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A NOVEL DETECTION OF A SINGLE *PLASMODIUM FALCIPARUM* IN INFECTED BLOOD

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SUMMARY : Detection of *Plasmodium falciparum* malaria by a specific DNA probe is a highly promising means for epidemiological surveillance of human malaria. However, none of presently available DNA probe methods could detect as little as a few parasites in infected blood. By amplification of a specific 206 base pairs *P.falciparum* DNA sequence using the polymerase chain reaction (PCR), as little as 0.01 picogram DNA or one-half of a parasite was sufficient for a specific detection. A PCR procedure for detection of *P.falciparum* in infected blood without prior DNA extraction was also developed which was sensitive for a single parasite. The procedure was simple and should be applicable for a large scale epidemiological study involving a very low parasitemia situation.

A highly sensitive detection of parasites is essential for epidemiological surveillance and in monitoring the effectiveness of therapeutic protocols. Nucleic acid hybridization is a promising means for specific and sensitive detection of parasites. Detection of parasites by nucleic acid hybridization depends for its specificity on the identification of parasite specific DNA sequence which is absent in the host and other closely related parasites. Several specific DNA probes for detection of *P.falciparum* have been developed (1-9) which could identify the parasites varying in number from 50 to 5,000. All these DNA probes except one (7) are based on specific nucleic acid hybridization to the target parasite chromosome which in blood stage is haploid. Although the DNA probes are usually derived from highly

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Abbreviations: bp, base pairs; PCR, polymerase chain reaction; pg, picogram.

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repetitive DNA sequences, none of the repeats exceeds one tenth of the total DNA content. A specific DNA sequence can be amplified by the polymerase chain reaction to millions of copies (10). Thus, it should be possible to amplify *P.falciparum* target DNA so as to increase the sensitivity of detection by DNA probe. Here we report the use of a set of primers derived from a cloned repetitive DNA specific to *P.falciparum* (8) to amplify the target DNA to the level that a single parasite in an infected blood was easily detected.

MATERIALS AND METHODS

Parasite material. *P.falciparum* K1 strain used for the DNA isolation was obtained by *in vitro* culture. The DNA was extracted from the parasites by pronase digestion and phenol extraction as previously described (8). The amount of DNA was calculated from the absorption spectra (20 O.D.$_{260nm}$ = 1 mg/ml). *P.falciparum* was also obtained from patients blood.

DNA amplification. DNA was amplified by the polymerase chain reaction as follows : varying amount of DNA was used in 50 ul Reaction Mixture containing Reaction Buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 200 uM each of dATP, dGTP, dCTP and dTTP, 1 uM each of the primers (5'-CGCTACATATGCTAGTTGCCAGAC-3' and 5'-CGTGTACCATACATCCTACCAAC-3') and 2.5 units of Taq polymerase (Cetus). The mixture was overlaid with mineral oil and the amplification cycle was 1 min at 94°C, 1 min at 45°C and 3 min at 72°C. The primers were designed from the 753 bp insert of pBRK1-14, whose sequence has been deposited with the UNDP/World Bank/WHO-TDR Malaria Sequence database.

Amplification of DNA in infected blood used a similar protocol as above. 20 ul infected blood of varying amount of the parasites were mixed with 200 ul Lysis Buffer (0.2% NaCl, 0.015% saponin, 1 mM EDTA), and centrifuged at 10,000xg at room temperature for 10 min to remove haemoglobin. The pellet was washed once with 250 ul of the Reaction Buffer. Following centrifugation at 10,000xg for 10 min, the pellet was directly suspended in 49.5 ul of the Reaction Mixture lacking Taq polymerase. After the mineral oil overlaying, the mixture was heated at 100°C for 10 min and then 0.5 ul of Taq polymerase was added. The first cycle was done at 2 min 94°C, 1 min 45°C, 3 min 72°C; subsequent cycles were at 1 min 94°C, 1 min 45°C, 3 min 72°C; and the last cycle was accomplished at 10 min 72°C.

Analysis of the amplified DNA. One fifth of the PCR product was analysed by agarose gel electropheresis and stained with ethidium bromide (11). When DNA probe detection was necessary, the DNA from agarose gel was Southern-transferred to nylon membrane (NEN) and hybridized with ^{32}P-labelled 753 bp DNA insert from pBRK1-14 (8). In the analysis of PCR product by dot-blot hybridization, 20 ul were spotted on the membrane and hybridized with the ^{32}P-labelled DNA or a digoxigenin-labelled 753 bp DNA (12). The ^{32}P-labelled probe was detected by an overnight autoradiography at -70°C. The digoxigenin-labelled probe was detected by color development using 5-bromo 4-chloro 3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as substrates (12).

RESULTS

Amplification of P.falciparum DNA. DNA extracted from *in vitro* cultured parasites was amplified by the polymerase chain reaction using the primers derived from a specific *P.falciparum* DNA sequence. As shown in Fig.1A, the PCR product showed a single band at the size of 206 bp corresponding to the size expected from the insert of the pBRK1-14 DNA probe specific for *P.falciparum* (8). With 30 PCR cycles, 1 picogram of a total *P.falciparum* DNA gave a visible band corresponding to approximately 10 nanogram of 206 bp DNA, indicating that the sequence had been amplified approximately 10^9 fold. Southern - blot hybridization of the PCR product as shown in Fig.1B using the 753 bp insert of pBRK1-14 as the probe demonstrated that the 206 bp had sequence homology to the *P.falciparum*. The probe gave detectable 206 bp band even when as little as 0.01 pg DNA was employed in the PCR. Considering that a parasite contains 0.02 pg DNA (4) this result demonstrated that as little as one - half of parasite DNA content was detectable.

Direct amplification of parasite DNA in infected blood. *P.falciparum* DNA in blood could be directly amplified by the PCR without the need to make a prior DNA extraction (Fig.2A). The PCR product

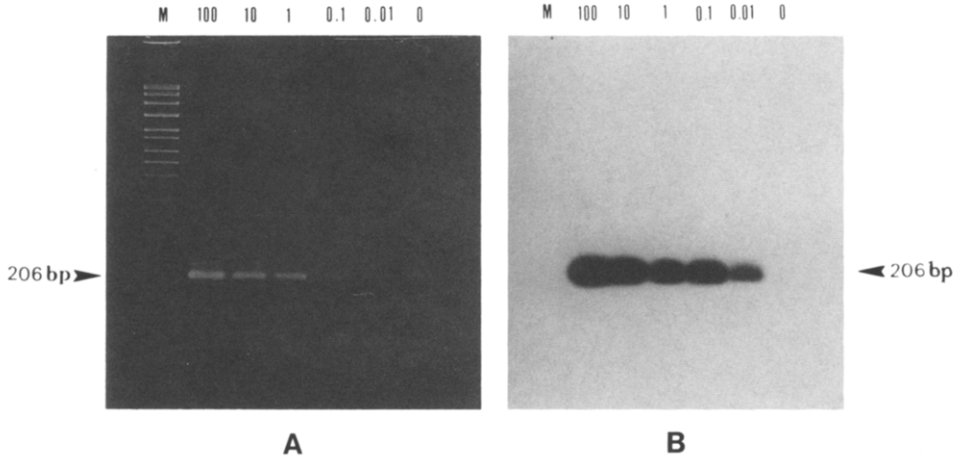

Figure 1 Analysis of PCR product by agarose gel electrophoresis and Southern-blot hybridization. [A]:ethidium bromide staining pattern ; M, DNA size marker ; 100 to 0 represents picogram of *P.falciparum* DNA template. [B]:autoradiographic pattern of [A] probing with ^{32}P-labelled 753 bp *P.falciparum* DNA.

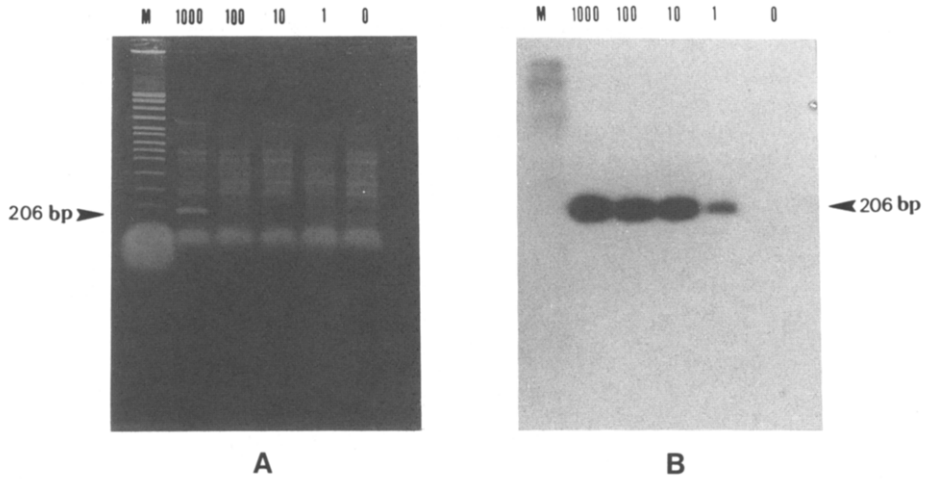

Figure 2 Detection of *P.falciparum* in the infected blood. [A]:ethidium bromide staining pattern ; M, DNA size marker ; 1000 to 0 represents number of *P.falciparum* in 20 ul blood. [B]:autoradiographic pattern of [A] probing with ^{32}P-labelled 753 bp *P.falciparum* DNA.

showed several bands in ethidium bromide-stained agarose gel. When this banding pattern was analysed by Southern - blot hybridization using the 753 bp insert as the probe, a single hybridization band corresponding to 206 bp was observed (Fig.2B). Amplification product from infected blood containing a single parasite clearly exhibited the 206 bp band. The PCR product could also be detected by dot-blot hybridization using the radioactive or non-radioactive DNA probe as

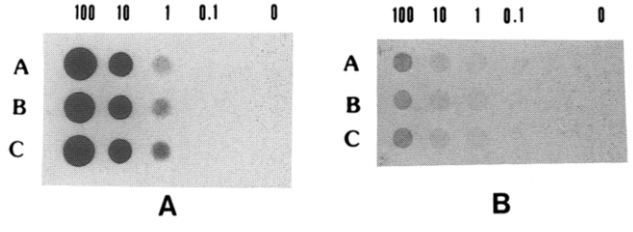

Figure 3 Dot-blot hybridization of PCR product from *P.falciparum* infected blood. A,B,C represents 3 specimens ; 100 to 0 represents number of parasites in 20 ul blood. [A] : autoradiographic signal from ^{32}P-labelled probe ; [B] color signal from digoxigenin-labelled probe.

shown in Fig.3. The hybridization signal was evident when the specimen contained as little as a single parasite.

DISCUSSION

We have demonstrated that a single *P.falciparum* in 20 ul of infected blood could be detected by amplification of a 206 bp DNA sequence specific to the parasite. This 206 bp DNA is an internal sequence of the 753 bp insert of *P.falciparum* moderately repetitive DNA probe, pBRK1-14 (8). A single band of PCR product indicated that the 206 bp was not tandemly repeated in the *P.falciparum* genome. The use of a moderately repetitive sequence as the amplification target gives an advantage over that of the highly repetitive 21 bp sequence (4) as the latter will be unlikely to produce a single PCR product which makes identification of the PCR product by agarose gel-electrophoresis difficult. The generation of 206 bp PCR product reported here should offer an alternative means of identification of *P. falciparum* by a simple agarose gel analysis.

None of the previous *P. falciparum* identification methods (1-9) could detect a single parasite in 20 ul blood. The most sensitive method was that based on ribosomal RNA detection which could detect approximately 50 parasites (7). The method reported here supplements the previous detection methods and makes possible studies in which a single parasite detection are required, viz., in an endemic area where people may carry a few parasites but remain asymtomatic, or in the determination of parasite clearance following drug treatment.

The detection procedure reported here of a single parasite in infected blood involved two major steps, amplification of target DNA and probe hybridization. The amplification step did not require a laborious and time-consuming DNA extraction. This reduces the detection procedure to the same level of that required for direct DNA probe hybridization. The non-radioactive detection should make the method applicable in a remote area or in a developing country where use of a short-lived

radioactive isotope is problematic. We are currently adapting the non-radioactive detection system for routine application.

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