Since no such fragment is seen with the *P. falciparum* DNA template, it is concluded that this results from non-specific amplification of the mammalian host DNA. The size of this non-specifically amplified fragment makes it readily distinguishable from the *P. vivax* specific fragment. This non-specific amplification is only occasionally observed (Fig. 3, *P. vivax* panel samples 271, 275, 280, 300, 305 and 355). A similar, albeit fainter, human-specific fragment (approx. 450 bp) is also sometimes seen following amplification with the *P. falciparum* specific PCR primers (rPF1 and rPF2).

PCR analysis of field samples. Blood from a total of 196 persons, attending the Malaria Clinic at the village of Borai in Thailand, was collected. DNA was purified from all the samples. PCR assays specific for *P. falciparum* and *P. vivax* were performed on all the samples, whereas only the 97 samples obtained on the second trip to Borai were assayed for *P. malariae*. *P. ovale* which is considered to be very rarely found in Thailand was not assayed for. However, one sample diagnosed by one of us (S.T.) following examination of the slide at a later date was identified as *P. ovale*. This sample was the only one assayed by PCR for the presence of *P. ovale*. In all PCR assays only 1 μl of template was used. This corresponds to the amount of human DNA and, if present, parasite DNA in approx. 5 μl of blood. The anti-coagulant heparin has been reported to be highly inhibitory to the *Taq* polymerase [6]. No significant inhibition was observed with the DNA from the heparinized blood samples used for this study. Presumably, the concentration of potential inhibitors had been minimized by the procedure employed for DNA preparation (see Materials and Methods).

A representative sample of the results obtained is presented in Fig. 3. The PCR assay is not quantitative, and consequently the product yield does not correlate with the level of parasitemia. The amount of product

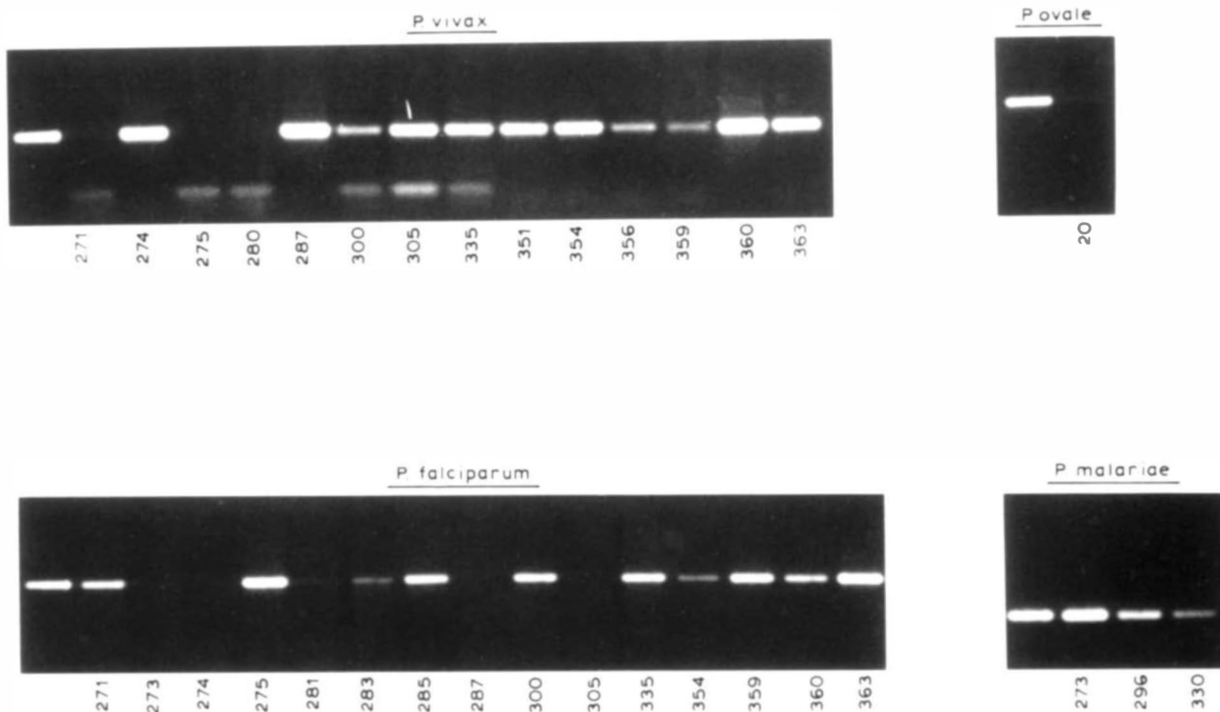

Fig. 3. A representative example of the results obtained using the PCR assay for the four human malaria species on samples from Thai patients. 1 μl of DNA, which corresponds to 5 μl of whole blood, was used in each assay. One fifth of the PCR product was electrophoresed in 1% agarose gels, and visualized by ethidium bromide staining. The number below each lane corresponds to the sample number. The left-hand lane in each panel contains the positive control in which genomic DNA from *P. falciparum*, *P. vivax*, *P. malariae* or *P. ovale* was used as a template for the PCR assay. Due to photographic reproduction, some of the fainter bands are difficult to discern. These are, for *P. falciparum* numbers 273 and 281 and for *P. ovale* number 20. The negative samples are numbers 274, 287 and 305 for *P. falciparum*, and numbers 271, 275 and 280 for *P. vivax*.

amplified, however, was sometimes much diminished when very low parasite numbers were present in the samples assayed. The sensitivity of the *P. malariae* and *P. ovale* primers appear to be less than that observed with the *P. falciparum* and *P. vivax* primers. This observation could not be confirmed since only 3 *P. malariae* and 1 *P. ovale* samples were available. Throughout the analysis, negative controls for the PCR assay were carried out for every set of amplification reactions. In addition, the majority of the results were derived from duplicate reactions. No evidence for cross-contamination or inconsistencies in the duplicate results was observed.

The results of microscopic diagnosis (Table I) which was carried out by the staff of the Malaria Clinic at the time of collection were as follows: 106 positive cases (54%) were detected, of which 81 (41%) were determined as *P. falciparum* infections, 23 (18%) as *P. vivax* infections, 1 (0.5%) as a *P. malariae* infection, and finally one case (0.5%) as a mixed *P. falciparum* and *P. vivax* infection.

The results of the PCR assays (Table I) were as follows: Plasmodia were detected in 129 people (66%). Infections due to *P. falciparum* alone were present in 89 cases (45%) and to *P. vivax* alone in 12 cases (6%). Two of the patients seen on the second collection harbored a *P. malariae* infection. The diagnosis of *P. ovale* malaria in one patient was confirmed. 24 patients (12%) were found to be simultaneously infected with *P. falciparum* and *P. vivax* and one patient had a *P. falciparum* and *P. malariae* double infection.

Seven of the samples which were diagnosed as *P. falciparum* and 2 as *P. vivax* in the

TABLE I

Diagnosis of patients from the Malaria Clinic in Borai, Thailand

	Positive							Negative
	F	V	M	O	F+V	F+M	Total	
Microscopy	81[a]	23[a]	1		1	–	106	90
PCR analysis	89 (114)	12 (36)	2 (3)	1[b] (1[b])	24 –	1	129 –	67[c]

F, *P. falciparum*; V, *P. vivax*; M, *P. malariae* and O, *P. ovale*. The numbers in these columns refer to infections caused by one species alone. Numbers in brackets include the mixed infection cases.
[a]Three false positives (1 *P. falciparum* and 2 *P. vivax*) were detected in the microscopic diagnosis.
[b]This was the only sample assayed by PCR for the presence of *P. ovale*.
[c]Six false negatives (*P. falciparum* by microscopy) were found by PCR analysis.

Malaria Clinic, proved to be negative following PCR analysis. In 3 of these (which include the two *P. vivax* diagnosed infections), no parasites could be detected by re-examination of the blood smears by one of us (S.T.). Thus, from microscopic examination, only 3 false positive cases were detected. Six PCR assay false negatives remain. DNA degradation was evident in one of the samples, and is probably the cause of the negative result observed with this sample. The level of parasitemia, as ascertained by prolonged microscopy, in the remaining 5 samples was very low. The presence of small amounts of PCR inhibitors in the DNA solution could be sufficient to reduce the yield of the amplified products to below the detection levels afforded by ethidium bromide staining. Furthermore, the possibility of a mis-labeling or mis-recording error in the field could not be discounted as the blood smears used by the Malaria Clinic staff were not available for re-examination.

In 32 cases (16%) the negative diagnosis following microscopic examination at the Malaria Clinic proved to be incorrect. *P. falciparum* was the species detected by amplification in these 32 samples. The presence of parasites was later confirmed by lengthy microscopic examination.

In only 6 (3%) patients was the infecting parasite species mis-diagnosed by routine microscopy. All these cases, 3 *P. falciparum* infections, 2 *P. malariae* infections and 1 *P. ovale* infection, as determined by PCR, had been identified as *P. vivax* by the field microscopists.

Significantly, 24 of the 25 mixed species infections detected by PCR were missed following blood smear examination. Of these (*P. falciparum* with *P. vivax*), 5 were deemed negative in the field. In 4 other cases (3 *P. vivax* and 1 *P. malariae* as determined by microscopy) the presence of *P. falciparum* was revealed by PCR. Finally, in 15 patients field diagnosed as infected with *P. falciparum*, *P. vivax* was also found by PCR.

Discussion

In this report we present a method in which species-specific sequences of the ssrRNA genes are exploited for use in a PCR assay which allows the unequivocal detection and identification of all four species of the human malaria parasite. The specificity of the assay was first established using control DNA samples from the four species and from the human host (Fig. 2).

The method was tested on blood samples obtained from the field, with the objective of establishing to what extent it compares with the diagnosis determined by the Malaria Clinic microscopists. Parasite counts were not performed on the samples used, since our aim was to develop a method of parasite detection that can replace routine microscopic examination, without any loss of reliability. An accurate determination of the sensitivity of the method is difficult to achieve. In view of the logarith-

mic DNA amplification afforded by PCR, and of the product detection method, namely ethidium bromide staining, exact quantification of the amplification reaction is unreliable. In addition, the precise enumeration of parasites in a given volume of blood is impractical for a large number of samples, since an accurate measure of erythrocyte numbers and parasitemia must be obtained. The latter is particularly arduous at low parasitemias. Calculation of the volume of blood examined by thick smear examination is also inexact, since it assumes a constant number of leucocytes per unit volume of blood, whereas in patients suffering from malaria this number varies considerably during the course of the infection. Furthermore the presence of human DNA in the reaction alters the efficiency of the reaction, significant inhibition has been observed when a large excess of human DNA has been included in the reaction (data not shown). Thus, amplification efficiency using in vitro cultured *P. falciparum* DNA, which is human DNA-free, cannot be compared accurately with that obtained from patient-derived material. Finally parasite material for quantification of detection must be derived from very highly synchronous cultures or infections, with the parasite being in the early ring stage when DNA synthesis is yet to be initiated. Multiply infected erythrocytes must also be totally excluded from the sample assayed.

The reliability of the PCR method presented in this article is very good. Only 5 false negative samples were observed following PCR amplification. It should be noted that the DNA product was detected visually. The sensitivity of detection could be considerably improved by the hybridization of labeled nucleic acid probes with the product. It was, however, felt that the minimum number of steps should be included in order to increase the practicability of the assay. Primer pairs resulting in a higher yield of amplification products might also improve the sensitivity of the assay. We are currently testing newly designed oligonucleotides for this purpose.

The results obtained from the field samples demonstrate that the PCR assay surpasses microscopy both in sensitivity and in diagnostic accuracy.

From the sample tested, 32 patients (16%) harbored parasites undetected by microscopy at the Malaria Clinic. This represents a substantial number of misdiagnosed patients, especially when one considers that *P. falciparum* was present in all these 32 patients. Apart from the serious medical implication of these missed diagnoses, the incidence of the parasite is underestimated. In the majority of these patients the parasite levels were low, so much so that in many instances examination of the thick smear for 40 min or more was needed before a parasite was observed, thus confirming the PCR diagnosis. Interestingly, 80 of the patients attending the Malaria Clinic were follow-up patients, who had been treated for malaria 1–3 weeks previously. 17 of the 32 cases (as discussed above) newly diagnosed positive by PCR belonged to this group. Following microscopy, 38 of these follow-up cases (47.5%) were still considered to harbor parasites. This proportion was increased to 55 patients (66%) by the PCR assay. These differences are quite important when assessing the efficacy of anti-malarial treatment.

Provided that infected cells are seen by the microscopist, the identification of the parasite is quite reliable. When missed and mixed infections are excluded, the accuracy of species diagnosis by the field microscopists was very high, only in 6 cases (3%) was the parasite species misdiagnosed. This underlines the high competence of the microscopists at the Malaria Clinic, since this level of accuracy equals that attained by senior malariologists [13].

The most dramatic result derived from the PCR analysis resides in the detection of mixed species infections. Simultaneous infections by 2 or more species have been observed in all the vertebrate hosts of Plasmodia [14]. In the human population, the percentage of these mixed infections has rarely been recorded to exceed a few percent of the total number of infected cases [13]. It was, therefore, unexpected to detect 25 mixed infections in the patients surveyed (13% of the total and nearly 20% of

the positive cases). The high proportion of mixed infections observed in patients from Borai has not been previously recorded in Thailand. In fact, figures obtained from the Malaria Division (Ministry of Health, Bangkok, Thailand) show that only 0.33% mixed infections were recorded by field microscopists for the period 1986–1988. It should be noted that the microscopic detection of *P. vivax* in an infection dominated by *P. falciparum*, is facilitated by the occasional presence of the distinctive late stages of the *P. vivax* asexual parasite (those of *P. falciparum* sequester and are not seen in the peripheral blood). In 15/25 PCR-determined mixed infections from our samples, the *P. vivax* parasite, which was superimposed on a microscopically detected *P. falciparum* infection, was brought to light by the PCR assay. In the reverse situation, a *P. falciparum* ring or early trophozoite amidst *P. vivax* parasites at a similar stage of development, is frequently overlooked. A diagnosis in which *P. falciparum* is missed is potentially of grave consequence to the patient. This is particularly so in Thailand where *P. vivax* infections are usually treated with chloroquine, an anti-malarial drug to which the local *P. falciparum* parasites are highly resistant. In 9/25 of the mixed infections detected by PCR from our samples, *P. falciparum* was overlooked by microscopy (superimposed on a *P. vivax* infection in 3 cases, on a *P. malariae* infection in 1 case, and missed along with *P. vivax* in 5 cases).

It is generally considered that an antagonism exists between the malaria species in mixed infections [13,15], where each species tends to dominate in turn through the course of the infection. This relationship has been somewhat bourne out by the very few experimentally induced simultaneous infections that have been described [16–18]. This phenomenon would explain the difficulty in detecting mixed infections by routine microscopy, especially when the patient is sampled only once. The PCR assay clearly offers an advantage in this situation. The detection of mixed infections is important not only for successful medical treatment, but also for ascertaining the true incidence of each species and consequently its transmission potential. Both of these factors are central to the elaboration of control measures, and for the assessment of their efficacy. Finally, there is evidence that the simultaneous presence of 2 or more species in a host can substantially alter the course of the infection [19–22]. In addition to the suppression of one of the species and the reduction of parasitemia, changes in the resulting pathology and mortality have been recorded. Very little is known about the effects on pathology when mixed infections occur in man. The possibility that the presence of a particular species can exacerbate or indeed alleviate the symptoms caused by another species merits investigation, particularly with respect to the *P. falciparum* parasite which is responsible for the majority of fatal malaria cases.

The detection protocol as presented in this article is neither sufficiently practical, nor rapid enough, for routine patient treatment in the field. We are currently developing further protocols in which sample collection and DNA preparation are simplified. The assay, however, is suitable for use in patients under hospital or clinical supervision. The PCR technology described provides a powerful tool in the study of malaria biology and epidemiology.

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