

Evaluation of DNA Recombinant Methodologies for the Diagnosis of *Plasmodium falciparum* and their Comparison with the Microscopy Assay

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Since 1984, DNA tests based on the highly repeated subtelomeric sequences of *Plasmodium falciparum* (rep 20) have been frequently used in malaria diagnosis. Rep 20 is very specific for this parasite, and is made of 21 bp units, organized in repeated blocks with direct and inverted orientation. Based in this particular organization, we selected a unique consensus oligonucleotide (pf-21) to drive a PCR reaction coupled to hybridization to non-radioactive labeled probes. The pf-21 unique oligo PCR (pf-21-I) assay produced DNA amplification fingerprints when was applied on purified *P. falciparum* DNA samples (Brazil and Colombia), as well as in patient's blood samples from a large area of Venezuela. The performance of the Pf-21-I assay was compared against Giemsa stained thick blood smears from samples collected at a malaria endemic area of the Bolívar State, Venezuela, at the field station of Malariología in Tumeremo. Coupled to non-radioactive hybridization the pf-21-I performed better than the traditional microscopic method with a $r=1.7:1$. In the case of mixed infections the r value of *P. falciparum* detection increased to $2.5:1$. The increased diagnostic sensitivity of the test produced with this homologous oligonucleotide could provide an alternative to the epidemiological diagnosis of *P. falciparum* being currently used in Venezuela endemic areas, where low parasitemia levels and asymptomatic malaria are frequent. In addition, the DNA fingerprint could be tested in molecular population studies.

Key words: *Plasmodium falciparum* - diagnosis - polymerase chain reaction - malaria

Plasmodium falciparum, the agent of the most lethal form of malaria, causes 1.5 to 2.7 million deaths each year, mostly among children. The incidence of malaria in the world is estimated to be 300-500 million clinical cases annually (WHO 1997). An approximate of 2,300 million people lived in areas with malaria risk, distributed in 100 endemic countries including Venezuela.

The Venezuela global incidence in 1997 was 28,056 malaria cases (Dirección de Endemias Rurales, Venezuelan Malaria Program, pers. commun.). Bolívar State located in the Amazon basin is responsible for 40-50% of the global inci-

dence for the whole country. The number of malaria cases has been in a constant rise during the previous years, due mainly to local economic activities (gold and diamond mines), changes in human migration patterns and a high prevalence of *P. falciparum* resistant malaria to chloroquine and pyrimethamine/sulfadoxine drugs.

Studies on epidemiology of parasitic infections, on measures to control disease and clinical evaluation of treatments, all require identification of the infecting species. The identification of malaria parasite is usually performed with traditional microscopic diagnosis of Giemsa-stained thick blood, which is inexpensive and of easy application in the field. Nevertheless, the sensitivity of microscopic method depends on highly trained examiners, is time-consuming when large numbers of samples must be examined, and is thus not the most appropriate method for large-scale epidemiological surveys.

Assay strategies have been proposed that directly detect abundant parasite nucleic acid sequences, including repetitive DNA (Franzen et al. 1984, Barker et al. 1986, Oquendo et al. 1986, McLaughlin et al. 1987, Zolg et al. 1987) or ribosomal RNA (rRNA) (Lal et al. 1989, Wathers & McCutchan 1989).

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In the *P. falciparum* genome there are several repetitive sequences. One of these consists of 21-bp blocks, imperfectly repeated in tandem clusters oriented in opposite directions (rep-20). These rep-20 sequences are found in all chromosomes of the parasite (Oquendo et al. 1986). DNA probes directed to these repeats hybridize with *P. falciparum* strains from South America, Africa, and South and Southeast Asia (Buesing et al. 1987), and their sensitivity is comparable to that of conventional microscopy (Lanar et al. 1989). The sensitivity of DNA and RNA approaches have been further increased through the amplification of the target DNA using the polymerase chain reaction (PCR).

Considering the high specificity of the rep-20 for *P. falciparum* and its genomic organization, our objective was directed to design a diagnostic PCR assay coupled to non-radioactive hybridization, for the detection of *P. falciparum* in human blood samples. In the present communication we report this diagnostic assay using a single oligo and digoxigenin labeled probes detected by photoluminescence. Its application to blood samples obtained and processed in the endemic areas, compared to traditional thick smear technique is discussed. We also report the application of the assay to isolated *P. falciparum* DNA to produce amplification patterns differentiating strains of several geographical locations and proposing its use as a tool in molecular epidemiological studies.

MATERIALS AND METHODS

Blood samples collection and treatment - The sample blood was obtained from patients with malaria symptoms assisting to the malaria diagnostic post of the Venezuelan Malaria Program in Tumeremo, Bolívar State. The samples were collected during two visits of one week each to the endemic area during July 1993 and March 1994.

Duplicate samples of fingerprick blood were collected from 33 individuals in heparinized capillary tubes; 50 µl were deposited in Whatman paper filter, dried at room temperature and stored in individual and labeled sealed plastic bag, in case it was necessary to repeat the PCR assay. Another 50 µl were transferred to a 1.5 ml centrifuge tube in which the *P. falciparum* DNA was isolated using the chelex-100-iron protocol (Wooden et al. 1993).

Microscopic examination - Two thick blood films were prepared for each patient during the blood collection process. The blood smears were stained with Giemsa, and one reading was performed at field site under routine conditions of work (100 fields examined under oil immersion optics before a slide was considered negative), by an expert microscopist at the diagnostic post of the

Malaria Program. For comparison purposes, a second microscopic diagnostic was done by one of us (L.U.); in this case, the sample was considered negative after 200 microscopic fields were examined. The parasite number was registered with respect to 200 white blood cells.

***P. falciparum* reference strain DNAs** - *P. falciparum* reference strain DNAs were a gift of the following researchers: (1) one Colombian strain from Dr Moises Wasserman (Instituto Nacional de Higiene de Colombia), (2) 12 Brazilian isolates from Dr Hernan Del Portillo (Universidade de São Paulo, Brasil) and (3) 2 cultured strains from Dr Erlinda Sánchez (Universidad de Carabobo and Malariología, Venezuela). The parasite genomic DNA was extracted using proteinase K, followed by phenol-chloroform extraction and ethanol precipitation.

Primers selection and PCR assays - Two oligonucleotides were designed: (1) a primer of 16 bp, pf-16 (5'-ACT AAC TTA GGT CTTA-3'), and (2) a primer of 21bp pf-21 (5'-ATG TTA GTC AAC TTA AGA CCT-3'), both derived from the pf-21 consensus sequence reported by Oquendo et al. (1986).

The *P. falciparum* assay described by Tirasophon et al. (1991), was included in this study as a reference test. This PCR uses primers K114-P1 (5'-CGC TAC ATA TGC TAG TTG CCA GAC-3') and K114-P2 (5'-CGT GTA CCA TAC ATC CTA CCA AC-3') for amplification of a 206 bp fragment and, primer K1-14 (5'-GCT ATA ACC ACT ATT GCA ACG-3') for hybridization. We designated the PCR assays I to III according to the oligonucleotid used: PCR I: pf-21 primer alone, PCR II: two primers pf-16 and pf-21, and PCR III: reference test using oligos K114-P1 and K114-P2.

PCR reaction conditions - Amplification reaction mixtures consisted of 50 mM KCL, 10 mM Tris HCL pH 8.8, 1% Triton X-100, 3mM dNTPs, and 3 mM MgCl₂. Primers pf-21 and pf-16 were at 4 µM. Primers K114-P1 and K114-P2 were at 1 µM. 1.25 units of Taq polymerase. The total volume was 25 µl. The specificity and sensitivity of the assays were tested against different *P. falciparum* DNA concentrations (200 ng/µl to 0.02 pg/µl), including in each case a negative control. Amplification products were analyzed by electrophoresis in 3% agarose gels and detected by staining with ethidium bromide.

PCR I and PCR II initial denaturation was done at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 2 min, and a final elongation at 72°C for 5 min.

PCR III initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, an-

nealing at 60°C for 1 min, and extension at 72°C for 1 min, and a final elongation at 72°C for 5 min.

Blots and DNA hybridizations - Amplified DNAs were size fractionated by electrophoresis in 3% agarose gel and transferred onto nylon membranes (Hybond N+, Amersham) using the VacuGene apparatus (Pharmacia LKB) following the manufacturer instructions. Slot blots were performed as described in Davis et al. (1986).

The filters were pre-hybridized in 2X SSC, 10X Denhardt's solution (Maniatis et al. 1982) and 1.5% blocking reagent (Boehringer-Mannheim) for 1 hr at 37°C. The hybridization with digoxigenin labeled oligonucleotides were incubated for 2 hr at 53°C for pf-21, and 50°C for K1-14. Filters were washed in 6X SSC-1% SDS at room temperature for 15 min, 2X SSC-0.1% at room temperature for 15 min, and finally 0.2X SSC-0.1% SDS at 37°C for 15 min. In the case of DNA probes, the filters were pre-hybridized in 6X SSC, 5X Denhardt's, 0.5% SDS, 5% blocking reagent and 50% formamide for 2 hr at 37°C. The hybridizations with digoxigenin labeled amplified products pf-21 and K114-P1/K114-P2 were incubated at 37°C for 12-18 hr. Filters were washed every 15 min in 2X SSC-0.1% SDS at room temperature for 1 hr, 1X SSC-0.1% SDS at 68°C each 15 min for 1 hr, and 0.1X SSC-0.1% SDS at 68°C twice each 15 min. The maximum concentration used for both the oligonucleotides and the probes was 20 ng/μl of hybridization solution.

DNA probe labeling and detection - The Genius Kit from Boehringer-Mannheim was used for DNA labeling and detection by photoluminescence. The oligonucleotides were labeled incorporating the dUTP-DGX using terminal transferase, following the manufacturer recommendations. The DNA probes were labeled by the multiprimer system incorporating the dUTP-DGX nucleotide. The photoluminescent substrate used was Lumi-phos 530 and the signals were detected using X-rays films.

RESULTS

PCR assays - Two PCR amplification systems were designed and standardized: PCR (I) pf-21 primer and PCR (II) pf-16+pf-21 primers. Both systems with target in the 21 bp repeat (rep 20). The reference system PCR (III) K114-P1/K114-P2 primers, was standardized to our conditions as described above. Fig. 1 shows the results of these amplification systems using DNA from two *P. falciparum* strains from Colombia. Both the PCR I and II directed to rep 20, yielded a broad range of amplified products, with band sizes from 200 bp to 3 Kb (Fig. 1A, C; lanes 1, 2), with higher intensity in the PCR II system. Similar results were reported by Barker et al. (1992), using complemen-

tary and degenerate primers directed against rep 20. Nevertheless, the use of two primers PCR II yielded nonspecific amplification with human DNA (Fig. 1C; lane 3) and negative control without DNA (Fig. 1C; lane 4). In the amplification with a single primer (PCR I) (Fig. 1A; lanes 1, 2), a broad range of reproducible amplified products was observed, clearly showing a 344 bp band for this *P. falciparum* isolate. No amplification was observed in the negative control (Fig. 1A; lanes 3, 4). The PCR III reference system yielded an expected single 206 bp band specific for *P. falciparum* (Fig. 1B; lanes 1, 2), without amplifying the human DNA (lane 3).

Considering the specificity and the amplification profile in *P. falciparum* DNA, the PCR I system was selected for detection of *P. falciparum* from field samples.

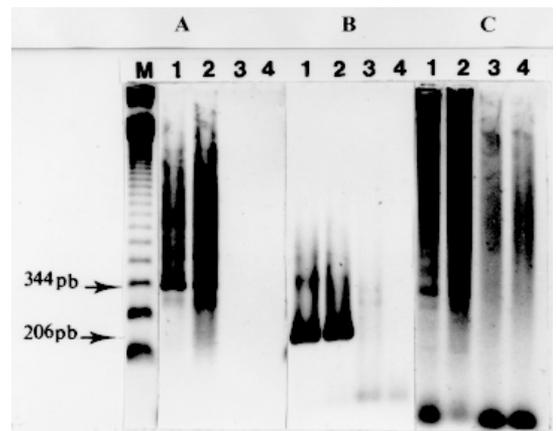


Fig. 1: PCR systems I, II and III. Electrophoresis in 3% agarose gel of amplified products by the systems. A: PCR I pf-21; B: PCR III K114-P1/K114-P2; C: PCR II pf-21+pf-16. Lanes - 1: 200 ng of *Plasmodium falciparum* purified DNA; 2: 50 ng of *P. falciparum* purified DNA; 3: negative control with human DNA; 4: negative control without DNA; M, 123 bp ladder.

Sensitivity of PCR I and PCR III systems - Once the specificity of these systems was determined, experiments were done to examine the detection sensitivity of both systems against serial dilutions of purified *P. falciparum* DNA. Fig. 2 shows the results of amplification products in 3% agarose gel electrophoresis for PCR I (Fig. 2A), PCR III (Fig. 2B) and Southern PCR III hybridizations (Fig. 2C). Both systems showed a detection level of 0.2 pg of *P. falciparum* DNA on agarose gels (Figs 2A, 2B; lane 7), equivalent to ten parasites. The South-

ern hybridization tests with digoxigenin labeled probe, yielded the following results: (a) the PCR I hybridized to the pf-21 primer showed a sensitivity level lower than the one observed in the electrophoresis, suggesting problems with the hybridization conditions (results not shown); (b) the PCR III hybridization (Fig. 2C) showed an increase in the sensitivity to 0.02 pg of DNA, equivalent to one parasite (Fig. 2C; lane 8).

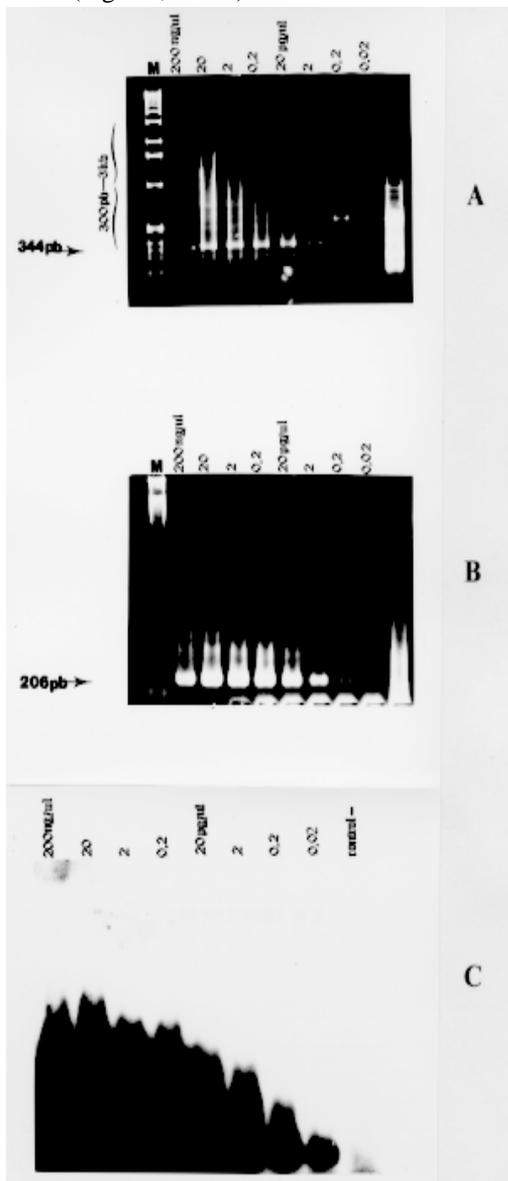


Fig. 2: sensitivity of PCR I and PCR II systems. Electrophoresis in 3% agarose gel of amplified products from serial dilutions of *Plasmodium falciparum* purified DNA. A: PCR I system pf-21; B: PCR III system K114-P1/K114-P2; C: Southern-blot hybridization of B against capture K-14 primer labeled with digoxigenin. The exposure time was 15 min. Lanes - 1: 200 ng/μl; 2: 20 ng/μl; 3: 2 ng/μl; 4: 0.2 ng/μl; 5: 20 pg/μl; 6: 2 pg/μl; 7: 0.2 pg/μl; 8: 0.02 pg/μl; M - Fig. 2A: ladder 1 Kb; Fig. 2B: ladder 123 bp.

Effects of anticoagulants on the PCR assays - Previous reports have revealed the inhibitory effect in PCR analysis of certain anticoagulants such as heparin (Barker et al. 1992, Tirasophon et al. 1991), citrate and EDTA (Tirasophon et al. 1994). We tested the PCR assay amplifying vertebrate rDNA included as a positive control for the PCR reaction (Premoli-de-Percoco et al. 1993), taking blood samples with different anticoagulants (heparin and citrate), including a sample in Wathman paper. The results showed the specific vertebrate's rDNA band of 126 bp in all the samples, indicating that there is no inhibition by the anticoagulants (results not shown).

PCR detection of P. falciparum DNA in human blood samples - Fig. 3 shows the agarose 2% electrophoresis of PCR III for a total of 32 samples. There is a 206 bp band (positive signal) in 11 samples (Fig. 3A; lanes 1, 2, 6, 7, 8, 9, 10, 11; Fig. 3B; lanes 2, 10, 14). There was inhibition of the amplification reaction in one sample (Fig. 3B; lane 6) as revealed by the absence of the vertebrate's rDNA amplification band of 126 bp. The same samples were examined using the PCR I system, with the single pf-21 primer (Fig. 4). Those samples

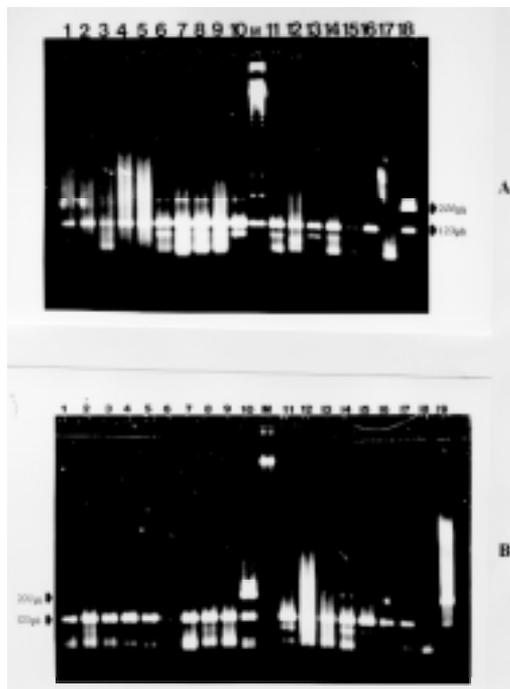


Fig. 3: PCR on human samples, PCR III system. Electrophoresis in 2% agarose gel of amplified products by PCR III. A: lanes 1 to 10 and 11 to 15, human samples 1 to 15; lane 16, negative control with human DNA; lane 17, negative control no DNA; lane 18, positive control with purified *Plasmodium falciparum* DNA. B: lanes 1 to 10 and 11 to 16, human samples 17 to 32; lane 17, negative control with human DNA; lane 18, negative control no DNA; lane 19, positive control with purified *P. falciparum* DNA; M, 123 bp ladder.

with a band pattern similar to the *P. falciparum* DNA positive control (Fig. 4A; lane 18), were considered positives. This positive pattern was observed in 24 samples (Fig. 4A; lanes 1, 2, 5, 6, 7, 8, 9, 10, 12, 13, 14; Fig. 4B; lanes 2, 4, 5, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17). The results showed the specific vertebrate's rDNA band of 126 bp in all the samples, indicating that there is no inhibition of the Taq polymerase by the anticoagulants used.

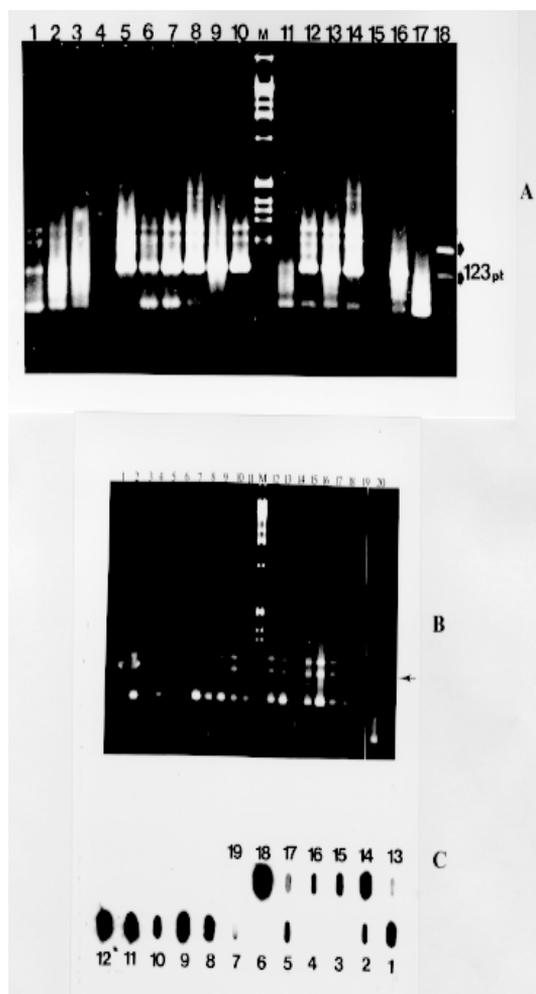


Fig. 4: PCR on human samples, PCR I system. Electrophoresis in 2% agarose gel of amplified products by PCR I pf-21. A: lanes 1 to 15, human samples 1 to 15; lane 16, negative control with human DNA; lane 17, negative control no DNA; lane 18, positive control with purified *Plasmodium falciparum* DNA. B: lanes 1 to 17 are human samples 16 to 32; lane 18, positive control with purified *P. falciparum* DNA; lane 19, negative control with human DNA; lane 20, negative control no DNA; M, 1 Kb ladder. C: slot blot hybridization of amplified products by PCR I pf-21 on human samples 16 to 32 against the pf-21 primer amplified product, labeled with digoxigenin. Exposition time: 30 min. Slots 1 to 17 are human samples 16 to 32; slot 18, positive control with purified *P. falciparum* DNA; slot 19, negative control with human DNA.

Slot-blot hybridization with PCR I pf-21 probe - Fig. 4C shows the slot-blot results of PCR amplifications with PCR I system on human samples. A positive signal was observed in slots 1, 2, 5, 8, 9, 10, 11, 12, 14, 15, 16, and 17.

DNA amplification fingerprint (DAF) of P. falciparum - Six of the 12 Amazonian Brazilian isolates were amplified by PCR I. Fig. 5 shows a different and specific polymorphic fingerprint for each isolate, within the range of 200 bp and 3 Kb. This method could be used as a molecular marker for genetic diversity studies in population genetic.

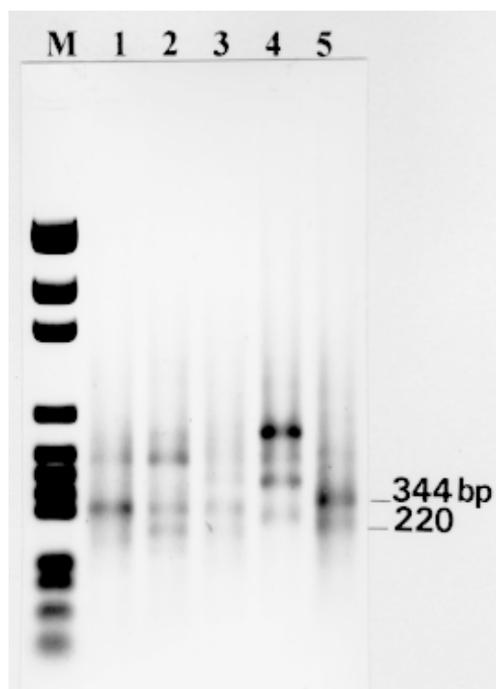


Fig. 5: DAF of *Plasmodium falciparum* isolates from Brazil and Colombia. Electrophoresis in 2% agarose gel of amplified products by PCR I pf-21. Brazilian isolates: 1 - 608; 2 - 365; 3 - 51; 4 - 54 and Colombian isolate: 5; M - PGEM molecular weight marker.

Microscopic diagnostic - Table I shows the comparative results of both microscopic readings (100 and 200 fields with immersion lens) from patients blood samples. Detection level was clearly improved when the number of fields was increased. Taking into account this improvement in the microscopic exam sensitivity, we used the 200 fields reading to compare the PCR and hybridization systems. The comparative results of the PCR I, PCR III and microscopy are presented in Table II. Table III illustrates the results of 16 samples derived from the PCR (I) pf-21 test coupled to a hybridization assay, in comparison with the microscopic diagnosis.

DISCUSSION

The current diagnosis of human malaria is achieved by microscopic examination of Giemsa stained blood smears. Although well adapted to the field situation, this methodology is not practical in terms of time and labor involved when large number of samples are required for epidemiological studies. The availability of DNA derived techniques for diagnosis of infectious agents with high specificity and sensitivity has represented an attractive alternative. Several groups have identified species specific repetitive sequences in the genome of *P. falciparum* (Franzen et al. 1984, Oquendo et al. 1986, Barker et al. 1986, McLaughlin et al. 1987) and derived DNA probes in an attempt to improve diagnosis. The highly repeated subtelomeric rep-20 sequence has been target of DNA probes as well as PCR diagnostic assays (Barker

et al. 1992). This late approach has increased the detection level but has not significantly surpassed the microscopy diagnosis probably due to self-complementation between the oligonucleotides used.

Exploiting the genomic organization of the pf-21 repeat, we have designed a PCR assay for *P. falciparum* based on a single consensus primer that coupled to non-radioactive hybridization, can be applied to the epidemiological diagnosis of *P. falciparum* with significant improvement over the microscopic analysis.

In order to improve the performance of the diagnostic PCR reaction it was necessary to optimize the protocol for blood sample preparation. The direct treatment of blood with chelex-100 Fe was adopted over the proteinase K incubation and sample preservation on filter paper. Consistent amplification of patient samples was obtained independently of the treatment with anticoagulants, as demonstrated by the vertebrate rDNA PCR assay.

We applied the PCR I system to 32 samples of malaria patients with moderate and low parasitemia. As a reference PCR, we used the PCR III system targeted on moderately repetitive sequences (Fucharoen et al. 1988, Tirasophon et al. 1991) that amplifies a unique band of 206 bp. The sensitivity of these DNA amplification assays was compared to the improved microscopic reading of 200 fields. This more extended examination of the slide increases the positive diagnosis of *P. falciparum* from 24% in 100 fields examined to 38% (Table

TABLE I

Comparative detection levels of two microscopic readings. Tumeremo Bolívar State, Venezuela 1993-94

Parasite species	100 field examined		200 field examined	
	Samples No.	%	Samples No.	%
<i>Plasmodium falciparum</i>	7	24	11	38
<i>P. vivax</i>	7	24	6	21
Mixed infection	-	-	2	7
Negatives	15	52	10	34
Total	29	100	29	100

TABLE II

Comparative detection levels between PCR I, PCR III and microscopy. Tumeremo Bolívar State, Venezuela 1993-94

Parasite species	PCR I		PCR III		Microscopy	
	No. samples	%	No. samples	%	No. samples	%
<i>Plasmodium falciparum</i>	19	66	11	38	11	38
<i>P. vivax</i>	-	-	-	-	6	21
Mixed infection	5	17	5	17	2	7
Negatives	5	17	13	45	10	34
Total	29	100	29	100	29	100

TABLE III

Comparative detection levels between PCR I coupled to an hybridization assay and microscopy. Tumeremo Bolívar State, Venezuela 1993-94

Parasite species	PCR I		PCR III		Microscopy	
	No. samples	%	No. samples	%	No. samples	%
<i>Plasmodium falciparum</i>	12	75	12	75	7	44
<i>P. vivax</i>	-	-	-	-	2	12
Mixed infection	1	6	2	12	-	-
Negatives	3	19	2	13	7	44
Total	16	100	16	100	16	100

D). Overall, examining the slides at 200 fields favored the diagnosis of mixed infections and the detection of low parasitemia over the 100 field's routine methodology.

Three of the 32 samples showed inhibition of the PCR reaction as evidenced by the absence of the 126 bp product of the vertebrate rDNA PCR assay, and were not included in the comparison analysis. The summary of the results from the 29 patients analyzed by microscopic examination and PCR systems I and III are shown in Table II. The detection ratio of the PCR I system in relation to both the PCR III system and the microscopic examination was 1.7:1. Five of the six diagnosed *P. vivax* infections were detected positive for *P. falciparum* by both PCR I and III systems, increasing the detection of mixed infections more than two fold.

When the PCR I analysis was coupled to hybridization (Fig. 4C) and these results were compared with the microscopic exam (Table III), a similar detection ratio of 1.7:1, with a higher sensitivity for the PCR was observed. The two reported cases of *P. vivax* infection were confirmed positive to *P. falciparum* by hybridization. In general the PCR I system had a higher detection level than both the PCR III system and the 200 field microscopic examination. This increment in the detection of *P. falciparum* infections is significant when sub-patent parasitemia and mixed infections are not detected by the traditional method.

Additionally, the single oligonucleotide amplification produces a DNA fingerprint specific for *P. falciparum* isolates of immediate application to population genetic studies. It was possible to observe a pattern of reproducible bands, specifically a band of 344 bp for the *P. falciparum* isolate from Colombia (Fig. 1A; lane 1, 2; Fig. 5; lane 5). The rep-20 repeat is found in the subtelomeric regions of *P. falciparum* chromosomes (Triglia et al. 1992). It is in this region where chromosome rearrangements responsible for chromosomal size changes observed is postulated to occur. The origin of these size changes is not well understood, but it is attributed to genetic exchange during the different types of cellular division (meiosis-mitosis). The possibility of obtaining an amplification pattern of these regions specific for each isolate (as the result of its chromosomal arrangement), would probably permit to follow in an epidemiological way the different circulating isolates, and the relationship of these DNA amplification fingerprints (DAFs) with other important characters, such as drug resistance and virulence.

In the group of patients studied, it was possible to distinguish at least three DAF variation patterns in the isolates circulating in the zone (Fig. 4A; lanes 1, 2, 8). A totally different pattern was observed in

the positive control DNA from Colombia strain (Fig. 4A; lane 18).

These results constitute an important tool in the epidemiology of *P. falciparum* malaria. This approach will allow the analysis of large number of samples for diagnosis, detecting mixed infections and low parasitemia. Concomitantly, circulating isolates can be identified through specific DAF for *P. falciparum*. We propose the combined use of this polymorphism detection analysis with other similar assays (RAPD, isoenzymes, and micro-satellites) that detect population changes, to further evaluate its discrimination capabilities in identifying different isolates circulating in endemic areas.

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