FIELD EVALUATION OF SIMPLIFIED RADIOACTIVE AND NONRADIOACTIVE DNA PROBE METHODS FOR THE DIAGNOSIS OF FALCIPARUM MALARIA

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Abstract. Specific DNA probe hybridization technique is one method of choice for detection of malaria infection. It provides an obvious advantage over conventional microscopy when large numbers of samples are simultaneously monitored. The method was simplified so that preparation and processing of blood specimens were all performed on membrane filters. Background signals generated from blood components were removed by treating samples spotted on the membrane with a series of buffer washes without the necessity of a protease digestion step. Hybridization was monitored using either ^{32}P-or digoxigenin-labeled DNA probe. 849 field samples collected from various malaria endemic areas in Thailand have been evaluated by this protocol and compared with microscopic examination. Sensitivity obtained by this procedure was comparable to that of microscopy at a malaria clinic. The specificities of both types of DNA probes were better than 93%, but digoxigenin-labeled probe performed better than ^{32}P-labeled one when the numbers of parasites were less than 25 per 200 white blood cells.

INTRODUCTION

The detection of infectious agents remains a basic requirement for the effective treatment of human parasitic diseases. In Southeast Asia including Thailand, the major cause of morbidity and mortality by parasitic diseases is still malaria (WHO, 1992). Detection of *Plasmodium falciparum,* the most virulent of the four human malaria parasites, needs a rapid, simple, sensitive and inexpensive diagnostic procedure. Today, the simplest method used in endemic area for diagnosis of human malaria is by microscopic observation. This method is adequate when the numbers of samples are limited. However, it is time consuming and very much dependent on individual microscopist evaluation.

Nucleic acid technology offers a promising means for routine diagnosis of malaria with the obvious advantage of its ability to investigate hundreds of samples simultaneously. This technique is based on hybridization of specific probes to parasite nucleic acid which always accompany parasite infection. Detection of the hybridized nucleic acid probes can be performed by employing either radioactive or non-radioactive system. Diagnostic DNA probes for *P. falciparum* have been developed and evaluated by many laboratories (Franzen *et al,* 1984; Guntaka *et al,* 1985; Pollack *et al,* 1985; Oquendo *et al,* 1986; McLaughlin *et al,* 1987a; Zolg *et al,* 1987; Boonsaeng *et al,* 1989). These comprise total parasite genomic DNA, regions of repetitive sequences and synthetic oligonucleotides. Probes against repetitive DNA sequences covering 1-10% of total genomic DNA exhibit high specificity and show advantage of higher sensitivity. The level of sensitivity detected by these DNA probes are between 50-500 parasites/μl blood or 10-100 pg when tested with cultured parasites or purified parasite DNA respectively. RNA probe synthesized from the small subunit of rRNA gene has also been developed and shown to be more sensitive than DNA probes in detecting blood parasites (Waters *et al,* 1989). However, the instability and difficulty in handling RNA have made DNA probes more favorable for field situations. Nevertheless, evaluation of DNA probes with clinical samples from the field remains limited with varied results (Holmberg *et al,* 1987; Delves *et al,* 1989; Sethabutr *et al,* 1988). Sensitivity of the DNA probes depends not only on the hybridization technique used but also on the method of sample preparation.

Extraction of parasite DNA for hybridization usually eliminates the problem of blood interference (Franzen *et al,* 1984; Guntaka *et al,* 1985; Holmberg *et al,* 1987; McLaughlin *et al,* 1987a). This procedure showed 68% sensitivity and 100% specificity

for *P. falciparum.* However, inclusion of additional laborious steps can cause loss of DNA. Lysis of blood by detergent and proteinase K treatment has been developed to replace DNA extraction (Mucenski *et al,* 1986; McLaughlin *et al,* 1987b; Barker *et al,* 1989). Although this method reduces labor and cuts down on the steps involved, additional equipment and chemicals are still required. Direct lysis of blood on the membrane has made the procedure more convenient and field tests by this method have been described (Pollack *et al,* 1985; Sethabutr *et al,* 1988; Boonsaeng *et al,* 1989). However, proteinase K, an expensive and unstable enzyme, was still needed.

We present here a simple blood sample preparation which does not require enzyme digestion and is suitable for nonradioactive detection system. Evaluation of this protocol was performed with 849 blood specimens collected from various malaria endemic areas in Thailand. The sensitivity, specificity and efficiency of this technique were explored with both radiolabeled and nonradiolabeled DNA probes in comparison with conventional microscopic examinations.

MATERIALS AND METHODS

Sample preparation

Since crude blood samples contain numerous substances which can interfere with both radioactive and nonradioactive DNA probe detection systems, sample preparation is a crucial step affecting overall sensitivity. Blood sample (10 µl) was drawn from a fingerprick into a nonheparinized capillary tube and directly applied onto nylon membranes (GeneScreen Plus, NEN) in duplicates (5 µl/spot). The samples were allowed to air dry for about 30 minutes. Thick blood film was prepared for microscopic determination at the same time. Membrane immobilized blood samples were sequentially soaked in the following solutions : 10 minutes in 10 mM Tris-HCl pH 7.4, 1 mM EDTA; 20 minutes in 0.5 M NaOH; 10 minutes in 1 M Tris-HCl pH 7.4; and 30 minutes in 0.25% SDS (for digoxigenin-labeled probe) or 10 minutes in 0.1% SDS (for ^{32}P-labeled probe). After being blotted dry, the membranes were soaked in prehybridization solution 5x SSC, 0.1% (w/v) lauroyl sarcosine, 1% (w/v) blocking reagent (Boehringer), 0.5% SDS, 50% formamide and 100 mg/ml sonicated salmon sperm DNA for digoxigenin-labeled probe or 5x SSC, 2% SDS, 2% skim milk, 5mM EDTA, 20mM Tris-HCl pH 7.4, 50% formamide and 100 mg/ml sonicated salmon sperm DNA for ^{32}P-labeled probe] at 42°C for 1-2 hours. At this point the blood color should have been completely removed. The membranes were then transferred to hybridization solution (same as prehybridization solution except that appropriate probes were added). Hybridization was carried out for another 4 hours at 42°C (both systems). Filters were then washed in 0.1 × SSC and 0.1% SDS at 55°C for 30 minutes (digoxigenin-labeled probe) or at 50°C for 1 hour (^{32}P-labeled probe). The non-radioactive detection system was carried out according to the manufacturer's protocol (The Genius™ system, Boehringer). Radiolabeled filters were exposed to either Kodak or Fuji X-ray film with an intensifying screen for 40 hours.

Parasite culture

Plasmodium falciparum strain K1 originally isolated from Kanjanaburi Province, Thailand, was maintained in continuous culture as described elsewhere (Fucharoen *et al,* 1988). Cultures were harvested at parasitemia of about 10% and the cells were washed before use. Dilutions with uninfected human blood were made to yield parasitemia of 0.004-0.5%. *In vitro* parasite cultures were used as positive and uninfected blood as negative controls.

DNA probe

A BlueScribe vector containing a 7 Kb *Hind* III fragment of 21 base pair repeats from *P. falciparum* (Rep 20) isolated from Gambian isolate HG-13 was used as a probe throughout the assays. The Rep 20 insert was kindly provided by the late J Scaife, University of Edinburgh, UK. The plasmid was linearized with *Hind* III before labeling. Amount of DNA used for ^{32}P and digoxigenin-labeling was 250 ng and 1 µg respectively. Both probes were prepared by using a standard random priming system. The ^{32}P-labeled probe was added into hybridization solution at a final concentration of $5 \times 10^5 - 1 \times 10^6$ cpm/ml while digoxigenin-labeled probe was diluted to 100 ng/ml.

Microscopic examination

The species and stages of parasites were identified by 2 microscopists. At the malaria clinic, a trained

microscopist reported the results from thick smear slides examination without counting. The second microscopist at the malaria center independently examined duplicate slides and reported parasite counts per 200 white blood cells.

RESULTS

Prior to processing blood samples, the effects of such treatment on extracted parasite DNA were first investigated. Color detection of serial dilutions of purified *P. falciparum* DNA using Rep 20 DNA probe was compared between treatment and nontreatment protocol (Fig 1A, 1B). The results showed no difference between the two methods indicating that chemicals in the buffers did not interfere with probe hybridization once the target DNA was attached onto the membrane. Sensitivity of detection of parasite DNA by colorimetric method was approximately 1 pg of DNA in both protocols.

Infected blood samples obtained from *in vitro* culture were then evaluated. Duplicate samples with

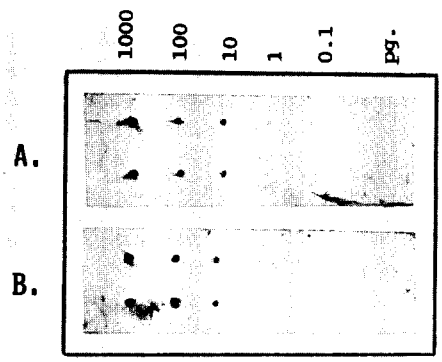

Fig 1–Chromogenic detection of extracted *P. falciparum* DNA by digoxigenin-labeled probe comparing between treatment (A) and nontreatment (B) protocol. Amounts of parasite DNA ranging from 0.1, 1, 10, 100 and 1,000 pg were spotted in duplicates onto the nylon membrane. Nontreatment protocol differed from treatment protocol only at the membrane processing prior to prehybridization step. Treatment protocol was as described in Materials and Methods. In nontreatment protocol, membranes were directly subjected to denaturation in 0.5 N NaOH and neutralization in IM Tris-HCl pH 7.4.

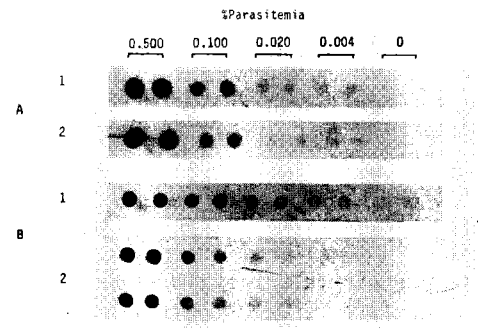

Fig 2–Detection of *P. falciparum* in blood specimens using ^{32}P-labeled DNA probe (A) and digoxigenin-labeled DNA probe (B). *In vitro* culture of *P. falciparum* was diluted with noninfected blood to yield 0.5, 0.1, 0.02 and 0.004% parasitemia and 5 µl of each were spotted on the membrane. Noninfected blood was used as negative controls (0% parasitemia). A1 and B1 were subjected to detection after the blood spotted membranes were processed with nontreatment protocol while A2 and B2 were processed using treatment protocol.

parasitemia ranging from 0.004 to 0.5% were compared between treatment and nontreatment protocols. Fig 2 shows that the treatment was necessary to remove the color background from blood specimens. No signals were detected in treated negative control blood samples whereas those from nontreated controls were visible. This protocol could be applied to both nonradioactive and radioactive probes because the same sensitivity was obtained when the same set of samples were tested in parallel (Fig 2A, 2B).

The question then arose concerning the duration during which blood samples could be kept on the membrane before processing. No background color was observed when blood spots were left to air dry for 30 minutes. However, background color was present if the blood sample was left over 1 hour on the membrane (data not shown). It should be noted that the protocol described here may not be suitable for other types of membranes.

The optimized protocol was then used on field specimens collected from various malaria endemic areas in Thailand. 849 blood samples were collected and examined for *P. falciparum* infection by both DNA probe hybridization technique and microscopy. As microscopic examination has always been assumed to be a gold standard for malaria diagnosis, sensitivity and specificity of DNA probe technique were

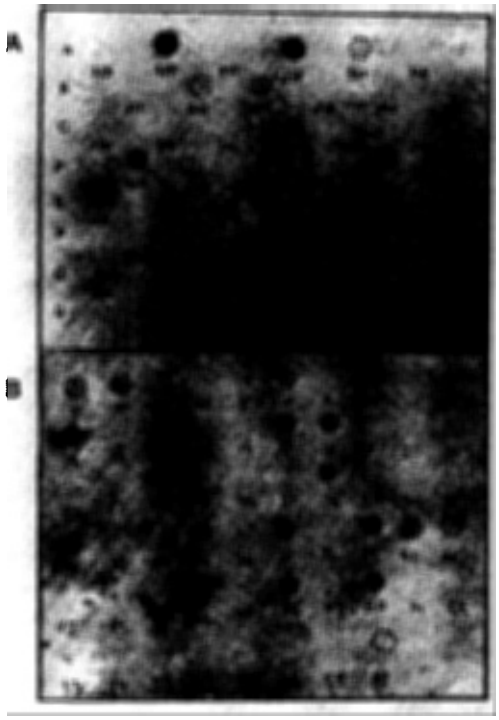

Fig 3–Examples of field evaluation by optimized DNA probe hybridization assays. A shows detection of blood specimens by ^{32}P-labeled probe whereas B shows detection by digoxigenin-labeled probe.

analysed in comparison with those of conventional microscopy. In addition, as a radioactive probe is widely used in DNA hybridization, it was tested in parallel with the nonradioactive probe. Blood samples collected in heparinized capillary tubes were found to generate background signals in negative controls (data not shown), therefore nonheparinized capillary tubes were used. Results from DNA hybridization analyses by both radioactive and nonradioactive probes are shown in Fig 3. The same positive and negative controls were included in each experiment to standardize the results. The color signal was clearly visible at the lowest parasitemia of control (0.004%) and there was no background in uninfected blood samples (data not shown). For ^{32}P-labeled probe, the results were evaluated after 40 hours of exposure. Nevertheless, it should be remarked that the sensitivity of radioactive system could have been increased by either longer exposure or increased specific activity. Fig 3A shows typical results using ^{32}P-Rep 20 probe; specimens number 331, 334, 337, 341, 344, 348, 349, 352, 353, 354, 361, 364, 366 and 375 were scored as being positive for *P. falciparum* infection while the rest were negative. Fig 3B shows typical results with digoxigenin-labeled probe; specimens number 1, 2, 6, 12, 14, 17, 18, 24, 28, 32, 35, 37, 39, 41, 42, 46, 48, 50 and 60 were recorded as positives. Both radioactive and nonradioactive DNA probes demonstrated reliable sensitivity when detecting blood containing at least 2,500 parasites in the samples.

Comparative studies between DNA-based assays and microscopy of clinical samples are summarized in Table 1. Digoxigenin and ^{32}P-labeled probes had sensitivity of 61% and 64% respectively when compared with clinical microscopy. However, when the reference was an expert microscopist, their sensitivity were decreased to 54% and 59%, indicating that the performance of DNA probes was more similar to clinical microscopy, which had a sensitivity of 73%.

We have also compared the sensitivities of both types of DNA probes and of clinical microscopy in situations of low (less than 25 parasites/200 WBC) and high (greater than 25 parasites/200 WBC) parasitemias (Table 2). The sensitivity of digoxigenin-labeled DNA probe (70%) was lower than that of ^{32}P-labeled DNA probe (81%) and clinical microscopy (89%) at high parasite density, whereas at low parasite level, nonradioactive probe showed better sensitivity (51%) than radioactive probe (10%) and was comparable to clinical microscopy (56%) using expert microscopy as reference. These numbers would be of more significance if the number of cases were higher and shared equally between high and low parasitemias. In our study, most of the parasite densities were higher than 25 parasites/200 WBC. We set up a minimum threshold level at a parasitemia of 0.004%, which is equivalent to 5 parasites/200 WBC (assuming an average of 5×10^6 RBC and 8,000 WBC in 1µl of blood) and should cover almost all ranges of sensitivity required. Despite their moderate sensitivity, specificity higher than 93% was always achieved by our DNA hybridization methods.

DISCUSSION

DNA hybridization assay has an obvious advantage over other detection techniques since hundreds of specimens can be collected and processed at one time. The procedure is simple and no special equipment

Table 1

Comparison between DNA probes and microscopy results.

	Sensitivity	Specificity	Efficiency
Dig / clinic	61%	93%	87%
Dig / expert	54%	97%	81%
^{32}P / clinic	64%	98%	89%
^{32}P / expert	59%	98%	82%
Clinic / expert	73%	99%	92%

Dig / clinic	Nonradioactive method compared with clinic microscopy.
Dig / expert	Nonradioactive method compared with expert microscopy.
^{32}P / clinic	Radioactive method compared with clinic microscopy.
^{32}P / expert	Radioactive method compared with expert microscopy.
Clinic / expert	Clinic microscopy compared with expert microscopy.
Sensitivity	Number of microscopy positives detected by DNA probe divided by total number of positive samples detected by microscopy.
Specificity	Number of microscopy negatives detected by DNA probe divided by total number of negative samples detected by microscopy.
Efficiency	Sum of number of microscopy positive and negative samples detected by DNA probe divided by total number of samples analysed.

Table 2

Sensitivity of detection. Results of digoxigenin-labeled probe, ^{32}P-labeled probe and clinical microscopy at parasite density higher and lower than 25 parasites per 200 WBC, using expert microscopy as a reference, were compared.

	Parasite No.	
	> 25/200 wbc sensitivity	< 25/200 wbc sensitivity
Digoxigenin	70%	51%
^{32}P	81%	10%
Clinic	89%	56%

or chemicals are needed. Though the whole process takes about 12 hours to complete, time taken per sample is minimal. DNA hybridization also possesses various methods of detections. With non-radioactive DNA probe, color visualization is the most convenient system requiring no extra facilities. Positive blue color signals were clearly visible even at parasitemia as low as 0.004%. We found that digoxigenin-alkaline phosphatase assay format gave clearer background than other enzyme-linked probes for blood samples. Direct lysis and sample processing on the membrane have simplified the DNA hybridization procedures since treatment of blood-spotted membranes can be performed conveniently in a plastic box. Omitting proteinase K in the treatment step has also reduced both cost and one step in the process. Treatment was necessary for blood samples but not for extracted DNA.

Another critical factor was the method of blood collection. Blood drawn into heparinized tubes created background color. This might be due to the high concentration of heparin which reacts with NBT/BCIP substrates to produce blue precipitation. Nonheparinized tubes were thus selected for use in blood collection when chromogenic detection was performed. Following the membrane treatment, samples could be kept for weeks until hybridization. The duration of the blood deposited on the membrane before treatment has to be less than 30 minutes otherwise a high background color remained.

Evaluation of this optimized protocol for *P. falciparum* detection in field trials revealed that the Rep 20 DNA probe obtained from Gambian isolate could detect *P. falciparum* parasites in Thailand probably indicating that the 21 base pair repeats exist in all parasite population. However, it should be noted that the signal strength did not always reflect the number of parasites; variation of signal pattern by Rep 20 DNA probe has also been observed when used to detect different cultures of *P. falciparum* (Oquendo *et al,* 1986). Comparison of malaria detection by DNA hybridization with expert microscopy showed that detection of *P. falciparum* by ^{32}P-labeled and digoxigenin-labeled DNA probes were of comparable sensitivity (59% and 54%, respectively). When the results of DNA hybridization were compared with clinical microscopy, which often screen slides for parasite in fields containing less than 200 WBC, the sensitivity was increased to 64% and 61%.

Relative to expert microscopic results, the specificity of detection was higher than 93% for all methods. At the lower limit of detection, DNA probes in this study did not always give consistent outcome. Occasionally, parasitemia of lower than 25 parasites per μl of blood could be detected whereas that containing a few hundred parasites per μl was not. One possible explanation may be attributed to inaccuracy in the number of parasites estimated by the microscopist.

The current study showed that an optimized protocol for DNA hybridization assay employing either nonradioactive or radioactive DNA probe could be performed on a large scale for diagnosis of malaria. With its practicality and ease of use, nonradioactive DNA probe hybridization assay should be suitable for epidemiological study.

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