asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

34. Since this report was submitted, A. P. Laudano and J. M. Buchanan (*Proc. Natl. Acad. Sci. U.S.A.*, in press) have reported that either Tyr^{511}, Tyr^{519}, or Tyr^{527} is phosphorylated in $pp60^{c\text{-}src}$. We thank R. Eisenman for advice and assistance with peptide synthesis, R. Wade for amino acid analysis, S. Courtneidge and D. Shalloway for discussions and communication of results before publication, S. Courtneidge for a synthetic peptide, J. Brugge for monoclonal antibody, T. Patschinsky and B. Sefton for sharing preliminary experimental results, R. Parker and D. Shalloway for cells, and C. S. King for technical assistance. Supported by grants from the American Cancer Society (BC-484) and National Institutes of Health (CA-28151, CA-17096 and CA-28458).

4 November 1985; accepted 28 January 1986

Specific DNA Probe for the Diagnosis of *Plasmodium falciparum* Malaria

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Malaria can be diagnosed either by direct microscopic examination of blood smears, which is time consuming and requires expertise, or by immunological techniques, which are effective but do not distinguish between past and present infections. In this study, a simple procedure was developed for spotting lysed blood from infected patients directly onto nitrocellulose paper and identifying the malaria species on the basis of hybridization of parasite DNA with a species-specific probe. A genomic DNA library of *Plasmodium falciparum* was screened to detect clones containing DNA sequences that are highly repeated within the parasite genome. Several such clones were further analyzed to identify those that hybridize specifically with *P. falciparum* DNA but not with DNA from humans, *P. vivax*, or *P. cynomolgi*. This technique appears to be sensitive enough to detect 10 picograms of purified *P. falciparum* DNA (equivalent to 100 parasites) and in field studies is able to detect approximately 40 parasites per microliter of blood.

APPROXIMATELY ONE THIRD OF THE world's population may be exposed to the risk of malaria infection (*1*). It is therefore not surprising that considerable effort has been directed toward the control, prophylaxis, and eventual eradication of this disease. Realization of these goals will ultimately depend on the availability of sensitive, simple, and inexpensive diagnostic means of detecting parasites in order to identify foci of infection and to evaluate the effect of various control programs.

Direct microscopic examination of blood smears continues to be the method of choice for diagnosing acute malaria. This method is both sensitive and specific since malaria can be differentiated from other potential infectious agents, and the various *Plasmodium* species can be readily distinguished. It is, however, time consuming to examine each slide, and a trained technician is required for accurate identification when low numbers of parasites are present. Immunodiagnostic procedures such as the enzyme-linked immunoabsorbant assay (ELISA) for detecting malaria antibodies are being developed and tested (*2*). These methods provide important information with regard to exposure to malaria, but, owing to the persistence of malaria antibodies after the disappearance of malaria parasites from the blood, such tests do not accurately discriminate between present and past infections. Immunodiagnostic methods for detecting antigen have also been developed (*3*). These are useful for detecting present infections, but antibody for malaria antigen from a past infection may interfere with this assay.

We report an alternative approach based on the use of cloned *P. falciparum* DNA for the specific detection of parasites in infected blood. This method detects current infections and should make it possible to measure

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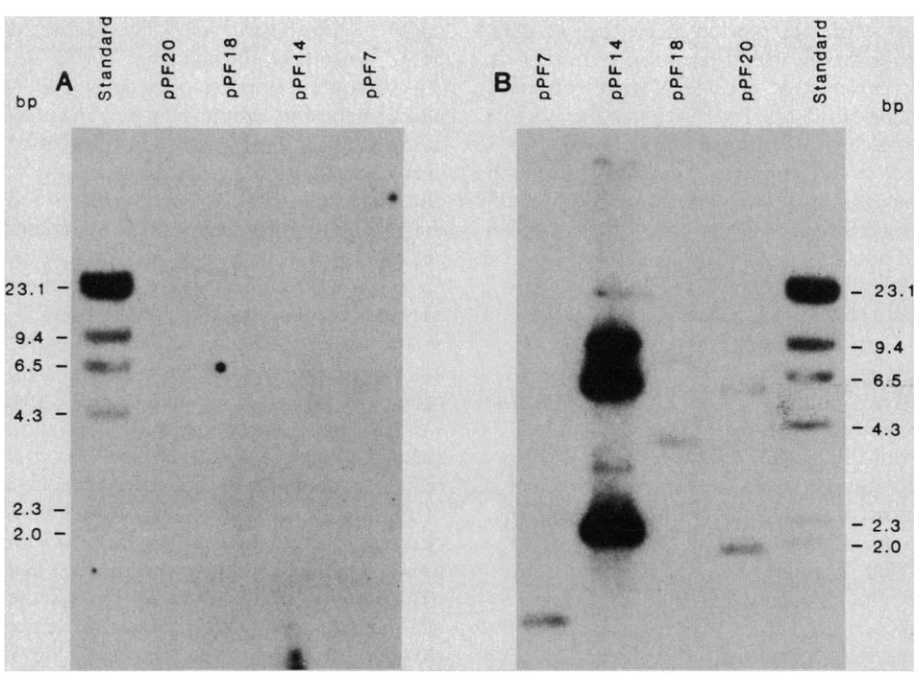

Fig. 1. Specific hybridization of recombinant plasmids with *P. falciparum* DNA but not with human DNA. Erythrocytes infected with the FCR3 strain of *P. falciparum* were maintained in vitro according to the method of Trager and Jensen (*12*). Infected erythrocytes were concentrated by gelatin sedimentation, lysed with detergent, treated with ribonuclease and proteinase K (Boeringer), and extracted with phenol-chloroform according to standard methods (*13*). *Plasmodium falciparum* DNA was then used to construct a genomic library. DNA was partially digested with Sau 3A (New England Biolabs) and was cloned into the Bam HI site of pBR322 before transformation of *Escherichia coli* strain HB 101. Transformed colonies were identified by sensitivity to tetracycline and were then screened by colony hybridization of duplicate nitrocellulose filters (0.45 μm, Schleicher & Schuell) using radiolabeled (nick-translated) DNA from either humans (placenta) or *P. falciparum*. Colonies were selected that hybridized strongly with *P. falciparum* DNA. Plasmid DNA was isolated from these colonies, digested, resolved, and transferred to nitrocellulose as described in the text (*14*). Filters were hybridized with either human (A) or *P. falciparum* (B) nick-translated DNA to confirm parasite specificity of cloned DNA. Numbers at the side are base pairs × 10^{-3}.

parasite density in infected blood. This method is readily adaptable to field use. Blood obtained from a digital puncture is lysed and applied directly to nitrocellulose, so that there is no need for extraction with phenol-chloroform or centrifugation of the sample (*4*).

A *P. falciparum* genomic DNA library was screened by colony hybridization using nick-translated *P. falciparum* DNA. From an initial screening of approximately 1000 colonies, 7 were chosen on the basis of hybridization intensity for further analysis. Plasmid was isolated by the alkaline miniprep procedure (*5*) and was double digested with the restriction enzymes Hind III and Sal I. The DNA was then analyzed by agarose gel electrophoresis and transferred to nitrocellulose filters by the Southern blot technique (*6*); the filters were probed with either radiolabeled human or *P. falciparum* DNA. In the experiment shown in Fig. 1, none of the clones hybridized to human DNA, whereas all clones hybridized with various intensities to radiolabeled *P. falciparum* DNA. In one case (pPF14), hybridization was notably more intense, suggesting that the cloned fragment is more highly repeated within the parasite genome.

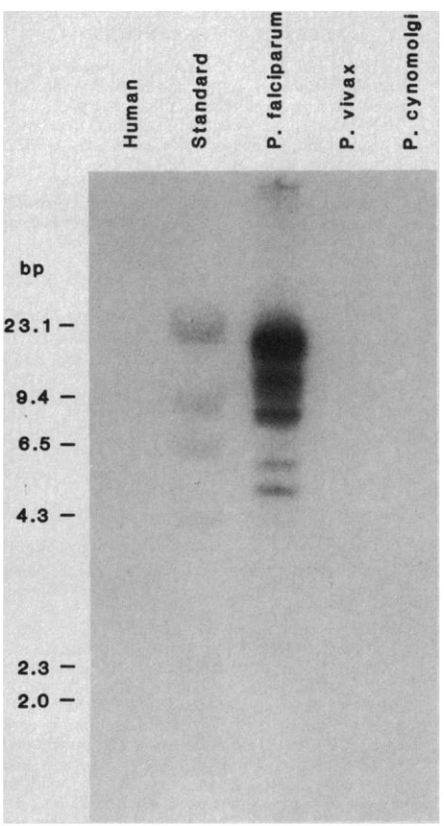

Fig. 2. Species specificity and the pPF14 DNA probe. Genomic DNA from humans, *Plasmodium falciparum*, *P. vivax*, or *P. cynomolgi* (2 μg each) was digested, resolved, and transferred to nitrocellulose all as described in the text. The filter was hybridized with nick-translated pPF14 and then exposed to x-ray film for 2 hours at −70°C using an intensifying screen. Longer exposure (up to 30 hours) did not reveal hybridization of the probe with either human, *P. vivax*, or *P. cynomolgi* DNA. Numbers at the left are base pairs × 10^{-3}.

Plasmid pPF14 was tested for species specificity. DNA from humans, *P. falciparum*, *P. vivax*, and *P. cynomolgi* was digested with Dra I, resolved by agarose gel electrophoresis, and transferred to nitrocellulose by Southern blotting. The filter was then probed with radiolabeled pPF14 cloned DNA. The results showed that pPF14 hybridizes only to *P. falciparum* genomic DNA (Fig. 2). This probe was also able to detect 10 pg of purified *P. falciparum* DNA spotted onto nitrocellulose (Fig. 3); this amount corresponds to the DNA content of approximately 100 *P. falciparum* parasites (*7*).

We then tested the ability of the clone pPF14 to detect parasite DNA in infected blood. Blood samples were obtained from infected patients at the Instituto de Medicina Tropical de Manaus in Brazil (Fig. 4B) or at the Malaria Clinic in Bangnamron, Thailand (Fig. 4A). Blood samples were diluted five times in dilution buffer and spotted directly on nitrocellulose paper as described above. Filters were then hybridized with pPF14, and the filters were exposed to x-ray film. This clone specifically hybridized with blood from patients infected with *P. falciparum* but not with blood from patients infected with *P. vivax* (Fig. 4 and Table 1). There was a correlation between parasite density and hybridization intensity, indicating that this method can be standardized, which would be useful in epidemiological and vaccine surveys.

These experiments suggest a general method for the development of highly specific DNA probes for diagnosis. By screening a genomic DNA library with radiolabeled genomic DNA and selecting the colonies that show the highest hybridization intensity, repetitive sequences can readily be selected. These sequences should provide the greatest sensitivity when used as probes because there are multiple copies in the genome. Clones containing repetitive DNA can then be screened for hybridization with DNA from other species or strains to identify clones of the desired specificity.

In the present experiments, we focused our efforts on developing a species-specific DNA probe for *P. falciparum*. This approach should not only yield probes specific for other malaria species but could also be used to develop probes that recognize finer distinctions, such as the difference between drug-resistant and drug-sensitive strains. DNA probes have been developed for the detection of a number of infectious agents (*8, 9*), and many of these will be developed into diagnostic techniques (*9, 10*). Because sample preparation and collection are especially important considerations for diagnostic techniques that will be used under field conditions in developing countries, our emphasis in this work has also been to devise a technique that requires a minimum of equipment and sample handling.

Like other workers (*4, 11*), we noted that when untreated, uninfected blood was spotted onto nitrocellulose there was nonspecific binding of the radiolabeled probe to elements in the blood. Therefore, we experimented with sample treatment and hybridization conditions to minimize nonspecific background associated with such elements. Four aspects of the protocol were found to be of particular importance. Lysis of blood (required for efficient use of the filtration manifold apparatus), in conjunction with proteinase K digestion, greatly reduced the background. Addition of 0.1 percent sodium dodecyl sulfate (SDS) to the prehybridization solution further reduced nonspecific binding. Finally, the time and temperature at which filters were baked was important. Baking for 2 hours at 50°C produced less nonspecific binding than did baking at 70°C for longer periods. These relatively minor changes in protocol made it possible to eliminate the extraction step.

Although we have been able to detect 10 pg of purified *P. falciparum* DNA, or approximately 100 parasites (*7*), with probe pPF14, in the present study we were able to

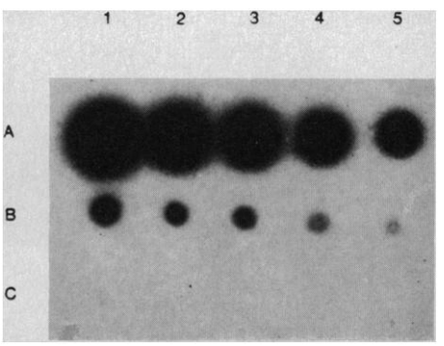

Fig. 3. Sensitivity of pPF14 for *P. falciparum* DNA. Purified *P. falciparum* DNA (strain FCR-3) was spotted onto nitrocellulose. The filter was treated as described in the legend to Fig. 4 and hybridized with radiolabeled pPF14 plasmid. The spot in (C), lane 1, represents our estimate of the limit of sensitivity; similar results have been obtained with *P. falciparum* DNA isolated from the Honduras strain. The amount of *P. falciparum* DNA spotted in each position is as follows: (A) lane 1, 6.25 ng; lane 2, 3.12 ng; lane 3, 1.56 ng; lane 4, 780 pg; lane 5, 390 pg; (B) lane 1, 195 pg; lane 2, 97 pg; lane 3, 48 pg; lane 4, 24 pg; lane 5, 12 pg; (C) lane 1, 6 pg; lane 2, 3 pg; lane 3, 1.5 pg; lane 4, 0.75 pg; lane 5, no DNA.

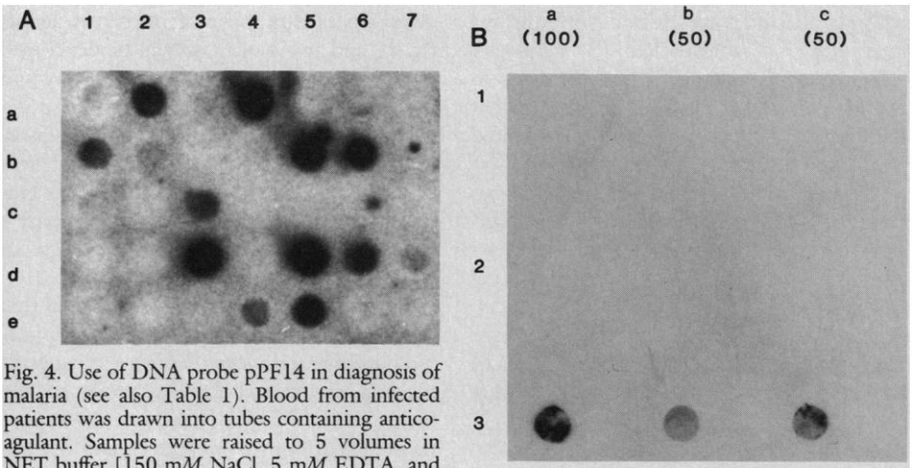

Fig. 4. Use of DNA probe pPF14 in diagnosis of malaria (see also Table 1). Blood from infected patients was drawn into tubes containing anticoagulant. Samples were raised to 5 volumes in NET buffer [150 m*M* NaCl, 5 m*M* EDTA, and 50 m*M* tris-HCl (*p*H 7.5)], and blood was lysed by incubation for 30 minutes at 37°C in the presence of 0.2 percent Triton X-100 and proteinase K_1 (100 μg/ml). Samples were then applied to nitrocellulose paper that had been wetted with NET buffer using a Minifold vacuum filtration apparatus (Schleicher & Schuell). Filters were air-dried (approximately 20 minutes), and DNA was denatured for 10 minutes by placing filters on a piece of Whatman paper saturated with 0.5*M* NaOH and 1.5*M* NaCl. The filters then were neutralized twice (10 minutes each) by transferring them to Whatman paper saturated with 3*M* tris-HCl (*p*H 8.0). After air drying, filters were baked for 2 hours at 50°C, then hybridized for 2 hours at 42°C in a solution containing 5× Denhart's solution (0.02 percent Ficoll, 0.02 percent bovine serum albumin, and 0.02 percent polyvinylpyrrolidone), 5× standard saline citrate (SSC, 0.56*M* NaCl, 75 m*M* sodium citrate, and 345 m*M* acetic acid), herring sperm DNA (100 μg/ml, Boeringer), 50 percent formamide, and 0.1 percent sodium dodecyl sulfate (SDS). Labeled pPF14 DNA (specific activity, 7×10^8 count min^{-1} μg^{-1}) was heated for 5 minutes at 100°C. Filters were hybridized for approximately 12 hours and then washed three times (30 minutes each) at 50°C in 0.1× SSC and 0.5 percent SDS before exposure to x-ray film. (A) Samples from 35 patients in Thailand; (B) samples from 3 patients in Brazil (numbers at top indicate microliters of blood).

Table 1. Use of DNA probe pPF14 in diagnosis of malaria (see also Fig. 4).

Origin of sample	Sample number*	Diagnosis†	Parasites (number) per 200 white blood cells‡		Parasites (number) per microliter of blood§	Intensity of hybridization‖
			P. falciparum	*P. vivax*		
Thailand (Fig. 4A)	a2	F	130		5200	+++
	a4	F	70		2800	+++
	b1	F + V	21	1	840	++
	b5	F	186		7440	+++
	b6	F	23		920	+++
	c3	F	25		1000	++
	d2	V		11	440	−
	d3	F	161		6440	+++
	d5	F	142		5600	+++
	d6	F	53		2120	++
	d7	F	<1		<40	+
	e1	F	<2		<80	−
	e2	V		216	8640	−
	e4	F	34		1360	+
	e5	F	210		8400	+++
Brazil (Fig. 4B)	1	V			2500	−
	2	V			1050	−
	3	F			3450	++
Normal blood			0			−
Controls						
Purified *P. falciparum* DNA (10 pg)						++
Purified *P. vivax* (50 pg)						−
Purified human DNA (50 pg)						−

*Refers to grid position in Fig. 4A and row number in Fig. 4B. †F, *P. falciparum* malaria; V, *P. vivax* malaria. ‡All other samples in Fig. 4 were negative as determined by microscopy and probe hybridization. §Values calculated on the assumption of 8000 white blood cells per microliter (Thai patients) or supplied by the clinic in Manaus (Brazilian patients). ‖Duplicate samples from Thai patients were prepared and probed on another filter; similar relative hybridization of the probe was observed.

detect 40 parasites per microliter of blood using a 50-μl sample (a total of 2000 parasites), corresponding to a parasitemia of 0.0009 percent. There are a number of possible explanations for the discrepancy between the sensitivity of the probe with purified DNA and with patient samples. Parasite DNA in lysed blood samples may not adhere as efficiently to nitrocellulose, or may be subject to degradation by nucleases. Further examination of problems associated with treatment and handling of samples may reveal ways to improve sensitivity.

Even though the probe technology in its current state of development may be less than optimal for the most sensitive diagnosis (*12*), its sensitivity compares favorably with that of the standard used in epidemiological surveys (*13*), and the method offers the major advantage of batchwise analysis of samples. As judged from earlier data (*13*), one microscopist can read 60 samples per day; for the probe technology, we estimate that one technician can process 1000 samples per day.

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15. We thank J. David for support and helpful discussions; M. Perkins for *P. falciparum* parasites used for DNA preparation and cloning; K. Mendis and P. David for *P. vivax* parasites; T. McCutchan for *P. cynomogli* parasites; W. Rogers and M. Lima for help with patient samples in Manaus; P. Yamakakul and M. Ettling for help with sample collection and microscopy in Thailand; R. Hoff and R. David for criticism of the manuscript; R. Gonski and D. Condon for technical assistance; and B. Sina for developing the *P. falciparum* library. Supported by the United Nations Development Programme–World Bank–World Health Organization Special Program for Research and Training in Tropical Diseases under the Scientific Working Group Chemotherapy of Malaria, grant 2 PO1 AI 16305-06, a grant from the Rockefeller Foundation, and a grant from the John D. and Catherine T. MacArthur Foundation. R.B. was supported by NIH training grant T32 AI 07167, and D.F.W. was supported in part by a Burroughs-Wellcome Fund Special Award in Molecular Parasitology.

30 October 1985; accepted 30 December 1985