DETECTION OF *PLASMODIUM FALCIPARUM* IN BLOOD USING DNA PROBE, pPF 14

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ABSTRACT

A DNA probe pPF14, which is species specific to *P.falciparum* have been used to identify malaria species. The blood specimens were pretreated before spotting onto nitrocellulose filter to eliminate non specific hybridization of DNA to blood components.

In this study, the DNA probe pPF14 was used for detection of *P.falciparum* in blood specimens spotted directly (a simple preparation of sample) onto nitrocellulose filter. Ninety two blood specimens from individuals with fever living in a *P.falciparum* endemic area were collected at the local Primary Health Centre in Lampung. Blood was collected by finger prick technique using heparinized capillary tubes. Twenty microliter of blood was plotted directly onto nitrocellulose filter, extracted, treated with proteinase K and tested with DNA probe pPF14. Another 20 microliter of blood was used for thick smear and examined microscopically. Nineteen out of 92 (20,7 %) of the blood specimens were found to contain *P.falciparum* parasites by DNA probing and by thick smear. The densities of parasites ranged from 350 to 28,775 per microliter.

The DNA probing technique was successfully done for the detection of *P.falciparum* in the laboratory. However, this technique seems not to be quite sensitive for parasitological survey (detects 40-50 parasites per microliter of blood); while using thick smear examination, 5-10 parasites per microliter of blood could be detected with confidence.

The important issue in this case is that no special treatment before spotting the blood onto nitrocellulose filter was needed to eliminate non-specific hybridization, when protease treatment was used. Collection of specimens would be easily done in the field.

INTRODUCTION

A DNA probe pPF14 had been developed by Barker et al for detection of *P.falciparum* in blood[1]. The DNA probe which contains a highly repeated DNA sequence of *P.falciparum*, was reported to be specific and sensitive to detect 40 parasites per microliter. The blood samples were lysed and digested by proteinase K before spotted onto nitrocellulose filter to minimize non-specific hybridization to blood components. The treatment performed before blood spotting would be unpractical if it should be done under field conditions. A simpler and more rapid technique should be developed if this DNA probe would be used in a larger scale under field conditions.

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Recently, other workers[2,3] were able to overcome the background problem by treatment with chitinase and proteinase K to eliminate non-specific hybridization of *B. malayi* DNA probe to mosquito components. The chitinase and proteinase K treatment was performed after the mosquitoes were squashed onto nitrocellulose filter.

The DNA probing assay using pPF14 was established in our laboratory and the procedure simplified by treating the blood samples with protein K after being spotted on nitrocellulose filter.

MATERIALS AND METHODS

Blood Samples

Blood samples were taken from individuals with fever living in a *P.falciparum* endemic area. Blood was collected by finger prick technique using heparinized capillary tubes and spotted (20 µl) directly onto nitrocellulose filters. Another 20 µl of blood from each patient was used to make a thick smear. Blood smears were stained with Giemsa and examined microscopically in 300 oil immersion fields for malaria parasites. The number of parasites per µl of blood was determined against white blood cells (WBC), assuming a WBC count of 7500 per µl. Filters with spotted blood were air-dried for 30 minutes. DNA was denatured by placing filters onto puddles of 0.5 M NaOH, neutralized with 1 M Tris pH 7.4 and with 1.5 M NaCl, 0,5 M Tris pH 7.4 and air-dried for 30 minutes. The filters were kept in a sealed plastic box and sent to the laboratory within 5 to 7 days after collection.

Enzymatic treatment of filters

The filters were baked for 90 minutes at 60^0C and then incubated with proteinase K (150 µg/ml 0,05 M EDTA) for 2 hours at 37^0C with gentle rocking, washed with 2X SSC and air-dried.

Hybridization

A clone pPF14, containing a highly repeated sequence of *P.falciparum*[1] was used as probe in the assay. It was ^{32}p-labelled by nick translation[4] to a specific activity of 5-6 X 10^8 cpm/µg. Hybridization procedures were as described by Barker et al[1]. The filters were pre-hybridized for 2 hours at 42^0C in pre-hybridization solution containing 10 X Denhard's, 5 X SSC, 100 µg/ml herring sperm DNA, 50 % formamide and 0,1 % SDS. The filters were then hybridized for 14 hours with 3-4 10^6 cpm/ml of ^{32}p labelled pPF14. Filters were washed 3 times for 30 minutes each at 50^0C in 0.1 X SSC, 0,5 % SDS and air-dried. Filters were then left in minus 20^0C exposed onto XAR-5 Kodak for 12-14 hours using an intensifying screen. Results seen on the X-ray film were evaluated. All experiments included a negative control (uninfected human blood) and a positive control (a synchronized ring form *P.falciparum* culture).

RESULTS

Hybridization to cultured *P.falciparum* and uninfected human blood spotted onto nitrocellulose filter without proteinase K treatment showed a false positive signal to uninfected human blood (fig. 1). When uninfected human blood was treated with proteinase K before probing, no false positives were obtained (Fig.2). This enzymatic treatment avoided non-specific hybridization to human blood components.

Hybridization to synchronized ring forms of *P.falciparum* culture (serially

diluted), *P.vivax* and uninfected human blood with prior proteinase K treatment showed that hybridization occured only on *P.falciparum*. This probe was able to detect 50 parasites per µl with a 50 µl cultured *P.falciparum* (fig. 3).

Nineteen out of 92 specimens (27.7 %) were shown positive for *P.falciparum* by blood smear and by probe (table 1). The parasite densities in these specimens varied between 350 to 28.775 parasites per µl of

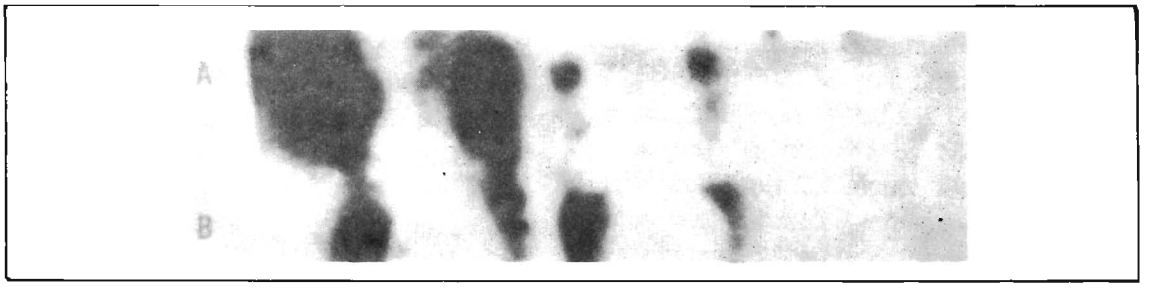

Figure 1. P. falciparum from culture, 800-50 parasites/µl (A) and uninfected human blood (B) were spotted directly on NCP and hybridized with 32P-pPF14. Non-specific hybridization show to uninfected human blood. (B)

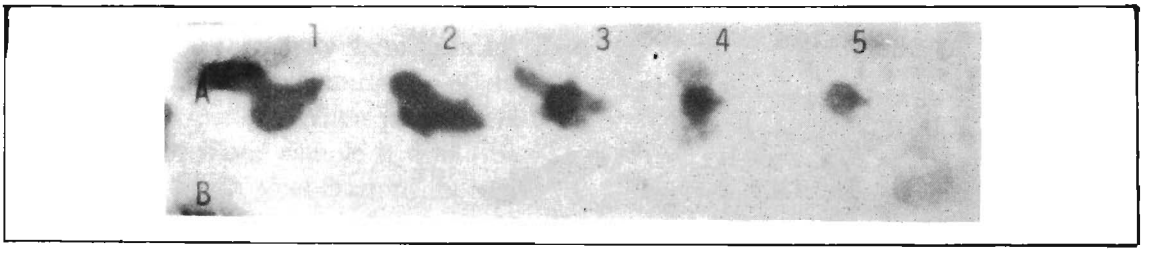

Figure 2. P. falciparum from culture, 800-50 parasites/µl (A) and uninfected human blood (B) were spotted directly on NCP, treated with 150 µg/ml proteinase K and hybridized with 32 P -pPF14. The enzymatic treatment eliminate non-specific hybridization to human blood components (B).

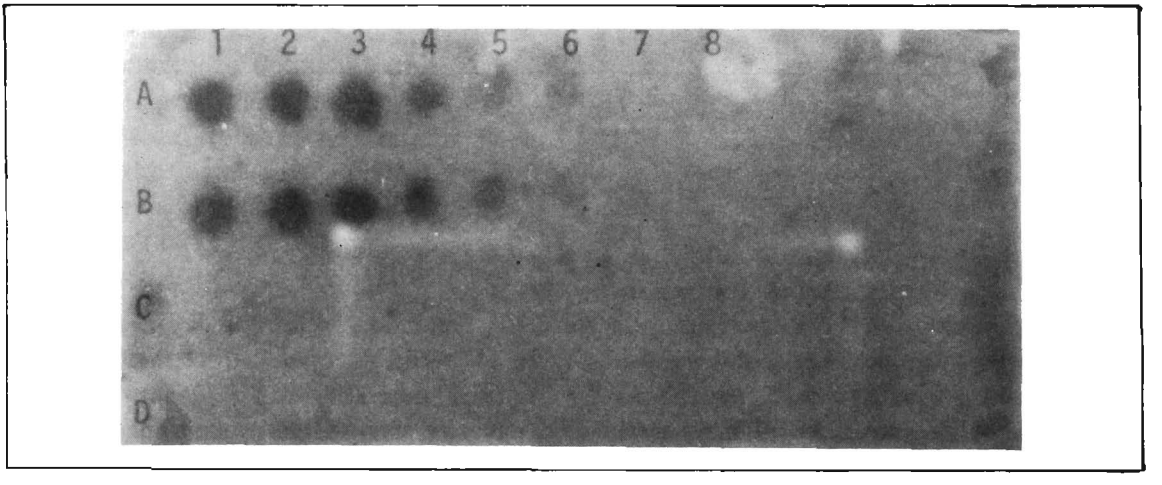

Figure 3. Sensitivity of pPF14 for P. falciparum. A and B; synchronized ring form P. falciparum from culture (50 µl) serially diluted (1600-10 parasites/µl. C; P. vivax and D; uninfected human blood.

Table 1. Detection of P.falciparum by DNA probe pPF14

Sample Number	Diagnosis*	Parasites/ 300 WBC	Parasites/ /µl of blood**	Hybridization
1.	F	186	4650	+
4.	F	285	7125	+
5.	F	76	1900	+
13.	F	183	4575	+
14.	V	-	-	-
15.	V	-	-	-
18.	F	1151	28775	+
20.	V	-	-	-
25.	F	70	1750	+
31.	V	-	-	-
33.	F	93	2325	+
39.	F	14	350	+
40.	F	169	4225	+
41.	F	72	1800	+
47.	F/V	5/-	25/-	-
50.	F	328	8200	+
52.	V	-	-	-
54.	F	1082	27050	+
55.	V	-	-	-
59.	F	161	4025	+
60.	F	284	7100	+
61.	V	-	-	-
69.	V	-	-	-
70.	F	378	9450	+
73.	F	63	1575	+
74.	V	-	-	-
86.	F	476	11900	+
88.	F	66	1650	+
89.	F	91	2275	+

* F, *P.falciparum* V, *P.vivax*
** Based on an average of 7500 WBC/ µl.

blood. One specimen contained a mixed infection of *P.falciparum* and *P.vivax*, but was found negative by probe. The density of *P.falciparum* in this specimen was 25 parasites per µl of blood. Nine out of 92 (9.7 %) were infected with *P.vivax* corfirmed by blood smear examination and negative by DNA hybridization.

DISCUSSION

Treatment with triton X and proteinase K of blood samples before spotting onto nitrocellulose filter in a *P.falciparum* detection assay using DNA probe pPF14, showed to be sufficient to eliminate non-specific hybridization to blood components[1]. In this study, efforts had been made to simplify the

elimination of the non-specific hybridization by treating the blood samples with proteinase K. The blood samples spotted directly onto nitrocellulose filter without prior treatment,were felt to be simpler and the samples could be carried to the laboratory with ease.

It was shown that the test was specific and could detect 50 parasites per µl of samples prepared from a synchronized ring form of *P.falciparum* culture (50 µl). This level of sensitivity corresponds to 0.001 % of parasitemia. This technique seemed to be inferior to the standard microscopic procedure whereby 0.0001% parasitemia (5 parasites per µl) could be detected[5].

All individuals who were shown positive microscopically were shown positive with DNA probe. This is because the samples that had been tested in this study were from individuals who had parasitemia of 350 to 28.775 parasites per µl. When parasitemia was lower in the blood sample it would be positive with microscopical examination and negative with DNA probe. In malaria surveys individuals with low grade parasitemia might be found, then this DNA probe might fail to detect the parasite.

The procedure available now needs some modification and improvement to reach the same sensitivity of microscopical examination. Furthermore, using ^{32}p as indicator is also a problem in Indonesia. We need a test with another indicator, that is long life, without many requirements for storage and which is practical for laboratories at the health centres.

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QUESTIONS AND ANSWERS :

1. Question: You have not presented an experiment with "criss-cross" test for both P.falciparum and P.vivax. Do you happen to know whether a DNA probe of P.vivax has no reaction on P.falciparum ?

   Answer : Yes, I have done the comparison with P.vivax.
   This probe is specific to P.falciparum and is not hybridized to P.vivax and other parasites.
   I have not heared about P.vivax probe. I use P.falciparum probe.