Detection of malaria in blood samples using radioactive as well as non-radioactive DNA probes complementary to repetitive DNA elements of the parasite

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ABSTRACT

We have utilized a battery of cloned repetitive DNA's as hybridization probes to detect P. falciparum parasites in blood. The repetitive element DNA probes are specific for the falciparum species, failing to hybridize with DNA of P. vivax. In addition the probes are strain-independent, since they give a positive hybridization signal regardless of the origin of the parasite stock (American, African, Indonesian). We demonstrate here the utilization of these probes in a non-radioactive assay based on the use of biotinylated DNA. The use of the biotinylated probes is made possible by a novel blood processing step consisting of SDS lysis followed by electrophoresis and electroblotting. This method eliminates the background problems that often plague the biotinylated probes, and yields a sensitivity of 60 pg of parasite DNA (300 parasites per million erythrocytes, or .03% parasitemia in a 50ul blood sample). Thus, the sensitivity of this non-radioactive hybridization assay is comparable to that of routine microscopic examination of thick smears.

INTRODUCTION

Malaria remains today one of the major health problems specially in tropical areas.The distinction between the 4 Plasmodium species that afect humans has important therapeutical implications.P.falciparum malaria causes the highest morbidity and mortality and is now posing a therapeutic problem

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due to its increasing chloroquine resistance. Prompt diagnosis and early treatment are essential.

At present malaria is diagnosed by identification of parasites in stained blood films under light microscopy. Only in the last decade have new serologic technics such as the indirect fluorecent antibody and hemagglutination test been aplied to the diagnosis of malaria (4,9,10).

The technics available now are not useful for large scale epidemiologic investigations nor for control measures, specially when high levels of sensitivity and specie specificity are required. To address these problems two approaches have been taken by different groups: a radioimmunoassay detection of parasite antigens and a DNA hybridization assay to detect sequences specific to the parasite total genome (2,5).

Here we report our results with a sensitive,specie-specific,non-radioactive and simple DNA-DNA hybridization assay based on the detection of P.falciparum DNA in blood samples(50 ul or less). This assay uses as a probe a mixture of the more abundant repetitive elements present in the P.falciparum total genome (2,5,7,10).

With this method more than 100 blood samples can be processed simultaneously, obtaining the results in 54 hrs.

MATERIALS AND METHODS

Preparation of labeled DNA probes: Plasmid DNA was extracted using standard methods (Doly version of alk SDS (1)).Radioactive DNA probes were prepared by nick-translation in the presence of [32]P-dCTP, followed by phenol/chloroform extraction and removal of unincorporated nucleotides in a centrifugal micro-column containing Sephadex G-50 (8).Biotinylated DNA probes were prepared by nick-translation in the presence of biotinylated dUTP (obtained from Bethesda Research Laboratories). After the reaction the unincorporated dUTP was removed using a centrifugal mini-column as above, omitting the phenol extraction step.

Hybridization: DNA from blood with or without malaria parasites was extracted using the following protocol: (1) To 50 ul blood sample ware added 0.42 ml of a mixture containing 0.12M NaCl, 30mM Tris, 10mM EDTA, 5% SDS,(pH 8.3)and 500 ug/ml proteinase K. After a 20 min. incubation at 40 deg. C, the material was extracted once with 0.6 ml of phenol/chloroform/isoamyl alcohol (1:1:1.02) and once with chloroform. The aqueous phase (approx. 0.4 ml) was used directly for dot blotting. DNA was denatured by adding 80 ul of 2M NaOH and incubating 15 minutes in hot water (50-70 deg. C). The solution was neutralized with 0.5ml of 2M ammonium acetate and then applied to nitrocellulose (which had been pre-wetted in 10X SSC) with the aid of a hibri-dot apparatus (Schleicher & Schuell, Keene, N. H.). Other purified DNA

standards were bound to nitrocellulose using a similar protocol. The nitrocellulose was baked for 1 hour at 80 deg. C and prehybridized for 2 hours at 50 deg. C in hybridization solution (50% formamide, 5X SSPE, 10X Dernhard's solution, 150 ug/ml heparin, 100ug/ml E. coli DNA. The labeled DNA probes were denatured by incubation in boiling water, added to hibridization solution, and allowed to hybridize for 3-12 hours at the temperatures specified in the figure legends. The hybridization washes were standard (4), and a concentration of 0.2% SDS was used throughout.
The final wash was for 30 min in 2X SSPE, 0.2% SDS, at 50 deg. C.
Radioautographic exposures were done at -70 deg. C using two intensifying screens. When biotinilated probes were used, the pre- and hibridization procedures were carried out in 45% deionized formamide pH 7, 5x SSPE, 0.5% SDS, 10x Denhart solution and 200 ug/ml denatured E.Coli DNA.The biotinylated probe is denatured heating it at 75 deg. C for 3 min.
When DNA separation from the blood samples was made by electrophoresis, the 50ul blood sample was digested with 75ul lysis mixture conteining 8 % SDS, 500ug/ml proteinase K in 50 mM Tris, 0.15 M Nacl, 20 mM EDTA pH 8.2 by incubation at 45 deg C for 30 min. After the lysis the sample was heated at 70 deg C for 3 min and 5ul sample buffer (70% glycerol, 0.05% xylene cyanol,0.05% bromophenol blue) were added before loading in the gel.

The gel was 1% agarose, 1.5% SDS in 1X TBE buffer (90 mM Tris-borate, 90 mM boric acid, 2 mM EDTA pH 8).The gel lengths were 25 x 20 x 0.5 cm with 120-150ul track capacity.
The gel was run at 150 V in 1X TBE for 45 min.Then the electrophoresis was stopes to load the purified parasite DNA standards and the gel was run again for 15 minutes more.
After the electrophoresis the gel was soaked in warm water for several hours to eliminate the SDS. Then the gel was incubated first in 250 ml 0.25 N HCL twice, for 15 min each time, second in 250 ml 0.5 N NaOH for 12 min ,twice, following by the incubation in 500 ml Tris-HCL 1M pH 7.5 for 15 min and finally in 500 ml 1x TAE buffer (10 mM Tris-base, 5 mM NaOAc, 0.5 mM EDTA pH 7.8) for 15 min.
The electrotrasferring was made to Nytran S&S 0.22uc (previusly soaked in 6X SSC in a electrotrasfer apparatus Hoeffer Scientific Products at 70 V for 1 hr.
After the electrotransfer the nylon membrane is baked in vaccum oven at 80 deg C for 1.5 hrs, before proceed to prehibridization conditions described above.
The color development reactions were carried out as specified in the BRL DNA detection system (6).

RESULTS

The isolation and partial characterization of the repetitive DNA clones

from total DNA of P. falciparum merozoites have been described in the manuscript in preparation (Binder et al., 1985). Of the eight clones that were characterized, we chose five for use in the dot blot hybridization experiments which will be described in this manuscript. Table I shows the size and a partial nucleotide sequence for each of the five clones. Knowledge of the nucleotide sequence allowed us to choose a hybridization temperature that was optimal for obtaining highly specific signals. As it turns out, the four repetitive sequences have a G+C content in the neighborhood of 46-48%, which is fairly high for P. falciparum DNA. This is in contrast to the repetitive element described by Franzen et al., which has a G+C content of 33%. We chose to carry out the hybridization reactions in 50% formamide, 5XSSPE, at 47 deg. C, which corresponds to a criterion of Tm=-20. It was observed that hybridization at 42 deg C produced signals that were about twice as strong, but also resulted in non-specific hybridization with samples containing relatively large inputs of human DNA (2-5 ug.). This background was objectionable in experiments in which relatively large human blood samples (200 ul) were to be analized.

Figure 2 shows a dot blot hybridization experiment which demonstrates the specificity of the P. falciparum repetitive DNA clones in the detection of parasite DNA. The blot includes relatively large inputs (50ng) of DNA from other human or animal plasmodia, including P. vivax, all of which fail to show detectable levels of hybridization. DNA from a Honduras stock of P. falciparum gives a signal that is about half as strong. This result could be explained by a somewhat lower content of these particular repetitive elements in the Honduras stock, or by the occurence of variants of the repeat unit where one or two nucleotides are mismatched relative to the sequence of the Gambia stock DNA used as probe. Other dots in this experiment demonstrate the lack of hybridization with DNA from several species of trypanosomes or leishmaniae, as well as human DNA negative controls at large DNA inputs (5 ug.). Three other repetitive clones have been tested for cross hybridization with Honduras DNA, and all gave similar results, that is, the signals were about 50-75% as strong as with the original Gambia DNA used to construct the library (data not shown).

The probes were then used in an experiment which attempts a rough quantitation of the number of parasites in in vitro cultures. The P. falciparum DNA standards show that with probes labeled at approx. 30 million CPM per ug of DNA the limit of detection is about 1 ng. Assuming an average haploid genome size of 0.033 pg for P. falciparum (3) , 1 ng of DNA represents approx. 30,000 parasites. The hybridization signals obtained in this experiment show a good correlation with the number of parasites wich had been determined on the basis of microscopic examination (see figure 3).

To explore the potential for designing non-radioactive hybridization probes for the detection of malaria parasites we labeled repetitive DNA with biotinylated deoxyUTP and used it as a probe for dot blotting experiments with reconstructed mixtures of human blood and in vitro parasite cultures, or with blood from infected monkeys. In this experiment the signal generating system is based on the utilization of strepavidin/alkaline phosphatase complexes which bind to biotinylated DNA, as described by Leary et al (1983). In the first part of these experiments (Panel A) we compared the signals generated by direct phenol extraction of 50 ul of blood with an alternative protocol where 200 ul of blood were treated with saponin in order to permit concentration of the parasites in a small volume (25 ul.). As seen in row A, columns 2 & 3, the sensitivity of the method is in the range of 5ng of parasite DNA. As expected, the saponin-concentrated sample does show a stronger signal (B2), but this gain is offset by an apparent increase in the background signal of a control negative sample which was also treated with saponin (C3). In the second part of these experiments we used the biotinylated DNA assay to detect P. falciparum in infected monkeys. As seen in panel B, the sensitivity of this assay was relatively low (about 10 ng of parasite DNA). However, the assay clearly detected a level of 1% parasitemia in the infected monkeys. The sensitivity of the assay could probably be increased by a factor of 4 (that is to about 2ng) by simply decreasing the diameter of the holes used to generate the dots on the nitrocellulose sheet, which has a relatively large DNA-binding capacity. An interesting observation regarding this experiment is the fact that the P. falciparum stock used to infect the monkeys was of Indonesian origin, thus validating the ability of the falciparum/Gambia probes to detect the falciparum species in parasite stocks from several continents (Asia, America).

DISCUSSION

We have isolated and characterized at least one representative repetitive sequence per family (homology in sequence pattern). We have seen that their abundancy in the parasite genome and a high their A-T content makes them better elements for use as signal or probe in hibridization assays for the specific detection of P. falciparum parasites in patients blood samples.

With the utilization of the four most abundant repetitive sequences of the Plasmodium falciparum DNA as a probe, plus the electrophoretic blood DNA separation and its electrotranfer to nylon membrenes, we have demostrated is possible that the disign of low and high sensitivity diagnostic assays for P. falciparum malaria is possible,both for clinical and epidemiological purposes.

All of the repetitive elements use have shown to be specie-specific.Those sequences are not present in all of the parasites tested,including parasites that can be present in the same geographical region, like leshmania or trypanosome.That means that this sequences are not present in P.vivax genome or those

are less abundant there.

The use of the biotinylated probes increase the posibility to apply this method in a large scale studies due the low cost and less methodology risk.

Table I : List of probes

Name	Size of insert	Sequence family	Partial Sequence pattern
pPFR-1	1200 bp	A	cacagtacccagtaactctacggatt
pPFR-2	800 bp	B	ctaacataggtcttaact
pPFR-5	340 bp	C	aaaccctaaaccctgaaccctaaa
pPFR-6	950 bp	B	aagtaagaccttagttattga
pPFR-7	210 bp	C	agggttc/t

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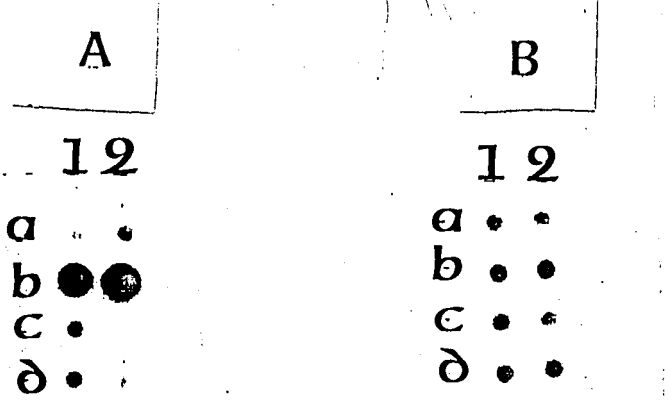

Figure 1: Hybridization of labeled P. falciparum or P. vivax DNA with dot blots containing cloned repetitive DNA. Equimolar concentrations of each clone were bound to nitrocellulose and hibridized with total labeled P.falciparum or P.vivax DNA. The hibridization was carried out in 50% formamide 5X SSPE, at 40 C. The results show the relative abundancy between each clone, and their A-T content.The clones pPFR-2 and pPFR-6 are the most abundant elements in P.falciparum genome and at the same time both have the richest A-T content.We observed that almost all of the clones are 5 to 6 fold less abundant in P.vivax.

A.Hibridization with P.falciparum DNA.
1, a-d pPFR-1 to pPFR-4
2, a-d pPFR-5 to pPFR-8

B.Hibridization with P.vivax DNA.
1, a-d pPFR-1 to pPFR-4
2, a-d pPFR-5 to pPFR-8

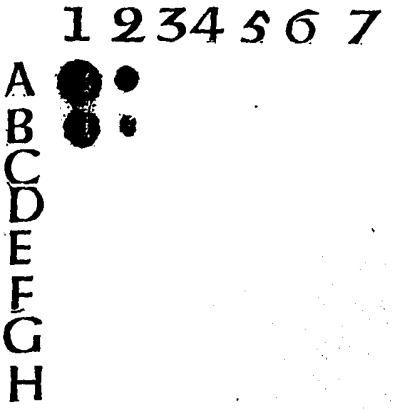

FIGURA 2. Hybradization of Plasmodium falciparum (Gambia) repetitive element probes with homologous and heterologous DNA's. Three different repetitive DNA clones were labeled by nick translation and used to probe dot blots containing DNA standards as indicated below:

A, 1-5, P. falciparum (Gambia) DNA, 50ng, 5ng, 500pg, 50pg, 5pg.
B, 1-5, P. falciparum (Honduras) DNA, 50ng, 5ng, 500pg, 50pg, 5pg.
C, 1-2, P. vivax (Central America) DNA, 50ng, 5ng.
D, 1-2, P. berghei DNA, 50ng, 5ng.
E, 1-2, P. invi DNA, 50ng, 5ng.
F, 1-2, P. gallinaseum DNA 50ng, 5ng.
G, 1-2, P. batrs DNA, 50ng, 5ng.
H, 1-4, Human DNA (from buffy coat), 5000ng, 1000ng, 100ng, 10ng.
B, 6-7, T. cruzi DNA, 100ng, 10ng.
C, 6-7, T. brucei DNA, 100ng, 10ng.
D, 6-7, T. b. gambiense DNA, 100ng, 10ng.
E, 6-7, L. tropica DNA, 100ng, 10ng.
F, 6-7, L. mexicana DNA, 100ng, 10ng.
G, 6-7, L. brasiliensis DNA, 100ng, 10ng.
H, 6-7, L. donovani DNA, 100ng, 10ng.

The specificity of the Plasmodium falciparum clones is shown by the unique positive signal to hibridize with Plasmodium falciparum DNA (A,1-2 Gambia and B,1-2 Honduras).

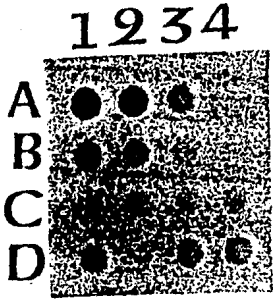

Figure 3: Detection of malaria parasites in erythrocyte culture using repetitive DNA probes.

DNA from the sources specified below was bound to nitrocellulose and hybridized with radioactive cloned DNA (a mixture of pPFR-1 and pPFR-5 DNA labeled by nick translation). The malaria parasite samples (rows C and D) were obtained from Dr. William Trager and prepared by mixing 10ul of erythrocyte culture with 40 ul of normal human blood. Samples are as follows:

A, 1-4, P. falciparum (Gambia) DNA, 10ng, 1ng, 0.1ng, 0.01ng.
B, 1-2, Phenol-extracted human blood (50ul) plus 1ng P. falciparum DNA
B, 3 , Phenol extracted human blood (50ul)
C, 1-4, Phenol-extracted malaria parasite cultures containing the following approximate parasite counts: 1, 240,000; 2, 80,000; 3, 240,000; 4, 600,000.
D, 1-4, Same as C, counts: 1, 160,000; 2, 48,000; 3, 160,000; 4, 370,000.

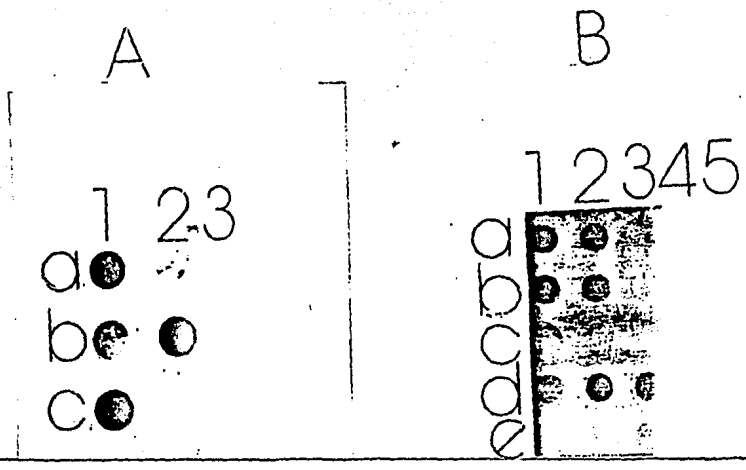

Figure 4: Detection of malaria parasites in blood using a biotinylated repetitive DNA probe.

Panel A. Detection of malaria parasites in human blood with or without a saponin concentration step. The hybridization assay was carried out in 45 % formamide, 5x SSPE at 50 C.The samples analized are as follows:

A, 1-3, P. falciparum DNA standards, 25ng, 10ng, 2.5ng.
B, 1-2, Phenol-extracted human blood samples (50ul) containing approx. 600,000 parasites.
C-1, Same as C-1, but 20ng of P. falciparum DNA added before dot blotting.
C-2, Phenol-extracted sample (50 ul) of normal human blood.

Panel B .DNA from clones pPFR-1, pPFR-5, and pPFR-8 was labeled with biotinylated dUTP and used in a standard hybridization assay. After washing off the excess probe, the nitrocellulose filter was treated with a BRL (Bethesda Research Laboratories) DNA detection system, which generates blue-purple dots in the presence of biotinylated DNA (see Materias and Methods). The samples analyzed are as follows.

A, 1-3, P. falciparum DNA standards, 250ng, 25ng, 2.5ng.
B, 1-3, P. vivax DNA standards, 250ng, 25ng, 2.5ng.
C, 1-3, Human DNA standards, 5ug, 1ug, 0.1ug.
D 1-3, Phenol-extracted blood (50ul) from a monkey infected with an Indonesian strain of P. falciparum, four samples processed in parallell. Parasitemia was 1 parasite per 100 erythrocytes.
E, 1-4, Phenol extracted blood (50ul) from normal monkey, four separate samples processed in parallell.

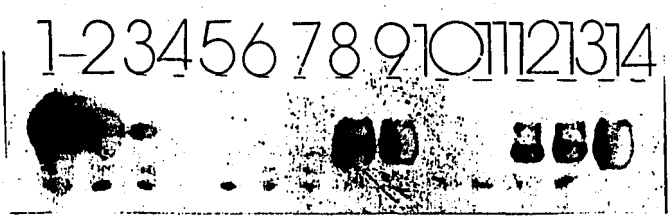

Figure 5: Detection of P. falciparum DNA in blood by electroblotting followed by hybridization.

A mixture of more abundant repetitive elements (2,6,10,11 clones) were used labeled by nick translation with radioactive or biotynylated deoxynucleotides as probe in the hibrydization of the electroblotted blood samples separated DNA from proteins by agarose gel electrophoresis. The samples analysed are as follows:

1-6 P.falciparum Gambia (FCR-3) DNA standars 100 ng to 0.01 ng.
7- Empty
8- 25ul monkey infected (P.falciparum) blood + 25ul monkey blood control.
9- 10ul monkey infected (P.falciparum) blood + 40ul monkey blood control.
10-11 -25ul human blood control.
12- 40ul human blood control + 10ul infected P.falciparum culture.
13-48ul human blood control + 2ul infected Plasmodium falciparum culture.
14-50ul human blood control + 50 ng FCR-3 P.falciparum DNA.