Diagnosis of *Plasmodium falciparum* infection by spot hybridization assay: specificity, sensitivity, and field applicability

**Martin Holmberg,1, 2 Anders Björkman,3, 4 Lena Franzén,1, 2 Lena Åslund,1 Marianne Lebbad,4 Ulf Pettersson,1 & Hans Wigzell2**

The spot hybridization assay for the detection of *Plasmodium falciparum* reported here uses as probe a repetitive DNA sequence from this species and exhibits a high degree of species specificity. Isolates from African, Asian, and South American patients were positive in the assay and gametocytes could be detected at the same level of parasitaemia as asexual parasites. An RNA probe containing the same repetitive sequence as the DNA probe has a detection limit of 1 parasite per 10^5 red blood cells. Comparison of the results of the assay with those obtained by microscopic examination of blood films indicated that the assay was more sensitive than microscopy if the blood films were examined for only 10 minutes; however, 40 minutes' examination by microscopy was slightly more sensitive than the assay.

Laboratory diagnosis of malaria most commonly involves identification of *Plasmodium falciparum* in blood films by light microscopy. This technique is time-consuming and tedious for use in large-scale epidemiological investigations. There is therefore a widely recognized need for improved diagnostic tests for malaria and rapid tests for the field assessment of potential malaria vaccines (1).

Recently, one of us described a spot hybridization assay for the detection of *P. falciparum* DNA that uses as probe a repetitive DNA sequence from the parasite (2). The probe consists of a DNA fragment containing 21 base-pair imperfect tandem repeats that constitute approximately 1% of the *P. falciparum* genome (3). In the assay, *P. falciparum*-infected blood from various geographical locations reacted positively, whereas DNA from *P. vivax*, *P. chabaudi*, and *P. yoelii* failed to hybridize. In a dilution experiment, parasitaemias at the 0.001% level could be detected in a sample of 50 µl packed red blood cells (2).

Here we report the results of investigations on the sensitivity and specificity of the hybridization assay and assess its applicability to epidemiological field studies.

**Materials and Methods**

**Parasite cultures**

A Tanzanian *P. falciparum* isolate (F-32 strain) and a *P. falciparum* clone from Honduras (HB-3 strain) were kept in continuous culture using the method described by Jensen & Trager (4). Cultures were harvested at parasitaemia levels of about 10% infected red blood cells and washed once before use. Samples were prepared using serial tenfold dilutions with uninfected blood, resulting in parasitaemia levels ranging between 10% and 0.0001%.

**Blood samples**

Venous blood samples (5–10 ml) were drawn from three patients with *P. falciparum* infections, originating from India and Uganda, upon admission to Roslagstull Hospital, Stockholm, and after initiation of treatment. Two of the patients received chloroquine, while the other was given sulfadoxine–pyrimethamine (Fansidar). Samples were washed in 0.9% sodium chloride solution and stored at −20 °C as
packed red blood cells; however, a few samples were stored for up to 7 days at room temperature.

Fingerprick blood samples (70 μl) from a sample group (28 adult Liberian men) living in a holoendemic area were collected in microhaematocrit tubes and stored at -20 °C. Before being assayed, the samples were divided into duplicates, each containing 35 μl blood. A blood film for microscopic determination of parasitaemia was prepared concomitantly. Uninfected samples of venous blood were obtained from healthy Swedish blood donors.

**DNA probes**

A clone, pRepHind, containing a 1.7-kb fragment of 21 base-pair repeats from *P. falciparum* was used as probe in the assay (2). This was 32P-labelled by nick translation (5) to a specific activity of 1–2 x 10^7 counts/min/μg. In order to produce a 32P-labelled RNA probe, the same *P. falciparum*-derived 1.7-kb fragment was also cloned into the HindIII-site of the SP64 vector (6). After linearization of the recombinant product, *in vitro* transcription was carried out using SP6 RNA polymerase, and the RNA probe obtained (designated SP6-RepHind) was labelled to a specific activity of 1–2 x 10^8 counts/min/μg.

**DNA extraction and hybridization**

The conditions used for DNA extraction, immobilization on nitrocellulose filters, and hybridization were the same as those described by Franzen et al. (2), except for the following modifications: proteinase K treatment was carried out for 30 minutes in the presence of 1% sodium dodecyl sulfate, and samples were immobilized using a dot-blot manifold. For the DNA probe, hybridization was carried out for 2 h at 65 °C, while for the RNA probe hybridization was conducted in 50% formamide solution for 3 h at 37 °C. The filters were washed in 2 x SSC (SSC = 0.15 mol/l sodium chloride, 0.15 mol/l trisodium citrate) for 5 x 15 minutes at 65 °C before being autoradiographed overnight using Kodak XAR-5 film and intensifying screens.

DNA from one helminth (Schistosoma mansoni) and several protozoa (Trypanosoma cruzi, Leishmania donovani, Entamoeba histolytica, and Giardia lamblia) was also extracted using the described procedure, resulting in yields of 10–100 ng DNA from each parasite. Samples of *P. vivax* and *P. malariae* from Aotus monkey and Plasmodium ovale from chimpanzee were kindly donated.  

**Microscopic examination of blood films**

Thick and thin blood films were treated with Giemsa stain. The species of parasites and their stages of development were then identified, while parasite counts were determined against leukocytes, assuming a leukocyte count of 10⁶ per μl. A blood film was considered negative if no parasite was detected in the thick film after scanning for 40 minutes in a microscope at ×1000 magnification. For a few films, thick ring forms were observed, which made it difficult to differentiate between *P. falciparum* and *P. malariae*. Diagnosis of *P. malariae* was therefore based on other, more specific criteria. Parasitological examinations were performed by a trained microscopist who was experienced in malaria diagnosis.

**RESULTS**

**Specificity of the assay**

No cross-reactions with other parasites. The results of earlier studies showed that the pRepHind probe described here does not cross-react with DNA from *P. vivax*, *P. chabaudi*, or *P. yoelli* (2). One sample each of blood infected with pure *P. ovale* (2% parasitaemia in chimpanzee) and of *P. malariae* (0.4% parasitaemia in Aotus monkey) was negative in the assay. Furthermore, up to 100 ng of purified DNA from *T. cruzi*, *L. donovani*, *E. histolytica*, *G. lamblia*, or *S. mansoni* were also negative in repeated assays. In contrast, *P. falciparum*, in both asexual and gametocyte forms, was positive.

**Ability to detect *P. falciparum* of different geographical origin and stages of development.** Two in vitro cultured strains of *P. falciparum* from different places of origin (the F-32 Tanzanian strain and the HB-3 clone from Honduras) were assayed using the hybridization technique. Serially diluted samples from both cultures gave positive signals down to a parasitaemia level of 0.001% when 50-μl samples were used. In a second experiment, samples of *P. falciparum* from India (2 samples), Uganda (1), and Liberia (28) were assayed and gave reactions whose intensity correlated with the observed parasitaemia levels (Fig. 1 and 2). Samples of *P. falciparum* from South American patients have previously been assayed with similar results (2). The two Indian patients in our study were also monitored daily during treatment: decrease in parasitaemia was reflected by a reduction in signal intensity in the assay (Fig. 1); however, both patients exhibited residual gametocytaemia at the end of the observation period and this was clearly detectable in the assay.

The hybridization assay can therefore detect *P. falciparum* DNA regardless of the geographical
Fig. 1. Spot hybridization assay with the pRepHind DNA probe for detection of *Plasmodium falciparum* infection in two patients undergoing treatment.

Row A: Controls. 1: positive control (*P. falciparum* from culture, 1% parasitaemia level); 3: negative control (uninfected blood).

Row B: Patient KS. 1: day 0 (before treatment), 12 000 parasites/μl; 2: day 1, 8800/μl; 3: day 2, 1400/μl; 4: day 4, gametocytes only; 5: day 5, gametocytes only; 6: day 6, gametocytes only; 7: day 7, gametocytes only.

Row C: Patient SS. 1: day 0, 19 000 parasites/μl; 2: day 1, 3700/μl; 3: day 2, 300/μl; 4: day 3, 10/μl; 5: day 4, gametocytes only; 6: day 5, gametocytes only.

Fig. 2. Hybridization with *Plasmodium falciparum* DNA probe of 28 samples from adult Liberian men from a holoendemic area.

Row A: Controls. 1, 2, 5: positive controls (*P. falciparum* DNA); 6: negative control (human DNA).

Level of parasites/μl (in parentheses) determined by microscopy (F = asexual *P. falciparum*; FG = gametocytes *P. falciparum*; M = *P. malariae*; neg = negative):

B1, (210 F); B2, (2 F); B3, (20 F); B4, (5 F); B5, (4500 F); B6, (145 F + M + 5 FG); B7, (10 F); B8, (20 F); C1, (260 M + F); C2, (2 F); C3, (25 F); C4, (30 FG + 10 F); C5, (10 F); C6, (neg); C7, (10 FG + 5F); C8, (2 F); D1, (neg); D2, (2960 F + M); D3, (70 M + 5 FG); D4, (40 F); D5, (170 F + M); D6, (neg); D7, (350 F + M); D8, (neg); E1, (neg); E2, (neg); E3, (5 M); E4, (15 F).

Fig. 3. Spot hybridization assay with pRepHind DNA and SP6-RepHind RNA probes. Two parallel serial dilutions of *Plasmodium falciparum* DNA were used: 10 ng; 1 ng, 100 pg, 10 pg, 1 pg. A control containing 10 ng human DNA was also assayed.

Row A: samples assayed with RNA probe.

Row B: samples assayed with DNA probe.
Fig. 4. Demonstration of detection limit in the spot hybridization assay (% parasitaemia) with various sample volumes. Hybridization was carried out using the RNA probe. Column A = 50 μl; B = 100 μl.

1 = 0.1% parasitaemia; 2 = 0.01%; 3 = 0.001%; 4 = 0.0001%; 5 = uninfected blood.

Fig. 5. Effects of storage time on signal intensity in the spot hybridization assay.

Row A: controls.

Row B: samples assayed after storage at room temperature for 7 days as packed red blood cells in plastic tubes.

Row C: samples dried on cellulose filter-paper (Whatman 3 mmChri).

Row D: samples dried on cellulose filter-paper and eluted after storage for 7 days.

Columns 1–4: parasitaemia levels of 0.1%, 0.01%, 0.001%, and 0.0001%, respectively.

Column 5: uninfected blood.
origin of the sample or whether the parasites are in asexual or sexual form.

**Sensitivity of the assay**

**Comparison between DNA and RNA probes.** Use of radiolabelled RNA probes rather than DNA probes increases the specific activity of the assay (6). In accord with this, the SP6-RepHind RNA probe was more sensitive than the corresponding DNA probe in detecting *P. falciparum* DNA (Fig. 3). Densitometric scans of the spots indicated that the RNA probe produced signals that were approximately three times more intense. Furthermore, a weak background signal from human DNA that was sometimes present with the DNA probe was not observed with the RNA probe.

**Influence of blood volume and leukocyte count.** Our results are consistent with previous findings which indicated that with the DNA probe a parasitaemia level of 0.001% could be detected in a 50-µl sample of packed red blood cells (2). Furthermore, with the RNA probe and 100 µl blood, we achieved a detection limit corresponding to 0.0001% parasitaemia, equivalent to around 10 parasites per µl (Fig. 4).

In order to test the sensitivity of the assay for samples that contained exclusively gametocytes, a sample of blood from a patient with pure gametocyteaemia was serially diluted. With 50-µl samples, gametocytes were detected down to a level of 40 per µl.

To analyse whether variation in leukocyte count influenced the assay, a fixed amount of DNA from *P. falciparum* was mixed with various amounts of human DNA (100 ng up to 10 µg). With the RNA probe, the added human DNA did not influence the hybridization assay, indicating that leukocyte count has no influence on the results of the assay.

**Stability of test samples**

For use in field trials, the stability of blood samples is of importance. Preliminary investigations suggested that when blood samples containing malaria parasites had been left for up to 7 days at room temperature or at 4 °C before being assayed there was no reduction in the sensitivity limit (Fig. 5). Similarly, after more than 6 months storage at –20 °C, there was no observable change in the ability of samples of parasitaemic blood to react in the assay.

Some alternative methods of storage have also been studied. For example, samples of blood blotted on to cellulose filter-paper and dried gave only a marginal loss of sensitivity when DNA was eluted after one week and assayed (Fig. 5).

**Comparison of the results of hybridization assay and microscopic examination**

In order to investigate whether small samples of blood obtained under field conditions could be screened as correctly and efficiently using the assay as by conventional microscopic examination by a skilled microscopist, we surveyed 28 asymptomatic adult Liberian men from a rural area with holoendemic malaria (7). The men were chosen because of their presumed low-grade parasitaemia. Use of the hybridization assay showed that 17 were carriers; in contrast, microscopic examination of each blood film for up to 40 minutes detected *P. falciparum* among 21 of the 28 subjects. Mixed infections with *P. malariae* were noted in 6 of the 21 individuals with *P. falciparum*, while pure *P. malariae* infection was found in one subject. Parasite densities varied from 2 to 4500 parasites per µl, with a median value of 20.

Of the four samples infected with *P. falciparum* that were negative in the hybridization assay, one had 10 asexual parasites per µl and 30 gametocytes per µl, another had 15 asexual parasites per µl, while two samples had 2 asexual parasites per µl. All samples without *P. falciparum* parasitaemia, after 40 minutes' microscopic examination, were also negative in the assay (see Fig. 2 and Table 1). Microscopic examination of blood samples for 40 minutes was therefore more accurate than the hybridization assay. However, if blood films were read for 10 minutes per slide, only 15 out of the 21 positive *P. falciparum* slides were identified.

**DISCUSSION**

A spot hybridization assay for the detection of low levels of *P. falciparum* in peripheral blood has been reported previously (2). Here, we have extended these

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investigations and confirmed the high degree of species-specificity of this assay as indicated by the absence of cross-reactions from samples infected with *P. malariae* or *P. ovale*. In addition, DNA from five other parasites also failed to cross-hybridize. We can therefore conclude that the hybridization test detects only *P. falciparum*-derived DNA.

To be truly useful, the assay should detect *P. falciparum* in blood, regardless of the geographical origin of the sample. This was the case for samples and isolates from patients in Africa, Asia, and South America, which all reacted positively, with signal intensities approximately correlating with the degree of parasitaemia. Gametocytes also reacted positively, demonstrating that the DNA sequence used as probe is preserved at the sexual stage. It is probable, however, that a more detailed analysis may indicate some variation in signal intensities. Support for this also comes from reports that certain strain variations may occur in the distribution and amount of repetitive DNA (7).

The high degree of sensitivity previously reported for the hybridization assay (2) has been confirmed and extended by the present study. Interference from human DNA in leukocytes was of no importance, and the sensitivity can be increased by the use of larger volumes of blood.

With parasite cultures, the sensitivity of the assay was 1 parasite per 10^6 red blood cells with 100-μl samples. In contrast, with the field samples from Liberia the lower limit of detectability was approximately 10 parasites per μl using a 35-μl sample of whole blood, corresponding to around 2 parasites per 10^6 red blood cells. The sensitivity of the hybridization assay is superior to that of microscopic examination of thick blood films on slides for 10 minutes by a highly skilled microscopist. However, for an examination time of 40 minutes per slide, microscopy was more sensitive than the present hybridization assay.

In two previously described immunoassays for the detection of *P. falciparum* the detection limits were 8 parasites (9) and 1 parasite (11, 13) per 10^6 red blood cells for cultured parasites. However, when evaluated in the field these levels could not be reproduced (10, 12). The present hybridization assay is therefore at least as sensitive as other assays for the detection of *P. falciparum*, particularly when single-stranded RNA probes are used in combination with blood volumes greater than 35 μl.

A spot hybridization assay, using as probe ^32^P-labelled total *P. falciparum* DNA, has recently been described (14), and its sensitivity is similar to that of our assay. However, its specificity has not yet been reported. Nevertheless, a sample of blood containing only gametocytes was negative in this assay but positive in our assay.

Preliminary results indicate that our assay permits the use of dried blood samples and that problems arising from storage and transport of samples during field trials are not expected. The time required for the hybridization assay is at present around 18 hours, which is adequate for field studies but unsuitable for use in clinical diagnosis.

A non-radioactive probe is essential for the widespread use of the spot hybridization assay described here. Several assays that use as probes enzyme-linked DNA or RNA have been reported (15-17), but their sensitivity has so far not matched that of ^32^P-labelled probes. For example, in our hands the probes labelled with biotin-streptavidin-alkaline phosphatase are 100-1000 times less sensitive than radiolabelled probes.

Another potentially useful assay involves the use of time-resolved fluorescence and europium chelates (18). This method has been successfully used in several immunoassays and is now being adopted for use in DNA hybridization assays. Both enzyme and fluorescent labels may play complementary roles in the future development of DNA hybridization assays.

ACKNOWLEDGEMENTS

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RÉSUMÉ

DIAGNOSTIC DE L’INFECTION À PLASMODIUM FALCIPARUM PAR HYBRIDATION-TRANSFERT:
SPÉCIFICITÉ, SENSIBILITÉ ET APPLICABILITÉ SUR LE TERRAIN

Environ 1% du génome de Plasmodium falciparum est constitué de répétitions en tandem imparfaites de séquences de 21 paires de bases. Une épreuve d’hybridation-transfert pour la détection de P. falciparum au moyen d’une sonde contenant ces séquences répétitives a été mise au point et possède une forte spécificité d’espèce. Des isolements prélevés sur des malades en Afrique, en Asie et en Amérique du Sud se sont montrés positifs et ont donné des signaux d’hybridation dont l’intensité était approximativement cor-rélée au taux de parasitémie. Les gamétocytes ont pu être décédés au même taux de parasitémie que les parasites asexués.

Une sonde d’ARN contenant les mêmes séquences répétitives a permis de déceler P. falciparum au taux extrêmement bas de 1 parasite pour 10⁶ érythrocytes. L’examen densitométrique des taches obtenues par hybridation-transfert montre que la sonde d’ARN est environ trois fois plus sensible que la sonde d’ADN. L’addition de grandes quantités d’ADN humain ne gênait pas l’épreuve, ce qui indique que des différences des nombres de lesocytes contenus dans les prélèvements de sang ne faussent pas les résultats.

L’effet de la conservation des échantillons sur la qualité de l’épreuve a été étudié après séjour de 7 jours à la température ambiante dans des tubes de plastique ou à l’état sec sur du papier-filtre; on n’a observé qu’une très faible baisse de la sensibilité.

On a ensuite comparé l’épreuve d’hybridation-transfert et l’observation au microscope pour examiner des étaliments sanguins obtenus sur le terrain chez 28 Libériens adultes de sexe masculin résidant dans une zone de paludisme holo-endémique. Vingt et un échantillons étaient positifs pour P. falciparum lorsque les lames de sang étaient examinées pendant 40 minutes par un microscopiste expérimenté; six de ces échantillons présentaient des infections mixtes avec P. malariae et un autre une infection simple à P. malariae. Dix-sept de ces échantillons positifs ont également été identifiés par hybridation-transfert. Les sept échantillons négatifs à l’examen microscopique étaient également négatifs par hybridation-transfert.

Ces résultats montrent que l’épreuve d’hybridation-transfert est plus sensible que l’examen microscopique de lames de sang pendant dix minutes; toutefois, si les lames sont examinées pendant 40 minutes, l’examen microscopique devient légèrement plus sensible. L’épreuve d’hybridation-transfert décrite ici est au moins aussi sensible que les autres épreuves de diagnostic et pourrait constituer un outil valable pour les études épidémiologiques. Néanmoins, pour pouvoir l’appliquer sur le terrain, il est indispensable de mettre au point des sondes marquées non radioactives.

REFERENCES
