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SPECIFIC DETECTION OF PLASMODIUM FALCIPARUM MALARIA BY A MOLECULARLY CLONED DNA PROBE

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SUMMARY: A highly repeated DNA sequence from Plasmodium falciparum was cloned and used as a probe in molecular hybridization to detect malaria. Our results indicate that the probe is specific to P. falciparum but not to other species of Plasmodium and is extremely sensitive. As little as a 20 pg parasite DNA, which is equivalent to about 1000 parasites can be detected. The cloned DNA can be used as a diagnostic tool to follow the course of infection of falciparum malaria.

Malaria is still one of the deadliest diseases in many developing countries. It is estimated that up to 100 million people are affected by this parasitic disease with annual deaths of about a million infected persons (1). It is caused by a protozoan parasite, Plasmodium and different species are prevalent in different parts of the world. For example, P. vivax predominates in Southeast Asia and Central America, whereas P. falciparum is common in Africa. In the life cycle of the parasite, asexual reproduction or schizogony takes place in humans and sexual reproduction or sporogony occurs in the mosquito (1).

The clinical manifestations include high fever and chills that are intermittent with a prodromal stage that lasts from one to a few days. The symptoms preceeding the malaria paroxysm are indistinguishable from some other viral diseases and therefore it is difficult to diagnose malaria during this prodromal period, during which time it is difficult to detect parasites in blood smears.

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The diagnosis of malaria is accomplished by demonstrating parasites in red blood cells (2). This is usually done in thick smears and more than one smear has to be examined in a day to confirm parasitaemia. Moreover, it is labor-intensive to carry out in large numbers and only can be detected at a higher parasite density by which time the patient is usually in the paroxysmal period. Immunodiagnostic methods are also available (3), but these are useful only in primary infections. Therefore, development of a rapid, specific and highly sensitive test will be immensely useful.

Here we describe a very sensitive technique that can detect malaria caused by P. falciparum at a very early stage. This method utilizes cloned DNA probes in molecular hybridization reactions with parasite DNA. We show here that as little as 0.02 ng of parasite DNA, which is equivalent to about 1000 parasites, can be detected.

METHODS

Parasites: Plasmodium falciparum FCR-3 was grown in human blood as described by Trager and Jensen (4).

Nucleic Acid Hybridizations: P. falciparum DNA was isolated by the CsCl method as described previously (5, 6). The DNA was boiled in 0.3 M alkali to denature and fragment DNA that is suitable for hybridization. Three-fold serial dilutions starting with 6 ng were made in 10 X SSC (1 X SSC is equivalent to 0.15 M sodium chloride and 0.015 M sodium citrate), containing 5 µg herring sperm DNA and spotted on nylon filter membranes (MSI) using dot blot apparatus. Human, mouse and calf thymus DNA were used as controls at 5 µg per dot. The filter was baked for 2 to 3 hours in vacuuo and hybridized in hybridization buffer (6 X SSC, 2 X Denhardt's buffer and 100 µg/ml salmon sperm DNA) containing 2 x 10^6 cpm/ml (5 X 10^7 to 1 x 10^8 cpm/µg DNA) pPL-7 DNA which was labeled with ^{32}P by nick-translation (7). After hybridizing for 12 to 15 hours at 60°C, they were washed at 60°C and exposed to X-ray film for 4 hours at -70°C with a Cronex intensifying screen.

Processing of Blood Samples: Blood samples containing 0.1%, 0.01%, 0.001%, and 0.0001% parasitaemia were processed either by the saponin method or by the direct phenol extraction. 0.1 ml blood samples were diluted to 0.4 ml with PBS and saponin was added at 0.025% for 10 minutes at 37°C, following which the released parasites were collected by centrifugation (10 minutes in a Eppendorf microcentrifuge), washed once with PBS (0.01 M phosphate buffer, pH 7.0, 0.15 M NaCl, 0.5% glucose) and resuspended in TE. Alkali was added to 0.3 M and boiled for 20 minutes. The solution was neutralized and applied directly on a filter or occasionally precipitated and the precipitated DNA was collected by centrifugation, resuspended and applied on filter membrane. In the direct phenol method (row P), 0.1 ml blood sample was diluted to 0.4 ml with TE and adjusted to 0.1 M sodium chloride, 0.5% sodium dodecyl sulfate and phenol extracted. To the solution, 0.3 M NaOH was added, boiled for 20 minutes and processed as for the saponin method.

RESULTS AND DISCUSSION

Plasmodium falciparum was grown in human blood as described (4). When parasitaemia reached about 10 to 15%, DNA and RNA were isolated by the CsCl

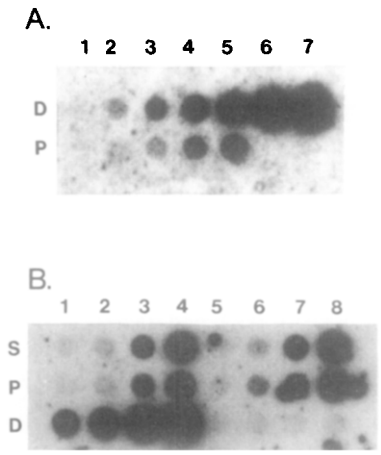

Fig. 1. Hybridization of pPL-7 probe to P. falciparum DNA.

A. Purified DNA (D) or DNA from parasites (P) serially diluted as described in the text. Row P, dots 6 and 7 contain calf thymus and human DNA.

B. Dots 1 to 4 and 5 to 8 in each row correspond to 0.0001, 0.001, 0.01, and 0.1% parasitemia, respectively and run in duplicates. S and P refer to the saponin and phenol methods. In row D, dots 1 to 4 contain increased amounts from 0.1 to 3 ng purified P. falciparum DNA (as in Fig. 1A). Row D spots 5, 6, 7 and 8 correspond to 1 and 4 µg calf thymus or human DNA.

method (5, 6). The DNA was denatured and fragmented to small size by boiling in alkali and adjusted to a concentration of about 5 to 6 µg per ml. Serial dilutions ranging from 6 ng (10^{-9} g) to 8 pg (10^{-12} g) were spotted on nylon membrane filter and hybridized with nick-translated pPL-7 probe. pPL-7 contains a 13.4 kb Plasmodium DNA fragment, cloned in pBR322 vector (6). The 13.4 kb fragment contains sequences that are highly repeated in the genome of Plasmodium falciparum (6).

Figure 1A shows dot blot hybridization of P. falciparum DNA with ^{32}P-labeled pPL-7 DNA probe, which was prepared as described in the legend. As little as 0.02 ng parasite DNA (row D, dot 2) can be detected. The lowest (spot 1) and highest (spot 7) concentration of purified DNA used were 8 pg and 6 ng, respectively (Fig. 1A, row D). In row P, we used serial dilutions of DNA directly isolated from 0.1 ml blood of 1% parasitaemia. Dot 5 (row P) contains DNA equivalent to 4 µl of 1% parasitaemia and dots 4 to 1 contain DNA after three-fold dilutions. That the probe is specific for Plasmodium DNA is shown by including 4 µg human (HeLa), mouse and calf thymus DNA as controls.

In order to demonstrate its applicability to detect malaria, 0.1 ml of infected blood with about 1% parasitaemia was serially diluted in blood (50% packed volume) to give rise to 0.1, 0.01, 0.001, and 0.0001% parasitaemia (Fig. 1B, rows S and P) and the DNA from each blood sample was isolated by two different methods. In the first method (Fig. 1B, row S), 0.1 ml infected blood was processed by the saponin method. Aliquots were spotted on nylon membrane filter using a dot blot apparatus. In the second method (Fig. 1B, row P), 0.1 ml blood samples were deprotenized by phenol, and the nucleic acids were either precipitated with ethanol, collected the precipitates, resuspended in 0.3 M alkali and boiled or directly boiled for 20 minutes in alkali followed by neutralization and precipitation. Aliquots of each dilution were spotted on nylon filter, baked and hybridized. These results indicate that as little as 20 µl blood with about 0.001% parasitaemia (dots 2 and 6 in rows S and P) is sufficient for a rapid and specific detection of the parasite. This amount of blood contains approximately 1000 parasites, therefore, we think that by processing 100 µl of blood samples, malaria can be detected at an earlier stage of infection.

Whether the molecularly cloned DNA probe is genus-specific or species-specific, is tested by spotting DNA from different species of Plasmodium and Babesia, another hemotropic parasite of cattle. Approximately 0.4 ml of infected blood at 0.1 to 4% parasitaemia was processed by the phenol method and the DNA from parasites in 0.1 ml blood was applied on each dot and hybridized with ^{32}P-labeled pPL-7 probe. This analysis shows that pPL-7 is species-specific because it hybridized to only P. falciparum but not to the other species of Plasmodium (Fig. 2). Although P. vivax and P. malariae are human pathogens, the probe does not hybridize to these species (Fig. 2). These results indicate that the cloned DNA sequence in pPL-7 is highly specific to P. falciparum and therefore can be employed in field trials.

Recently other groups (8-13) have also identified repeated sequences in Plasmodium and some investigators have used these as DNA probes to detect P. falciparum (8, 9, 11, 12). The nucleic acid sequence analysis of our clone indicates that it probably contains at least two different repeat elements, one of which appears to correspond partly to a sequence obtained by other groups (8). Since our clone

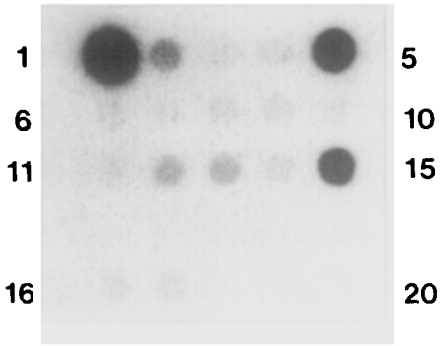

Figure 2. Dot blot hybridization of DNA from different Plasmodium species. DNA was isolated by the phenol method and fragmented with alkali as described above. It was spotted on nylon filter, baked and hybridized with pPL-7 probe at 60°C. The species include P. falciparum (5 and 15), P. berghii (2), P. brazilianum (3), P. coatneyi (4), P. fragile (6), P. inui (7), P. malariae (8), P. simium (9), and P. vivax (10 and 11), Babesia bigemini (12 and 17), B bovis (13 and 16). Spot 1 contains 2 ng of purified P. falciparum DNA. Spot 5 and 15 contains DNA equivalent to 10 µl of 0.1% and 20 µl of 0.01% parasitaemia of P. falciparum. The parasitaemia ranged from 0.2% (P. vivax) to about 5% (P. coatneyi) and 15 to 20% for B. bovis and B. bigemini. At higher concentrations of Babesia, there is slightly higher background (spots 12 and 13) than when they are diluted to 4 to 5% (spots 16 and 17). P. berghii also gave higher background at higher concentrations because it is about 15% paraeitaemia.

contains more than one repeat element (Rao, Guntaka and Green, manuscript in preparation), it offers selective advantage over the synthetic oligonucleotide probes (8, 13) because of the 'tailing effect' of the long DNA that is used as a probe.

The technique described here is also rapid and extremely sensitive and many samples can be tested at one time. Availability of simple tests for rapid and specific diagnosis of malaria in developing countries should facilitate institution of early chemotherapy, and will permit monitoring the levels of parasitaemia in malaria vaccine trials.

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