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Screening donor blood for malaria by polymerase chain reaction

Vu thi Ty Hang[1,2], Tran Van Be[2], Phan Ngoc Tran[1,2], Le Thiet Thanh[1], Luong Van Hien[3], E. O'Brien[4] and G. E. Morris[1] [1]*Research Division, North-East Wales Institute, Deeside, Clwyd, CH5 4BR, UK;* [2]*Blood Transfusion and Haematology Centre, 118 Hung Vuong, Quan 5, Ho Chi Minh City, Viet Nam;* [3]*Tram Y Te, Nam Cat Tien, Tan Phu, Dong Nai, Viet Nam;* [4]*Department of Haematology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK*

Abstract

In countries where malaria is endemic, its transmission is a hazard of blood transfusion. The microscopical and immunological methods in current use for malaria diagnosis are unsatisfactory for low levels of parasitaemia in blood donations. The polymerase chain reaction (PCR) can be 100-fold more sensitive than thick blood film examination when appropriate primers are used and can detect and distinguish *Plasmodium falciparum* and *P. vivax* in a single tube. A study of 1506 blood donations in Ho Chi Minh City (3 of which were positive) suggests that PCR can provide an effective screen for *P. falciparum* under local conditions. Studies in a region of Viet Nam where malaria is common showed that PCR detects many more cases of low-level parasitaemia (19/30) than thick blood films (4/30).

Keywords: malaria, *Plasmodium falciparum*, donor blood screening, polymerase chain reaction

Introduction

Transmission of malaria by blood transfusion is a significant problem in regions of the world where this disease is endemic; *Plasmodium falciparum* in transfused blood may lead to fatalities. The standard test for *Plasmodium* spp. is the microscopical identification of parasites in Giemsa-stained thick and thin blood films. While this is ideal in many ways as a rapid and inexpensive diagnostic test, it is not very suitable for screening large numbers of donor blood samples. This is partly because parasitaemias are likely to be much lower in asymptomatic donors than in symptomatic patients and partly because the very high proportion of negative samples in donor blood screening renders the microscopical method subject to lapses of concentration by technical staff; a similar problem arises with samples taken for epidemiological studies (BARKER *et al.*, 1992). The sensitivity limit of the thick blood film examination has been estimated at about 5 parasites per microlitre and this requires counting 100 microscope fields (BARKER *et al.*, 1992). Immunological tests (OAKS *et al.*, 1991) and hybridization tests using labelled deoxyribonucleic acid (DNA) probes (WATERS & MCCUTCHAN, 1989) have been developed but have shown only modest improvement in sensitivity over the use of blood films. When large volumes of blood are being delivered to a transfusion recipient, a very sensitive test is required to minimize the risk of parasite transmission.

The polymerase chain reaction (PCR) has been used to amplify *Plasmodium* DNA in genes for surface antigens (SCHRIEFER *et al.*, 1991; BROWN *et al.*, 1992; WOODEN *et al.*, 1992) and repetitive genomic DNA (TIRASOPHON *et al.*, 1991; BARKER *et al.*, 1992). The PCR can detect a single *P. falciparum* in 20 μL of human blood when primers based on the moderately-repetitive DNA probe pBRK1-14 are used (TIRASOPHON *et al.*, 1991), 2 orders of magnitude more sensitive than the standard thick blood film. We set out to determine whether this method is suitable for screening donor blood and whether simultaneous screening for *P. falciparum* and *P. vivax* is possible. We also wished to discover whether the cost of reagents or the incidence of false positive reactions would limit the usefulness of PCR for blood screening in a developing country like Viet Nam.

Materials and Methods

Sample treatment

Blood samples from malaria patients were collected in hospitals in Ho Chi Minh City and *Plasmodium* parasites were counted by conventional microscopical examination of thick blood films stained with Giemsa's stain; the remainder of each sample was freeze-dried and stored at −20°C. Samples of donor blood collected at the Ho Chi Minh City Blood Transfusion Centre were subjected to PCR immediately after collection. Human erythrocyte cultures infected with *P. falciparum in vitro* were stored at −20°C after counting parasites microscopically.

Freeze-dried blood samples were reconstituted with water. Dilution, if necessary, was done with uninfected human blood after reconstitution. 50 μL of blood were added to 200 μL of lysis buffer (0·2% NaCl, 1% Triton X 100®, 1 mM ethylenediaminatetraacetic acid) and the mixture was centrifuged at 13 000 ***g*** at room temperature for 10 min. The pellet was washed in 250 μL of PCR buffer (10 mM Tris-HCl, pH 8·8, 40 mM KCl, 1·5 mM $MgCl_2$, 0·1% bovine serum albumin) and centrifuged at 13 000 ***g*** for 5 min.

PCR amplification

The washed pellets were resuspended in 40 μL of PCR buffer containing adenosine, guanosine, cytidine and thymidine diphosphates (dATP, dGTP, dCTP and dTTP; Pharmacia Ltd; 200 μM each), primers (0·25 μM each) and 1·5 mM extra $MgCl_2$. For *P. falciparum*, oligonucleotide primers (forward, 5′-CGCTACATATGC-TAGTTGCCAGAC-3′ and reverse, 5′-CGTGTAC-CATACATCCTACCAAC-3′) which amplify a 206 base pair (bp) sequence were used (TIRASOPHON *et al.*, 1991). For *P. vivax*, novel primers which amplify a 131 bp sequence from the gene for the small subunit of ribosomal ribonucleic acid (RNA) (WATERS & MCCUTCHAN, 1989) (forward, 5′-GCAACGCTTCTAGCTTAATC-3′ and reverse, 5′-ACAAGGACTTCCAAG CCGAAGC-3′) were used.

The samples were overlaid with mineral oil and incubated for 10 min at 100°C before adding 0·75 units of Taq polymerase in 10 μL of PCR buffer plus supplements. They were then amplified for 30 cycles, each cycle consisting of 0·5 min at 55°C, followed by 1 min extension at 72°C and 0·5 min at 94°C. DNA amplified by PCR was detected by visualization under ultraviolet transillumination of the appropriate size band in a 2% agarose gel (molecular biology grade) containing 0·53 μg/mL ethidium bromide.

Results

The detection limit for *P. falciparum* was between 1 and 4 parasites in 50 μL of blood (Fig. 1a), using primers based on the moderately-repetitive DNA sequence, pBRK1-14 (TIRASOPHON *et al.*, 1991). This sensitivity was somewhat lower than the 1 parasite per 20 μL obtained by TIRASOPHON *et al.* (1991) since we omitted the subsequent Southern blot with a ^{32}P-labelled oligonucleotide probe because of the limitations of cost and availability of radiolabelled probes in Viet Nam. The detection limit for *P. vivax*, using primers based on the

Address for correspondence: Dr G. E. Morris, Research Division, N. E. Wales Institute, Deeside, Clwyd, CH5 4BR, UK.

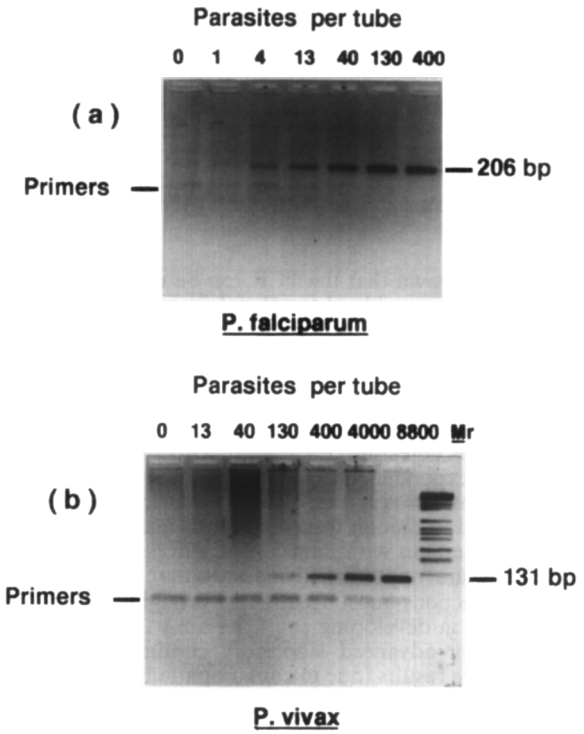

Fig. 1. PCR detection of *P. falciparum* and *P. vivax* in malaria patients. PCR primers for *P. falciparum* amplify a DNA band of 206bp (a), while primers for *P. vivax* amplify a band of 131bp (b). The M_r markers (Boehringer, Mannheim: VI, pBR328 digested with *Bg*lI and *Hin*fI) range from 145bp to 2176bp. Before dilution with normal human blood to the concentrations shown, smear counts indicated parasite levels of 108 000 per 50 μL for (a) and 8800 for 50 μL for (b), assuming that 1 μL of blood is equivalent to 400 microscope fields (BARKER *et al.*, 1992).

small subunit of ribosomal RNA (SSUrRNA) was between 40 and 130 parasites in 50 μL of blood (Fig. 1b). This difference was probably due to the different primers and target DNA sequences, so alternative primers are being studied. The concentration of parasites in the infected blood samples used was determined by microscopical examination, assuming that 400 microscope fields are equivalent to 1 μL of blood (BARKER *et al.*, 1992). Table 1 shows that, for *P. falciparum*, threshold dilutions of about 1–4 parasites per 50 μL were obtained using blood samples with different levels of parasitaemia, including those from 2 patients with low level parasitaemias after drug treatment (nos. 3 and 4). A visual assessment of PCR band intensity was used to produce this Table, but an attempt was made to measure the yield of PCR product by microdensitometry of a photographic

Table 1. Detection limit of *P. falciparum* by ethidium bromide PCR

	Blood samples[a]			
	1	2	3	4
No. of parasites per test[b]				
800				++
400	+++	+++	++	
130	++(+)			
40	++	++(+)		
13	+(+)	++		
8				+
4	+	+(+)	+	
1	−	+		
0·8				−
0·4			−	

[a]Four blood samples (1–4) from malaria patients (*P. falciparum*) with different initial parasitaemias (270, 114, 3 and 4 parasites/50 microscope fields, respectively) were serially diluted with normal human blood and each dilution was subjected to PCR. The PCR band intensities obtained from sample no. 1 are shown in Fig. 1a. The low parasitaemias in samples 3 and 4 did not allow an accurate estimate of parasite numbers.

[b]No. of parasites per 50 μL sample of diluted blood required to give a PCR band of the intensity shown (graded from +++ to −).

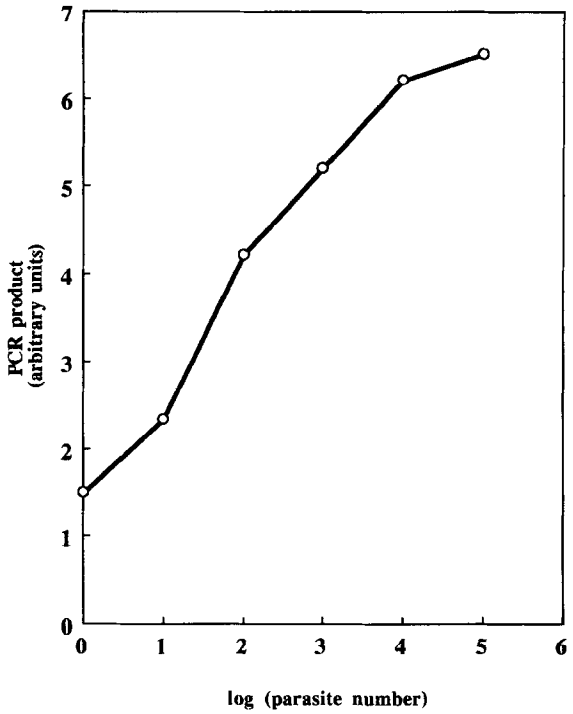

Fig. 2. Dose–response of PCR using 1–10^5 cultured *P. falciparum*. Photographic negatives were scanned with an LKB® laser microdensitometer and printed peaks of the 206bp band were cut out and weighed. Arbitrary units of PCR product were derived from these weights. Dilution experiments showed an approximately linear relationship between amount of PCR product and densitometer peak size up to 5 arbitrary units.

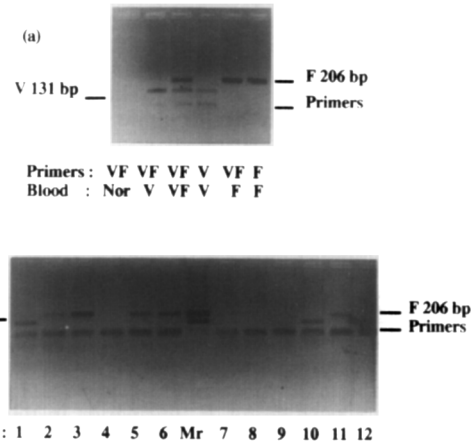

Fig. 3. Screening blood by PCR for both *P. falciparum* (F) and *P. vivax* (V) infections in a single test using mixed primers. (a) Single (V, F) or mixed (VF) primer pairs were used, each at the standard concentration. A 50:50 mixture of the blood samples used for Fig. 1 was used undiluted for the PCR (VF). Uninfected human blood (Nor) was used as a negative control. (b) Blood samples from 3 *P. vivax* patients (lanes 1, 10 and 12), 5 *P. falciparum* patients (lanes 2, 3, 5, 6 and 11) and 4 suspected dengue fever patients (negative controls: lanes 4, 7, 8 and 9) were subjected, undiluted, to PCR with mixed primers. The M_r markers are a mixture of previously amplified 206bp and 131bp DNA.

negative, using *P. falciparum* parasites obtained from cell cultures (TRAGER & JENSEN, 1976) (Fig. 2). As few as 1–10 parasites could be detected by ethidium bromide staining alone after PCR.

By using a mixed set of 4 primers, we could detect both *P. falciparum* and *P. vivax* in the same blood sample (Fig. 3a). The 206bp *P. falciparum* band was produced in all tubes containing both *P. falciparum* DNA and the *P. falciparum* primers, while in all tubes containing both *P. vivax* DNA and *P. vivax* primers the 131bp product was obtained; the primers were entirely species-specific. The practical use of the screening method is illustrated in Fig. 3b. Although simultaneous detection of both species was possible, the 206bp *P. falciparum* band was sometimes produced in reduced amounts when all 4 primers are used in the PCR (Fig. 3a, and results not shown). Because *P. falciparum* is more dangerous to the transfused patient than *P. vivax*, it may prove preferable in practice to omit *P. vivax* primers in order to maintain maximum sensitivity for *P. falciparum* until this problem is overcome.

Table 2. Field surveys of malaria using *P. falciparum* primers for PCR

	No. of samples		
	Tested	PCR positive	Positive by blood film
Blood donors in Ho Chi Minh City	1506	3 (0·2%)	0
Volunteers from Binh Chanh (rural area near the city)	239	6 (2·5%)	0
Forest wardens at Nam Cat Tien	30	19 (73%)	4 (13%)

The results of 3 field surveys are summarized in Table 2. In August and September 1993, a carefully controlled survey of 1506 blood donations was carried out in which each malaria-positive donor was recalled, resampled and retested both by PCR and by thick blood film. Only 3 donations (0·2%) were infected with *P. falciparum* and, in each case, a rather weak 206bp band suggested a low level of parasitaemia. On retesting a fresh blood sample taken the following day all 3 were positive by PCR and negative by the thick blood film examination. All 3 donors were asymptomatic, but lived in areas where malaria transmission is known to occur: 2 in rural areas of the outskirts of Ho Chi Minh City and one in Ben Tre province in the Mekong delta. Subsequently, 239 blood samples were obtained from volunteers in Binh Chanh district on the western outskirts of Ho Chi Minh City, a region where some malaria transmission was known to occur. Six samples (2·5%) were positive for *P. falciparum* in the PCR, though the 206bp band was weak, and all 6 had negative thick blood films. (The 239 volunteers had presented at a public health clinic where free vitamins and medicines were distributed.)

The study was extended to tests on blood samples taken in a hyperendemic area. The Nam Cat Tien forest national park (150–300 m above sea-level) lies in Dong Nai province to the north of Ho Chi Minh City. The park wardens, who live at the edge of the forest, have histories of repeated illness due to malaria, which is monitored locally and treated promptly. Blood samples were taken from 30 wardens, 16 from those undergoing treatment (quinine plus chloroquine) at the time and 14 from asymptomatic individuals. Over 80% (13/16) of the former were positive for *P. falciparum* in the PCR and 43% (6/14) of the latter, compared with 3 of the former and 1 of the latter who had positive thick blood films. It is well established that low levels of parasitaemia, even detectable on thick blood films, can occur in asymptomatic individuals in endemic areas (OAKS *et al.*, 1991), so it is perhaps not surprising that more such cases are detected by PCR than by thick blood film examination. Of the 3 patients undergoing treatment who were negative for *P. falciparum*, one was undergoing treatment for a known *P. vivax* infection. *P. vivax* may also have been responsible for the other 2 cases, since both parasite species cause malaria in this region (alternatively, the quinine treatment may already have reduced *P. falciparum* below the threshold level of detection).

All blood samples which gave thick blood films with detectable *P. falciparum* were also positive in the PCR; these included the 4 samples in Table 1, the 9 in Fig. 3, 4 at Nam Cat Tien, and 5 patients (data not shown) who presented with fever at Ho Chi Minh City hospitals.

Discussion

We have shown that the PCR can be used for routine screening of donor blood for *P. falciparum* under conditions found in developing countries. We believe this to be the first successful medical application of the PCR within Viet Nam. Sensitivity can be 100 times greater than the microscopical examination of thick blood films and refinement of the method may eventually permit simultaneous detection of *P. vivax* with similar sensitivity. In our experience in Ho Chi Minh City, thick blood film examination very rarely detects malaria parasites in donor blood, although transmission of malaria by transfusion, sometimes with fatal consequences, has occurred in recent years (unpublished observations).

The objections most frequently brought against the use of PCR in developing countries are its high cost and the need for advanced laboratory conditions to avoid false positive results (due to contamination of samples by previous PCR products). To obtain useful epidemiological data on malaria, avoidance of false positive results is important. However, from the point of view of preventing malaria transmission by transfusion, occasional false positive results are of little importance since the only consequence is that one less unit of donor blood is taken, and a much higher proportion of blood donations are rejected for other reasons (e.g., nearly 10% for hepatitis B [TRAN VAN BE *et al.*, 1993]). It is very difficult to rule out entirely the possibility that some false positive results in the PCR were obtained in the surveys when the blood film was negative (Table 2), but there is a good correlation between the positive samples and the prevalence of malaria in the region of origin of the donors. In the surveys, the proportion of blood donors positive for *P. falciparum* cannot be interpreted to indicate the local prevalence of malaria infection, because those who present themselves as donors may not be representative of the total population. No false negative result was observed insofar as all samples giving a positive thick blood film were also positive in the PCR. It is clear, however, that the use of PCR detects more cases with low-level parasitaemia than thick blood film examination. Predictably, a high proportion (43%) of asymptomatic individuals at Nam Cat Tien were positive for *P. falciparum* in the PCR. They had presumably acquired a tolerance of low-level parasitaemia which might not be shared by a transfusion recipient. The minimum number of parasites required to transmit malaria by blood transfusion may vary for individual recipients, but it is known that a single parasite can transmit the disease in mice. Even if a single parasite can be detected in a 50 μL blood sample, several thousand parasites might be present in a full unit of blood and still pass undetected. This illustrates the importance of using as sensitive a test as possible to minimize, though never eliminate, the risk of malaria transmission by blood transfusion.

The marginal cost of reagents for a single, 'in-house' PCR test as described here is less than US$ 0·5, the thermostable DNA polymerase being by far the most expensive component. This is comparable to the cost of commercial enzyme-linked immunosorbent assay (ELISA) kits for hepatitis B, for example, though much more than the cost of thick blood films or 'in-house' ELISA kits; inadequate sensitivity of the latter tests, however, limits the value of this comparison. The calculation does not take into account any additional costs that might arise from patents held on the PCR process. In practice, PCR

fits in well with the method for sampling and testing blood currently operating in the Ho Chi Minh City blood transfusion centre; blood samples are taken in the early morning and results from the PCR are available within 5–6 h, whereas donors return the following morning to provide a full unit of blood. Our studies have been carried out with DNA stained with ethidium bromide only. Although subsequent Southern blot hybridization with a radioactive probe and overnight exposure to X-ray film would undoubtedly increase the sensitivity of the method (TIRASOPHON *et al.*, 1991), it would also increase its cost and reduce its practicality under local conditions in Viet Nam. Using ethidium bromide detection, PCR requires little specialized laboratory equipment other than a microcentrifuge and flat bed electrophoresis apparatus. A thermal cycler is a useful labour-saving device, but can be replaced by one technician and 3 water baths.

Acknowledgements

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References

Barker, R. H., Banchongaksorn, T., Courval, J. M., Suwonkerd, W., Rimwungtragoon, K. & Wirth, D. F. (1992). A simple method to detect *Plasmodium falciparum* directly from blood samples using the polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene*, **46**, 416–426.

Brown, A. E., Kain, K. C., Pipithkul, J. & Webster, H. K. (1992). Demonstration by the polymerase chain reaction of mixed *Plasmodium falciparum* and *P. vivax* infections undetected by conventional microscopy. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86**, 609–612.

Oaks, S. C., Mitchell, V. S., Pearson, G. W. & Carpenter, C. C. J. (1991). *Malaria: Obstacles and Opportunities*. Washington, DC: National Academy Press, pp. 73–89.

Schriefer, M. E., Sacci, J. B., Wirtz, R. A. & Azad, A. F. (1991). Detection of polymerase chain reaction-amplified malarial DNA in infected blood and individual mosquitoes. *Experimental Parasitology*, **73**, 311–316.

Tirasophon, W., Ponglikitmongkol, M., Wilairat, P., Boosaeng, V. & Panyim, S. (1991). A novel detection of a single *Plasmodium falciparum* in infected blood. *Biochemical and Biophysical Research Communications*, **175**, 179–184.

Trager, W. & Jensen, J. B. (1976). Human malaria parasites in continuous culture. *Science*, **193**, 673–675.

Tran Van Be, Buu Mat, Nguyen Thi Man & Morris, G. E. (1993). Hepatitis B in Ho Chi Minh City, Viet Nam. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **87**, 262.

Waters, A. P. & McCutchan, T. F. (1989). Rapid, sensitive diagnosis of malaria based on ribosomal RNA. *Lancet*, **i**, 1343–1346.

Wooden, J., Gould, E. E., Paull, A. T. & Sibley, C. H. (1992). *Plasmodium falciparum*: a simple polymerase chain reaction method for differentiating strains. *Experimental Parasitology*, 75, 207–212.

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Announcement