FINAL REPORT

DNA PROBE FOR THE DIAGNOSIS OF VIVAX MALARIA

A RESEARCH PROJECT

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SUMMARY

Genomic DNA libraries of *Plasmodium vivax* were constructed in several plasmid vectors, i.e., pUC13, pUC19 and pUN121 then transformed into *Escherichia coli* host cells and screened for the clones containing repetitive DNA sequences for use as the specific and sensitive DNA probes for the diagnosis of vivax malaria.

For the pUC plasmids, purified DNA obtained from pools of local isolates of the parasites enriched by the sulfoethyl cellulose-Sephadex G25-Percell technique was partially digested with the restriction enzyme Sau3AI and ligated to the BamHI site of the plasmid vectors. The recombinant plasmids were transformed into *Escherichia coli* strains JM109 and DH5α host cells for the pUC13 and pUC19 vectors, respectively. The transformants were then screened with $^{32}$P-labelled *P. vivax* and human genomic DNA. One clone, VPL101, which gave relatively strong hybridization signal against only the labelled *P. vivax* but not *P. falciparum* or human DNA probes was obtained from the genomic DNA library constructed in the pUC13 plasmid vector and transformed into *Escherichia coli* strain JM109 host cells in Australia. VPL101 was subcloned and partially characterized for its DNA sequenced. VPL101 and its subcloned VPL101/5 which contained a 205 basepairs (bp) tandem DNA repeat sequence were used successfully for detection of *P. vivax* DNA samples obtained from vivax malaria patients in Australia, but they did not give satisfactory results when tested against isolates of the
Therefore, the genomic DNA library was similarly constructed in pUC19 plasmid vector at Mahidol University and the *E. coli* DH5α transformants derived from the library were screened for the clones containing the repetitive sequences by the same procedure. Eleven bacterial clones, namely, pVM-1 through pVM-11 which gave strong hybridization signals against the $^{32}$P-labelled *P. vivax* genomic DNA but not the human DNA were obtained and further characterized. These clones contained *P. vivax*-specific DNA inserts with the sizes ranging from 0.6 to 2.8 kilobasepairs (kb). Cross hybridization studies revealed that they could be divided into 4 main groups, namely, groups I, II, III and IV which contained the common repetitive DNA fragments with the sizes of approximately 0.6, 1.2, 1.0 and 0.6 kb, respectively. $^{32}$P-labelled purified DNA inserts were used as probes for hybridization against *P. vivax* DNA extracted from blood samples of 67 vivax malaria patients. It was found that the 4 different probes hybridizes to more than 95% of the samples with similar patterns indicating that these DNA sequences were common among the *P. vivax* isolates from different endemic areas in Thailand. The DNA probes did not give any hybridization signal to the relatively high amounts of human genomic DNA or falciparum DNA samples. This study demonstrated that more than one repetitive DNA sequences may be present in *P. vivax*. Limited DNA sequencing data revealed that these *P. vivax*-specific DNA inserts contain high (approximately 70%) AT base content. These DNA inserts have high potentials for development of the
specific and sensitive DNA probe for vivax malaria.

Construction of *P. vivax* genomic DNA library was also carried out using pUN121 plasmid vector at Mahidol University. Purified *P. vivax* genomic DNA was completely digested with the restriction enzyme EcoRI, ligated to the EcoRI site of the plasmid vector and transformed into the *E. coli* JM107 host cells. Four bacterial clones which gave strong hybridization against the 32P-labelled *P. vivax* genomic DNA were obtained. These clones contained *P. vivax*-specific DNA inserts with the sizes ranging from 0.6 to 8.0 kb. These clones are presently being characterized for the DNA sequences and their uses as the probes for diagnosis of vivax malaria are being explored.
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INTRODUCTION

Malaria is still an important public health problem plaquing the economic development of countries of the third world (1). The disease threatens 300 million people and causes 2-4 million deaths a year (2). The prevalence of the disease has created a great loss of public health and socioeconomic of the country. Inspite of a century of extensive attempts to eradicate the disease, the incidence showing little change over the last 20 years (3). The resistance of the anopheline mosquitoes to the insecticides and malaria parasites to the drugs are implicated. The lack of the efficient large scale screening of the carriers is also lead to the inefficient control and eventual elimination of the disease.

Malaria in man is caused by a protozoan of the *Plasmodium* species, namely *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Among these, *P. falciparum* and *P. vivax* are the two most prevalent human malaria parasite species. Although *P. vivax* is second to *P. falciparum* in its world-wide frequency, it is also widespread causing more than 90% of malaria in regions as diverse as the Middle East, Central America and China, and is the major species responsible for relapsing malaria (4). Maintenance of vivax malaria has been attributed in part to the relapsing nature and the presence of asymptomatic cases which serve as the source of infection. Therefore, one of the effective measures in the control of the disease is to establish a technique suitable for a large scale screening of the carriers which would lead to the early treatment and the elimination of the sources of infection.
Currently, specific diagnosis of malaria is routinely made by microscopic examination. Although this method of diagnosis which requires well-trained personnel is satisfactory in terms of sensitivity and specificity, however, it is time-consuming, labor-intensive, difficult to reproduce quantitatively and not suitable for surveillance or for large scale epidemiological studies. Furthermore, it will not be cost-effective in malaria control in an area with very low but persistent transmission, because the cost to diagnose one positive slide will be high. Thus, there is a need to develop alternative methods for detection of the patients especially those with low grade parasitemia to supplement and perhaps eventually to replace microscopy. Recently, techniques utilizing DNA probes have been developed for diagnosis of many infectious agents including viruses, bacteria and protozoa including *Leishmania* species and *P. falciparum* (5-20). Limited field applications of the DNA probes for the diagnosis of falciparum malaria have been reported (21, 22). The DNA probes of *P. falciparum* were cloned from the parasite genomic DNA or synthesized (12, 16, 17, 23). In addition, there was also a report on the use of radio-labelled oligonucleotides derived from ribosomal RNA (rRNA) sequences of *Plasmodium* species including *P. vivax* for detection of very low number of the parasites (24, 25). However, their field applications on the clinical samples has yet to be tested comprehensively.
Overall Objectives

The objectives of the present study were the followings:

1. To construct genomic DNA library of *P. vivax* in appropriate vectors of *Escherichia coli*.

2. To identify and isolate the appropriate vectors containing *P. vivax* DNA inserts for use as probes for detection of *P. vivax* in clinical specimens.

3. To apply the selected DNA probes as a tool for specific diagnosis of *P. vivax* infection in the field.

MATERIALS AND METHODS

**Blood Samples**

1.1 *P. vivax* infected blood

Approximately 5 to 10 ml of blood were obtained from vivax malaria patients who admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University during July 1988 to September 1991. The blood samples were collected in heparin tubes (15 units/ml) before any medical treatment from 820 patients with parasitemia higher than 0.1% during the time period. These blood samples were used for *P. vivax* infected cell enrichment for further DNA extraction as the "purified *P. vivax* DNA" which was suitable for the DNA cloning work as well as for hybridization with the $^{32}$P-labelled DNA probe. The blood samples which were collected and kept at 4°C
for more than 18 hours were not suitable for the enrichment. These blood samples were directly extracted for the "crude P. vivax DNA" which was used for hybridization with the $^{32}$P-labelled DNA probe.

1.2 Normal blood

Approximately 10 ml of blood were obtained in the heparin tube from 6 normal healthy donors for further extraction of human DNA.

2 Enrichment of $P. \text{vivax}$ infected red blood cells

Enrichment of the $P. \text{vivax}$ infected cells was performed according to the method described by Tharavanij et al. (25). Briefly, the blood was collected in a heparinized tube (15 units/ml). One ml aliquots of the blood were passed through several 5 ml syringe columns each of which containing an equal volume of sulfoethyl cellulose (Serva Feinbiochemica, Heidelberg, Germany) and Sephadex G-25 (Pharmacia, Uppsala, Sweden) previously equilibrated with phosphate buffer pH 7.5. The columns were washed with the same buffer until the eluates were free of red blood cells. The eluates were centrifuged at 500 x $g$ for 10 minutes at room temperature. After removal of the supernatants, the cell sediments were pooled and restored to 50% hematocrit in the above buffer and approximately 2 ml aliquots of the cell suspension were layered on a discontinuous Percoll gradients comprising successive layers of 75%, 60%, 50% and 40% Percoll in the above buffer. After centrifugation at 1,450 x $g$
for 15 minutes at 4°C, the upper bands of cells containing enriched P. vivax infected cells were collected, washed by centrifugation at 4,000 x g for 15 minutes at 4°C. The cell pellets were reconstituted in the same buffer. Small aliquots of the cells were counted in a hemocytometer, Giemsa stained and examined by the light microscopy. The final cell suspensions were centrifuged at 10,000 x g for 3 minutes at room temperature and the packed cells were stored at -70°C until use. This procedure consistently yielded more than 50% P. vivax infected cells with less than 1% contamination of the white blood cells.

3 Genomic DNA

3.1 Purified P. vivax DNA

Pooled purified P. vivax DNA was isolated from each batch of at least 30 enriched P. vivax infected blood samples by phenol extraction procedure (27). Briefly, the pack cell samples were digested in a small volume of digestion buffer containing 0.01 M Tris-HCl pH 8.0, 0.01 M NaCl, 0.001 M EDTA, 2% sodium dodecyl sulphate (SDS) and 200 µg/ml proteinase K at 56°C for 3 hours. The samples were then pooled in a polypropylene centrifuge tube. The DNA was separated from the protein by extraction with the water saturated phenol solution, pH 8.0. An equal volume of the phenol solution was added to the samples and the tube was gently mixed by inverting for 5 minutes. After centrifugation at 1,500 x g for 10 minutes at 4°C, the upper aqueous layer containing the DNA was collected. The phenol extraction was repeated two more times then an equal volume of 4%
isoamyl alcohol in chloroform was added. The tube was gently mixed and centrifuged as above. The upper aqueous phase was reextracted with the isoamyl alcohol/chloroform two more times and the aqueous extract was collected. DNase-free RNase A (Sigma Chemical Company, St. Louise, Mo., USA) was added to the extract at a final concentration of 50 µg/ml. The solution was incubated at 50°C for 1 hour before extracted with the phenol and isoamyl alcohol/chloroform solutions as above. The aqueous extract was dialysed against a large volume of 50 mM Tris-HCl, 10 mM EDTA and 10 mM NaCl at room temperature for 2-3 hours. The dialysed DNA solution was added with a stock 3 M NaCl solution to a final concentration of 0.1 M. The *P. vivax* DNA was precipitated by adding 2 volumes of cold absolute ethanol and kept at -20°C for 18 hours. The DNA was precipitated by centrifugation at 10,000 x g for 20 minutes at 4°C. The DNA sediment was washed once with 70% ethanol and briefly dried at room temperature. The DNA pellet was dissolved in a small volume of 10 mM Tris-HCl, 1 mM EDTA buffer pH 8.0 (TE) and stored at 4°C. The total DNA concentration was estimated by comparing the ethidium bromide (EtBr) fluorescence of a small aliquot of the DNA electrophoresed in 0.8% agarose gel against standard DNA (27). Purified *P. vivax* DNA was also isolated from the individual samples, and quantitated by the methods as above.

3.2 **Crude *P. vivax* DNA**

Crude *P. vivax* DNA was prepared from blood of vivax malaria patients which was kept at 4°C for more than 18 hours and was not suitable for the infected cell enrichment. The blood was
washed once in 0.01 M phosphate buffer saline pH 7.4 (PBS) by centrifugation at 500 x g for 10 minutes at room temperature. The blood cells were directly lysed by addition of 5 volumes of PBS containing 0.2% Triton X-100. The lysed samples were centrifuged at 10,000 x g for 20 minutes at 4°C and the supernatants were discarded. The pellets were digested and phenol extracted and the isolated DNA was precipitated with ethanol washed with 70% ethanol and reconstituted in TE as described for the purified P.vivax DNA samples above. The total DNA concentrations was estimated either by EtBr fluorescences as above or by measuring the optical densities at 260 nm. Twenty OD260 unit was equivalent to 1 mg DNA/ml (27). These DNA preparation contained high, undetermined amounts of contaminating human DNA.

3.3 **Human DNA**

Human DNA was isolated from 10 ml of whole blood obtained from normal healthy donors by the method described by Professor David I. Hoar (Department of Medical Biochemistry, Faculty of Medicine, University of Calgary, Canada). Briefly, the heparinized blood was added with 5 volumes of ammonium chloride-Tris solution and incubated at 37°C for 5 minutes. The lysed cells were centrifuged at 1,000 x g for 7 minutes at 4°C and the supernatant poured off. The cell pellet was washed once with 3 volumes of 0.85% NaCl and centrifuged at 1,000 x g for 7 minutes at 4°C. The pellet was collected and resuspended in a small volume of high TE buffer and mixed with an equal volume of
sodium dodecyl sulphate lysis mixture. The lysate was kept at 4°C for 14 hours before extraction for two times with the equal volume of water-saturated phenol solution pH 8.0 then isoamyl alcohol/chloroform as described above. After the last centrifugation, the volume of aqueous layer was measured and 1/10 volume of 4 M ammonium acetate was added. The solution was mixed and added with 2 volumes of cold absolute ethanol. Long strands of high molecular weight DNA were collected with a curved end pasteur pipette. The DNA was washed with 70% ethanol, dried briefly at room temperature and redissolved in a small amount of TE buffer pH 8.0. The total DNA concentration was determined by measuring the optical density at 260 nm as above.

4 Restriction endonuclease digestion

Digestion of genomic DNA or plasmid DNA was done according to the conditions recommended by the manufacturers. For the complete digestion, generally 1 µg of DNA was restricted with 2-5 units of the enzyme in the supplied buffer at the optimum temperature for 1-2 hours. The reaction was usually terminated by heating at 65°C for 10 minutes or adding EDTA to a final concentration of 10 mM.

5 Agarose gel electrophoresis

For routine analysis of intact genomic DNA, plasmid DNA and separation of DNA fragments, horizontal electrophoresis in 0.8% to 2% agarose gel depending on the DNA sizes was commonly used. Weighted DNA grade agarose (Bio-Rad, Richmond, California, USA) was dissolved in Tris-acetate-EDTA (TAE) buffer and heated
to dissolved and cooled to 50°C before pouring into the tape-sealed gel tray. Appropriate gel comb was inserted into the gel and the gel was allowed to set at room temperature for 20-30 minutes. DNA samples were mixed with a one-fifth volume of the gel loading buffer containing 0.25% bromophenol blue and 0.25% xylene cyanol and 30% glycerol before loading into the wells. Electrophoresis was carried out at 7 V/cm in the TAE buffer for 1-2 hours or until bromophenol blue dye front reached 75% across the gel. The gel was removed and stained with 0.5 μg/ml EtBr for 20-30 minutes. Visualization of the DNA bands was done at 300 nm by a UV transluminator (Fotodyne Incorporated, New Berlin, Wisconsin, USA).

6 Recovery of DNA fragments from agarose gel

After separation of the DNA fragments by the agarose gel electrophoresis, gel slices containing the desired DNA fragments were cut out and isolated by the glass milk method (28). Briefly, the gel slices were weighed for an estimation of the gel volumes. Two and a half volumes of 6 M NaI stock solution were added and the tube was heated at 50°C for 5 minutes to dissolve the agarose. Approximately 2-5 μl of the glass milk (Gene Clean™ kit, Bio 101 Inc., La Jolla, California, USA) per μg of the DNA were added to the dissolved agarose solution. The tube was incubated with constant mixing at room temperature for 10-15 minutes. The glass milk/DNA complex was sedimented by centrifugation at 1,500 x g for 10 minutes and the supernatant discarded. The pellet was reconstituted in a small volume of the washing solution and transferred to a microcentrifuge tube, washed
3 times with the washing solution by centrifugation at 10,000 x g for 5 seconds. Following the last washing the DNA was eluted two times in a small volume of TE buffer or water at 50°C for 5 minutes. The eluates were pooled and the DNA content quantitated by the method described above.

7 Transfer of DNA to nylon membrane (Southern blotting)

The DNA fragments separated by the agarose gel electrophoresis were transferred onto a modified charged nylon membrane (Zeta-Probe membrane, Bio-Rad or Hybond N+, Amersham, Aylesbury, Buckinghamshire, England) by the alkali blotting procedure (29). After the electrophoresis and EtBr staining, the gel was ready for the DNA transfer. The DNA transfer setup was as follows: a plastic box was filled with the alkali transfer buffer (0.4 N NaOH). A platform was made by placing a glass plate across the top of the box and covering it with a wick made from three sheets of Whatman 3MM filter paper saturated with the transfer buffer. The gel was placed on the platform and surrounded with a clear plastic wrap to prevent the blotting buffer being absorbed directly into the paper towels above. A sheet of the nylon membrane cut to an exact size of the gel was placed on top of the gel then covered with three sheets of the 3MM filter paper cut to size and wetted with the blotting buffer. Care was taken not to trap any air bubble between each layer. A stack (approximately 5 cm of height) of the absorbent paper towels was placed on top of the 3MM paper. A glass plate was placed on the absorbent paper followed by a 0.5 kg weight on top. The
transfer was allowed to proceed for 18 hours. The setup was carefully dismantled, the membrane was marked with a pencil to allow later identification of tracks, rinsed in 2X SSC, air-dried, baked at 80 °C for 30 minutes in a vacuum oven and kept at 4°C until use.

8 Plasmid DNA

8.1 Plasmid vector

The pUC13 plasmid vector which was previously cut with the restriction enzyme BamHI and treated with bacterial alkaline phosphatase was purchased from Pharmacia Biotechnology International AB, Uppsala, Sweden. The pUC19 plasmid vector was purchased from Life Technologies, Inc., Bethesda, Md. Two and a half micrograms of the pUC19 plasmid vector was cut with 10 units of BamHI (Life Technologies, Inc.) in a volume of 20 μl of 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂ and 100 mM NaCl at 37°C for 1 hour. After the incubation, the cut DNA was purified by the glass milk method as above. The cut vector was further treated with bacterial alkaline phosphatase (Life Technologies, Inc.) in a volume of 100 μl of 0.05 M Tris-HCl pH 8.0 at 65°C for 1 hour. After the incubation, a stock of 0.5 M EDTA pH 8.0 was added to a final concentration of 10 mM. The alkali phosphatase-treated DNA was purified by the glass milk method as above. Agarose gel electrophoresis of the cut DNA revealed a single band of approximately 2.7 Kb with the yield of approximately 40%.
8.2 Recombinant plasmids

Plasmid DNA was prepared from *E. coli* strain DH5α or JM109 harboring the recombinant plasmids by modification of the alkali lysis procedure (27). A single bacterial colony was picked from the LB agar culture and inoculated to 100 ml of LB medium containing 100 μg/ml ampicillin. The bacteria were grown with shaking at 37°C for 18-24 hours. The bacterial cells were harvested by centrifugation at 5,000 x g for 15 minutes at 4°C, resuspended and pooled in 2 ml of lysis solution containing 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA and 1 mg/ml lysozyme. The suspension was incubated at 37°C for 1 hour before 4 ml of 1% SDS in 0.2 N NaOH solution was added and mixed thoroughly by gently inverting the tubes several times. The tube was kept at 26°C for 5 minutes before 3 ml of 5 M potassium acetate solution pH 4.8 was added. The tube was stored in an ice bath for 1 hour before pelleted by centrifugation at 10,000 x g for 15 minutes at 4°C. The supernatant was collected, extracted with an equal volume of phenol solution, phenol/chloroform-isoamyl alcohol and chloroform-isoamyl alcohol and treated with RNase A as above. The RNase A treated DNA solution was re-extracted with phenol/chloroform-isoamyl alcohol solutions before the DNA was precipitated by ethanol. The DNA pellet obtained after centrifugation was resuspended in a small volume of TE buffer and kept at 4°C until use.

Preparation of small amounts of the plasmids was also carried out using the smaller scales of the bacterial cultures and reagents.
Escherichia coli competent cells

Escherichia coli strain JM109 was maintained at the Queensland Institute of Medical Research, Brisbane, Australia. E. coli strain DH5α was obtained from Life Technologies, Inc. They both are ampicillin sensitive and can be host for other pUC and α-complementation vectors (30). The bacteria were grown in LB agar at 37°C for 16-18 hours and kept at 4°C until use. Long term storage of the bacteria was made both in LB agar stab cultures at room temperature or in LB broth with 15% glycerol at -70°C.

The Escherichia coli competent cells were prepared by the method described by Hanahan (30). Briefly, a bacterial colony was picked and inoculated to 5 ml of LB broth and the bacteria were allowed to grow at 37°C for 18 hours. The bacterial culture was then diluted 1:100 in 100 ml of sterile SOB medium and grown with vigorous shaking at 37°C until the optical density (O.D.) was between 0.4-0.8. The bacteria were quickly cooled in an ice bath for 15 minutes, harvested by centrifugation at 1,000 x g for 12 minutes at 4°C. The supernatant was completely discarded and the bacterial cells was reconstituted in 32 ml of RF1 solution (30) and kept in the ice bath for 15 minutes. The bacteria were pelleted by centrifugation and the supernatant was discarded as above. The cells were reconstituted in 7.7 ml of RF2 solution (30) and kept in the ice bath for 15 minutes. These competent cells were distributed in small aliquots (225 μl) in chilled 1.5 ml microcentrifuge tube, flash freeze in liquid N₂ and kept at -70°C until use.
Construction of *P. vivax* genomic DNA library

The genomic DNA library of *P. vivax* was constructed in the pUC13 or pUC19 plasmid vectors previously cut with BamHI and treated with the bacterial alkali phosphatase (pUC/BamHI/BAP). Approximately 500 ng of *P. vivax* DNA were partially cut with 1 unit of Sau3A1 (Life Technologies, Inc.) in 20 mM Tris-HCl pH 7.4, 5 mM MgCl$_2$ and 50 mM KCl at 37°C for 5, 10 and 15 minutes. At the end of the digestion, 0.5 M EDTA pH 8.0 was added to the reaction mixture to a final concentration of 10 mM. The DNA fragments generated were purified by the glass milk technique and the purified DNA in a small volume of TE was analysed by agarose gel electrophoresis and EtBr staining. Approximately 200 ng of the partially Sau3A1 cut *P. vivax* DNA were ligated to an equal amount of pUC/BamHI/BAP in a 20 µl volume by T4 DNA ligase (Life Technologies, Inc.) in 0.05 M Tris-HCl pH 7.6, 10 mM MgCl$_2$, 1mM ATP, 1 mM DTT and 5% (w/v) polyethylene glycol-8000 at 14°C for 18-24 hours. At the end of the incubation period, EDTA was added to a final concentration of 10 mM. The *P. vivax* genomic DNA library was kept at 4°C until use.

Transformation of *E. coli* competent cells

Transformation of the *P. vivax* genomic DNA library to the competent cells of *E. coli* strain JM109 or DH5α was as follows: Frozen aliquots of the competent cells were thawed at 26°C until the cell suspension was just liquid. The tubes were placed on ice and split into a 100 µl aliquot in a 13 x 100 plastic tube. One microliter of the DNA library previously warmed at 50°C for 10
minutes was added to the cell suspension. The cells were mixed gently and kept in an ice bath for 30 minutes before heat shocked at 42°C for 90 seconds. The cells were chilled in the ice bath immediately after the heat treatment then 0.9 ml of warm (37°C) SOC medium was added. The cells were incubated at 37°C with shaking for 60 minutes and 200 μl aliquots were spreaded on LB agar containing 100 μg/ml of ampicillin previously spreaded with 40 μl of 20 mg/ml of 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal). The plates were incubated at 37°C for 18-24 hours and examined for the bacterial colonies.

12 DNA labelling

Purified DNA from *P. vivax*, human, vector and recombinant plasmids was labelled with ^32^P-dATP (Amersham International plc, Buckinghamshire, England) by the random primer labelling (multiprime DNA labelling system; Amersham Internation plc). Briefly, approximately 50 ng of the DNA were mixed in a 50 μl volume of reaction buffer containing Tris-HCl pH 7.8, MgCl₂ and 2-mercaptoethanol, unlabelled dCTP, dGTP and dTTP, random hexanucleotide primers, 2 units of Klenow fragment of DNA polymerase I and 50 μCi of ^32^P-dATP. The reaction mixture was incubated at 37°C for 30 minutes. At the end of the incubation period, EDTA was added to the final concentration of 10 mM and the labelled DNA was separated from the unincorporated ^32^P-dATP by passing the reaction mixture onto Sephadex G-50 previously equilibrated in 1X SSC containing 0.1% SDS in a 1 ml syringe column by centrifugation at 1,500 x g for 4 minutes at 4°C. The first three 100 μl aliquots were collected and 1 μl of each
sample was counted in a liquid scintillation counter. Normally the specific activity of the labelled DNA was between $1-1.5 \times 10^9$ cpm/μg DNA.

Screening for bacterial clones containing *P. vivax* specific DNA inserts

Screening for bacterial clones containing *P. vivax* specific DNA insert was done by *in situ* hybridization (27). Nitrocellulose filters (Micron Separation Inc., Westboro, MA, USA) were placed onto the *E. coli* colonies derived from the *P. vivax* genomic DNA library grown on the LB agar plate containing ampicillin and X-gal. After marking the filter orientation for further identification, the filters were lifted and placed colony side up on the fresh LB agar containing ampicillin and the plates were incubated at $37^\circ C$ for 4-6 hours until bacterial colonies appeared. The bacterial colonies were lysed, DNA denatured and neutralized by placing the filters on Whatman 3 MM papers wetted with 10% SDS for 5 minutes, twice with 0.5 M NaOH in 1.5 M NaCl for 10 minutes and twice with 0.5 M Tris-HCl pH 7.5 in 1.5 M NaCl for 10 minutes, respectively. The filters were briefly rinsed in 2X SSC, air-dried, baked at $80^\circ C$ for 90 minutes in vaccuum oven and kept at $4^\circ C$ until use. On the day of screening, the filters were soaked for 5 minutes in 2X SSC, washed for 30 minutes at $50^\circ C$ in 5X SSC containing 0.5% SDS. The bacterial debris on the filters were scraped out using gloved fingers and the filters were ready for hybridization with the $^{32}$P-labelled probes.
14 DNA hybridization

The nitrocellulose filters or nylon membranes containing the blotted denatured DNA were prehybridized in the prehybridizing solution containing 6XSSC, 5X Denhardt's solution, 0.5% SDS, 1 mM EDTA and 100 μg/ml of sheared, denatured salmon testis DNA with shaking in 90 cm-crystalizing dishes or in the sealed plastic bags. The volume of the prehybridizing solution was kept at 0.1 ml per 1 cm² of the filter. For hybridization, the ³²P-labelled probes denatured by heating at 100°C for 5 minutes and quick cooling were added to the prehybridizing reactions and the hybridization was allowed to proceed for 18 hours at 60°C with shaking. After the incubation period, the hybridizing solution was carefully poured into a ³²P-waste container and the membranes were rinsed in 2X SSC containing 0.1% SDS, washed twice in the same solution at 26°C for 5 minutes, once in 0.5X SSC containing 0.1% SDS at 60°C for 15 minutes. Finally the membranes were mounted onto sheets of paper, sealed in plastic bags and subjected to autoradiography on Kodak X-Omat XK or AR X-ray films with light intensifying screens at -70°C for 0.2-24 hours.

15 DNA dot blotting

Appropriate amounts of DNA samples were denatured by heating at 100°C for 5 minutes and quickly cooled in 0.5 ml volumes of 0.4 M NaOH or TE. Dot blotting of DNA samples was done using a dot blotting apparatus (Bio-Rad, Richmond, VA, USA). A sheet of the nylon membrane was wetted with distilled water
before assembled in the apparatus. Denatured DNA samples were placed into each appropriate well and the vacuum was applied until the wells were just dry. The wells were rinsed once with 0.5 ml of 0.4 M NaOH. The membrane was disassembled, rinsed with 2X SSC, air dried, baked at 80°C for 30 minutes in a vacuum oven and kept at 4°C until use.

16 Deprobing of DNA blots

The hybridized nylon membranes were not allowed to dry to prevent irreversible binding of the DNA probe. They were deprobed as soon as possible after autoradiography by boiling in a large volume of 0.5% SDS at 100°C for 10 minutes, cooled at 26°C and kept at 4°C until reuse.
RESULTS

1 Construction of the first *P. vivax* genomic DNA library

Construction of *P. vivax* genomic DNA library was first done in Australia at the Queensland Institute of Medical Research. Enriched *P. vivax* infected cells from a pool of 4 *vivax* malaria patients were lysed and digested with proteinase K and the DNA was extracted and purified. The purified DNA was partially cleaved with the restriction enzyme Sau3A1 and the DNA fragments generated were ligated to the BamH1 site of the plasmid vector pUC13 (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden). The ligated plasmids were transformed into *Escherichia coli* JM109 host cells and the transformed colonies were selected on culture plates containing 100 µg/ml of ampicillin. The colonies were then transfered to nitrocellulose filters and screened with $^{32}$p-labelled nick-translated *P. vivax*, *P. falciparum* or human DNA. Approximately 1,500 transformant colonies were screened and an *E. coli* clone (VPL101) was found to contain a 3.2 kb specific *P. vivax* DNA insert.

Further characterization of the clone was made in our laboratory at the Faculty of Tropical Medicine, Mahidol University and at the Queensland Institute of Medical Research, Australia. Purified VPL 101 plasmids were cut by the restriction enzyme BamH1 yielding a 3.2 kb *P. vivax* DNA insert as shown by agarose gel electrophoresis and ethidium bromide staining. The BamH1-cleaved DNA insert was purified, cleaved with HpaII and the resulting DNA fragments subcloned into the AccI site of pUC19.
These subclones from VPL 101 were prepared for dideoxy DNA sequencing (31, 32). It was found that this insert contained at least 2 copies of a 205 bp tandem repeat sequence which judged from partial sequencing of VPL 101 and sequence data from its subclones VPL101/5 and VPL101/7. Comparison of VPL 101/5 and VPL 101/7 shows that some base substitutions, insertions and deletions occur in the repeat sequences (33, a copy of the paper is herewith attached below).

VPL101 and its subclone, VPL101/5, containing a 240 bp DNA insert with the *P. vivax* specific DNA repeat sequence were used to hybridize against a number of *P. vivax* DNA extracted from infected blood samples and *P. falciparum* and human DNA in Australia. It has been claimed that the VPL101 and VPL101/5 were highly specific (33).

2 Hybridization of VPL101 and VPL101/5 to *P. vivax* infected blood samples and *P. vivax* DNA at Mahidol University.

Because of the previous successful results with the probes when testing against the *P. vivax* DNA isolated from vivax malaria blood samples in Australia, we hereby attempted to determine whether the probes could hybridize to the local parasite's DNA. VPL101 and VPL101/5 plasmid DNA were prepared by the method described above and the DNA inserts were separated from the vectors by cutting with the restriction enzymes BamHI and BamHI/Hind III, respectively. The digested DNA samples were subjected to agarose gel electrophoresis and the specific bands of 3.2 kb and 240 bp were cut, the gel strips removed and
purified by the glass milk method as described above.

2.1 Hybridization of VPL101 and VPL101/5 to small aliquots of the whole blood of infected patients

Purified VPL101 and VPL101/5 plasmids were labelled with $^{32}$P-dATP by nick translation or the random primer labelling and used to directly detect the parasite's DNA in the 50 ul aliquots of whole blood from the patients. The whole blood samples from 78 patients were either heated in 0.4 M NaOH and 10 mM EDTA at 100°C for 5 minutes or digested with proteinase K solution before the alkaline heat treatment as above then blotted to a nylon membrane by the dot blotting apparatus (Bio-Rad Laboratories). Some of the samples were also extracted with the phenol/chloroform solutions after the enzyme digestion and the alkaline heat treatment. However, all of the samples gave negative results with both VPL101 and VPL101/5. Purified VPL101 and VPL101/5 DNA inserts labelled with the isotope by either nick translation or random primer labelling which gave a higher specificity also gave negative hybridization to all of the samples.

2.2 Hybridization of VPL101 and VPL101/5 DNA inserts to *P. vivax* DNA

Due to the negative hybridization reaction of VPL101 and VPL101/5 to the small aliquots of the *P. vivax* infected blood, it was thought that it might be due to the interference of
the crude undefined blood cell's components or the relatively insensitivity of the DNA probes in the hybridization. Therefore, 10 purified \textit{P. vivax} DNA as well as 57 crude \textit{P. vivax} DNA were prepared from individual blood samples and tested with both probes. The amounts of the individual purified and crude DNA samples blotted onto each well of the nylon membrane by the dot blotting apparatus varied from approximately 10 ng to 200 ng and 40 ng to more than 1,000 ng, respectively. Table 1 summarizes the amounts of the \textit{P. vivax} DNA blotted onto the membrane. Pooled \textit{P. vivax} DNA and human DNA were also included in the blots. The results (Figures 1 and 2) showed that VPL101 DNA insert hybridized to most of the vivax DNA samples and the VPL101 plasmids. However, the DNA insert also gave some reactivity against human DNA. The reactivity signals between the vivax samples and the human DNA were not significantly different. Additional 18 vivax malaria DNA samples and human DNA extracted from whole blood of 5 normal volunteers were also tested, but a similar cross reactivity between the \textit{P. vivax} DNA and human DNA was obtained.

The cross hybridization of the VPL101 insert with the human DNA was also shown by Southern blotting. DNA samples prepared from a vivax malaria patient and a normal volunteer were cut with the restriction enzymes EcoRI, BamHI, PstI and/or Hind III and the DNA fragments were separated by agarose gel electrophoresis. Southern hybridization revealed that the VPL101 DNA insert gave positive bands with both vivax malaria DNA and normal human DNA (Figure 3). VPL101/5 also gave a similar
Table 1  Amounts of the *P. vivax* DNA from vivax malaria patients
don't-blotted onto the nylon membrane.

<table>
<thead>
<tr>
<th>Sample no</th>
<th>DNA blotted (ng)</th>
<th>Sample no</th>
<th>DNA blotted (ng)</th>
<th>Sample no</th>
<th>DNA blotted (ng)</th>
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<td>&gt;1,000</td>
<td>51</td>
<td>&gt;1,000</td>
<td></td>
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</tr>
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</table>

*P. vivax enriched samples.
Figure 1. The autoradiogram of hybridization of VPL101 DNA insert to *P. vivax* DNA isolated from 36 vivax malaria patients. Well numbers A1 to A12, C1 to C12 and E1 to E12 are DNA from patient numbers 1 to 12, 13 to 24 and 25 to 36 denatured by heating at 100°C for 5 minutes before blotting (boiled only), respectively. Wells numbers B1 to 12, D1 to D12, and F1 to F12 are DNA from patients 1 to 12, 13 to 24, and 25 to 36 heated in 0.4 M NaOH at 100°C for 5 minutes before blotting (alkaline treated and boiled), respectively. Well numbers G1 to G6 and G8 to G12 are 5 µg, 1 µg, 0.2 µg, 0.04 µg and 0.008 µg of human DNA boiled only and alkaline treated and boiled, respectively. Well numbers H1 to H10 are 25 ng, 5 ng, 1 ng, 0.2 ng, 0.04 ng, 0.008 ng, 0.0016 ng, 0.32 pg, 0.064 pg and 0.0128 pg of VPL101 DNA alkaline treated and boiled, respectively.
Figure 2. The autoradiogram of hybridization of VPL101 DNA insert to crude *P. vivax* DNA isolated from 31 vivax malaria patients. Well numbers A1 to A12 and C1 to C11 are DNA from patient numbers 37 to 48 and 49 to 59 denatured by heating at 100°C for 5 minutes before blotting (boiled only), respectively. Well numbers B1 to B12 and D1 to D12 are DNA from patient numbers 37 to 48 and 49 to 59 heated in 0.4 M NaOH at 100°C for 5 minutes before blotting (alkaline treated and boiled), respectively. Well numbers E1 to E8 are DNA from vivax malaria patient numbers 60 to 67 alkaline treated and boiled, respectively. Well numbers F10 to F12 are 100, 50 and 80 ng of 3 different batches of pooled *P. vivax* DNA boiled only. Well numbers G8 to G12 are 60, 12, 2.4, 0.48, and 0.08 ng of another pooled purified *P. vivax* DNA boiled only, respectively. Well numbers H1 to H10 are 25 ng, 5 ng, 1 ng, 0.2 ng, 0.04 ng, 0.008 ng, 0.0016 ng, 0.32 pg, 0.064 pg and 0.0128 pg of VPL101 DNA boiled only, respectively.
The autoradiogram of hybridization of VPL101 DNA insert with DNA extracted from blood of a vivax malaria patient and normal human DNA cutting various restriction enzymes. Lanes 1 to 4: vivax DNA cutting with Pst I, Hind III, Bam HI and EcoRI, respectively. Lanes 5 to 7: human DNA cutting with PstI, BamHI, and EcoRI, respectively.
result. Therefore, it was concluded from these experiments that VPL101 and VPL101/5 were not suitable for use as the DNA probes for detection of the malaria parasites in the local blood samples.

3 Screening of the first *P. vivax* genomic DNA library at Mahidol University

The *P. vivax* genomic DNA library constructed in Australia was used to transform *Escherichia coli* DH5α and screened by $^{32}$P-labelled purified *P. vivax* genomic DNA for any new bacterial clones which might contain *P. vivax* specific DNA insert suitable for use as the probe for detection of the parasite. Up to 8 transformation experiments with a total of approximately 24,000 bacterial colonies derived from the library were screened. Although several clones were positive when screened with the $^{32}$P-labelled *P. vivax* genomic DNA probe, subsequent testing revealed either cross-reactive with the $^{32}$P-labelled human genomic DNA or non-reactive with the former *P. vivax* genomic DNA probe used. Therefore, they were not further used in subsequent experiments.

4 Construction of *P. vivax* genomic DNA library using pUC19 plasmid vector at Mahidol University.

Due to the unsuccessful attempts to obtain any bacterial clone containing *P. vivax* specific DNA insert from the first genomic DNA library constructed in Australia, construction of the genomic DNA library was tried at Mahidol University using pUC19 plasmid vector (purchased from Life Technologies, Inc.).
4.1 Preparation of partially digested *P. vivax* genomic DNA and construction of the *P. vivax* genomic DNA library

Approximately 1.5 μg of purified *P. vivax* genomic DNA was partially restricted with 3 units of Sau3A I at 37°C in a 45 μl volume. Aliquots (15 μl) were taken at 10, 15 and 20 minutes and the reaction was stopped by adding 0.5 μl of 0.5 M EDTA pH 8.0. The DNA fragments generated were purified by the glass milk method and each of the DNA aliquot was reconstituted in a 15 ul volume. One microliter of each aliquot was loaded on a 0.7% agarose gel and electrophoresed along with the 1 kb DNA molecular size markers. EtBr staining of the separated DNA fragments revealed that all of the three Sau3A I partially digested *P. vivax* DNA samples appeared similarly as the faint smeared bands with the sizes ranging from approximately 0.5-2 kb (Figure 4). The DNA patterns in the gel were further blotted onto the nylon membrane and probed with $^{32}$P-labelled purified *P. vivax* genomic DNA. The autoradiogram of the Southern blot is shown in Figure 5. It was found that the Sau3A I partially digested genomic DNA showed 6 repetitive DNA bands with the sizes of approximately 1.7, 1.6, 1.3, 1.2, 0.7 and 0.6 kb over the smeared background. The blot was stripped, reprobed with $^{32}$P-labelled human genomic DNA and autoradiographed. The autoradiogram of the blot revealed 3 repetitive bands with the sizes of approximately 3.2, 1.6 and 1.2 kb over the smeared background (Figure 6).

Approximately 200 ng of the Sau3A I partially digested
Figure 4 The EtBr staining patterns of Sau3AI partially digested *P. vivax* genomic DNA after agarose gel electrophoresis. Lane 1, 2 and 3 are the DNA cutting for 10, 15, and 20 minutes, respectively. Lane 4 is the DNA molecular size standards. Numbers at right indicate molecular sizes in kb.
Figure 5 The autoradiogram of hybridization of $^{32}$P-labelled *P. vivax* genomic DNA to the Sau3AI partially digested *P. vivax* genomic DNA after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 4 for samples descriptions.
The autoradiogram of hybridization of $^{32}$P-labelled human genomic DNA to the Sau3AI partially digested *P. vivax* genomic DNA after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 4 for sample descriptions.
P. vivax DNA was ligated at 14°C for 18 hours with an equal amount of the plasmid vector pUC 19 treated with BamHI and BAP in a 20 μl volume as described in the Materials and Methods. One microliter of the library was used to transform 0.2 ml of competent Escherichia coli DH5α and the transformed cells were plated onto LB agars with ampicillin, X-gal and IPTG (see Materials and Methods). The ratio of the blue/white bacterial colonies which grew on the medium was approximately 1:1 indicating that about 50% of the bacteria contained the DNA inserts. Approximately 30,000 colonies were obtained from 10 transformation experiments. The bacterial colonies were lifted from the plates by the nitrocellulose membranes, lysed, their DNA denatured and probed with ³²P-labelled purified P. vivax genomic DNA for the clones harboring the P. vivax specific DNA inserts. More than one hundred colonies which gave relatively strong signals against the P. vivax genomic DNA probes were picked from the master plates and rescreened with either ³²P-labelled P. vivax genomic DNA or human genomic DNA. Only eleven colonies, namely, pVM-1, pVM-2, pVM-3, pVM-4, pVM-5, pVM-6, pVM-7, pVM-8, pVM-9, pVM-10 and pVM-11 which gave the strongest signals with the P. vivax genomic DNA probe but not with the human genomic DNA probe were selected for further testings.

4.2 Cross-hybridization among the P. vivax-specific DNA inserts

The pVM-1 through pVM-11 plasmids were purified, cut with restriction enzymes and the insert sizes determined by agarose gel electrophoresis. It was found that these recombinant
plasmids may or may not contain the BamHI site, therefore, combinations of the enzymes which cut at the left and the right sides of the BamHI site in the multiple cloning region of the pUC19 were tried for cutting out the DNA insert with the minimum portion of the vector. The recombinant plasmids, the restriction enzymes used for cutting the DNA inserts and the sizes of the *P. vivax*-specific DNA inserts are summarized in Table 2. The sizes of the *P. vivax*-specific DNA inserts ranged from approximately 0.6 kb to 2.8 kb. The *P. vivax*-specificities of these DNA inserts were confirmed by Southern blotting. These recombinant plasmids were cut with the appropriate restriction enzymes and approximately 200 ng of the DNA were subjected to agarose gel electrophoresis (Figure 7). They were then blotted onto the nylon membrane and probed with the $^{32}$P-labelled *P. vivax* genomic DNA (Figure 8). It was shown that only the DNA inserts gave reactivities against the probe. $^{32}$P-labelled human genomic DNA did not give any hybridization signal against the DNA blot (data not shown).

The individual DNA insert was then prepared by cutting the recombinant plasmid with the appropriate restriction enzymes, separated from the vector by agarose gel electrophoresis and purified using the glass milk method described above. These DNA inserts were further tested whether any of them contained the common DNA sequences. Purified DNA inserts were labelled with $^{32}$P by the random primer labelling and used to probe the Southern blots similarly prepared as shown in Figure 7. It was shown that cross-hybridization occurred among these DNA inserts. They
Table 2. The pUC19 recombinant plasmids, restriction enzymes used for cutting the DNA inserts and the sizes of the *P. vivax*-specific DNA inserts.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Restriction enzymes used</th>
<th>Sizes of DNA inserts (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVM-1</td>
<td>BamH I/EcoR I</td>
<td>1.7</td>
</tr>
<tr>
<td>pVM-2</td>
<td>BamH I/Pst I</td>
<td>1.2</td>
</tr>
<tr>
<td>pVM-3</td>
<td>BamH I/EcoR I</td>
<td>1.7</td>
</tr>
<tr>
<td>pVM-4</td>
<td>Xba I/Kpn I</td>
<td>1.0</td>
</tr>
<tr>
<td>pVM-5</td>
<td>EcoR I/Pst I</td>
<td>1.4</td>
</tr>
<tr>
<td>pVM-6</td>
<td>BamH I/EcoR I</td>
<td>1.7</td>
</tr>
<tr>
<td>pVM-7</td>
<td>EcoR I/Pst I</td>
<td>1.4</td>
</tr>
<tr>
<td>pVM-8</td>
<td>EcoR I/Pst I</td>
<td>1.2</td>
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<td>pVM-10</td>
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<tr>
<td>pVM-11</td>
<td>Xba I/Kpn I</td>
<td>0.6</td>
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</table>
Figure 7  The EtBr staining of the 11 selected pUC19 recombinant plasmids containing \textit{P. vivax} specific DNA inserts cutting with appropriate restriction enzymes after agarose gel electrophoresis. Lane A is molecular weight size standards. Lanes B, C, D, E, F, G, H, I, J, K and L are pVM-1, pVM-2, pVM-3, pVM-4, pVM-5, pVM-6, pVM-7, pVM-8, pVM-9, pVM-10 and pVM-11, respectively. Lane M is the pUC19 plasmid vector. Numbers at left indicate the molecular sizes in kb.
Figure 8 The autoradiogram of hybridization of $^{32}$P-labelled P. vivax genomic DNA against the 11 pUC19 recombinant plasmids containing P. vivax specific DNA inserts cutting with appropriate restriction enzymes after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 7 for sample descriptions.
could be divided into 4 groups according to the cross-hybridization results. These 4 groups of the plasmids containing the common DNA sequence are summarized in Table 3. pVM-1, pVM-2, pVM-8, and pVM-9 DNA inserts were selected as the representatives for the plasmids groups I, II, III, and IV, respectively, because it was found in our preliminary studies that they were easily cut out from the vector and showed similar reactivities with the other plasmids of the same group when hybridized with the $^{32}$P-labelled *P. vivax* genomic DNA probe. Figures 9 to 12 show the hybridization reactions of $^{32}$P-labelled purified pVM-1, pVM-2, pVM-8, and pVM-9 DNA inserts against the Southern blots containing all of the 11 recombinants plasmids, respectively.

4.3 Characterization of the *P. vivax* specific DNA inserts

4.3.1 pVM-1

The purified 1.7 kb pVM-1 DNA insert was $^{32}$P-labelled by the random primer labelling and used to hybridize against the *P. vivax* DNA samples derived from the 67 vivax malaria patients on the same blots which were previously probed with VPL101 (the autoradiograms are shown in Figures 1 and 2). Unlike the VPL101 probe which gave rather low hybridization signals to both *P. vivax* and human DNA samples, pVM-1 gave relatively strong signals against only *P. vivax* DNA samples, not at all to the human DNA and a minor signal against VPL101 which might be due to the small part of the pUC19 vector at one end of the probes (Figures 13 and 14). At least 63 of 67 *P. vivax* DNA samples were judged positive after 2 hours of autoradiography at
Table 3. The 4 groups of 11 *P. vivax*-specific pUC19 recombinant plasmids containing the common DNA sequences

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasmids</th>
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<tr>
<td>I</td>
<td>pVM-1, pVM-3, pVM-6, pVM-11</td>
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<tr>
<td>II</td>
<td>pVM-2, pVM-4, pVM-8</td>
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<tr>
<td>III</td>
<td>pVM-5, pVM-7</td>
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<tr>
<td>IV</td>
<td>pVM-9, pVM-10</td>
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Figure 9  The autoradiogram of hybridization of $^{32}$P-labelled purified pVM-1 DNA insert against the 11 pUC19 recombinant plasmids containing *P. vivax* specific DNA inserts cutting with appropriate restriction enzymes after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 7 for sample descriptions.

A B C D E F G H I J K L M

4.0—
3.0—
2.0—
1.6—
1.0—
0.5—
Figure 10  The autoradiogram of hybridization of $^{32}$P-labelled purified pVM-2 DNA insert against the 11 pUC19 recombinant plasmids containing *P. vivax* specific DNA inserts cutting with appropriate restriction enzymes after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 7 for sample descriptions.
Figure 11 The autoradiogram of hybridization of $^{32}p$-labelled purified pVM-8 DNA insert against the 11 pUC19 recombinant plasmids containing *P. vivax* specific DNA inserts cutting with appropriate restriction enzymes after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 7 for sample descriptions.
Figure 12 The autoradiogram of hybridization of $^{32}$P-labelled purified pVM-9 DNA insert against the 11 pUC19 recombinant plasmids containing $P.\text{ vivax}$ specific DNA inserts cutting with appropriate restriction enzymes after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 7 for sample descriptions.
Figure 13 The autoradiogram of hybridization of $^{32}$P-labelled pVM-1 DNA insert to *P. vivax* DNA from 36 *vivax* malaria patients. Please see legend to Figure 1 for sample descriptions.
Figure 14 The autoradiogram of hybridization of $^{32}$P-labelled pVM-1 DNA insert to crude *P. vivax* DNA from 31 *vivax* malaria patients. Please see legend to Figure 2 for sample descriptions.

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44
-70°C. Extended autoradiography revealed that all of the samples were judged positive without any increase in the signal against the human DNA. Additional 18 *P. vivax* DNA, 6 human DNA and 1 *P. falciparum* DNA samples were also tested. It was found that all 18 *vivax* samples were positive whereas the human and falciparum samples were negative (data not shown). Therefore, pVM-1 gave positive results with at least 81 of 85 (95%) of the *P. vivax* DNA samples and was highly specific. Although the sensitivity of the pVM-1 in the detection of the parasites has not been evaluated, it can detect as low as 0.08 ng of the purified DNA (Figure 14).

Hybridization of $^{32}$P-labelled pVM-1 against the Southern blot containing the Sau3AI partially cut *P. vivax* genomic DNA library (which was previously probed with the $^{32}$P-labelled *P. vivax* genomic DNA and human DNA as shown in Figures 5 and 6) revealed 3 strong bands with the sizes of approximately 1.7, 1.2 and 0.6 kb (Figure 15). An attempt was made to determine the minimum size of the DNA fragment which gave the highest reactivities. When the 1.7 kb purified pVM-1 DNA insert was completely digested with Sau3AI, it gave at least 4 bands with the sizes of approximately 0.7, 0.6, 0.2 and 0.1 kb as shown by the agarose gel electrophoresis (data not shown). Probing of the DNA fragments by the $^{32}$P-labelled *P. vivax* genomic DNA revealed that only the 0.6 kb fragment gave strong hybridization signal against the probe. Therefore, the 0.6 kb fragment was purified, ligated into the BamHI site of the pUC19 plasmid vector and transformed into the host *E. coli* DH5α. The subclone, pVM-1S3, contained the 0.6 kb DNA insert was isolated and found to
Figure 15  The autoradiogram of hybridization of $^{32}$P-labelled pVM-1 DNA insert against the Sau3AI partially digested P. vivax genomic DNA after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 4 for sample descriptions.
hybridize to all of the group I plasmids, particularly the pVM-11 which also contained approximately 0.6 kb DNA insert. pVM-1S3 was also found to hybridize with the similar pattern and sensitivity to the 67 *P. vivax* DNA samples from the vivax malaria patients. Both pVM-1S3 and pVM11 are presently determined for their DNA sequences. Partial DNA sequence data revealed that they contained high AT base content with almost identical sequences.

4.3.2 pVM-2

The purified 1.2 kb pVM-2 DNA insert was \(^{32}\)P-labelled by the random primer labelling and used to hybridize to the *P. vivax* DNA samples derived from the 67 vivax malaria patients and the Southern blot containing the Sau3AI partially cut *P. vivax* genomic DNA. It was found that the hybridization patterns of the labelled pVM-2 against the 67 *P. vivax* DNA samples from the vivax malaria patients were almost identical to those of pVM-1. Hybridization of the pVM-2 probe against the Sau3AI partially cut *P. vivax* genomic DNA revealed at least 3 bands with the sizes of approximately 1.2, 1.6 and 1.7 kb (Figure 16). Presently, pVM-2 which contained no further Sau3AI cleavage site is being analysed for its DNA sequence. Partial DNA sequencing data revealed that most of the sequences were different from those of pVM-1.

4.3.3 pVM-8

The purified 1.2 kb pVM-8 DNA insert was \(^{32}\)P-
Figure 16 The autoradiogram of hybridization of $^{32}$P-labelled pVM-2 DNA insert against the Sau3AI partially digested P. vivax genomic DNA after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 4 for sample descriptions.
labelled by the random primer labelling and used to hybridize to the *P. vivax* DNA samples derived from the 67 vivax malaria patients and the Southern blot containing the Sau3AI partially cut *P. vivax* genomic DNA. It was found that the hybridization patterns of the labelled pVM-8 against the 67 *P. vivax* DNA samples from vivax malaria patients were also almost identical to those of pVM-1. Hybridization of the pVM-8 probe against the Sau3AI partially cut *P. vivax* genomic DNA revealed at least 3 bands with the sizes of approximately 1.0, 1.2 and 1.7 kb (Figure 17). Complete Sau3AI digestion revealed that pVM-8 was cut into a 1.0 and 0.2 DNA fragments. Presently, pVM-8 and pVM-4 which contained the 1.0 kb DNA fragment are being analysed for its DNA sequence. Partial DNA sequencing data revealed that they were almost identical and had certain oligonucleotide sequences common to pVM-1S3.

4.3.4 pVM-9

The purified 1.7 kb pVM-9 DNA insert was $^{32}$P-labelled by the random primer labelling and used to hybridize to the 67 *P. vivax* DNA samples derived from the vivax malaria patients and the Southern blot containing the Sau3AI partially cut *P. vivax* genomic DNA. It was found that the hybridization patterns of the labelled pVM-9 against the 67 vivax malaria patient were almost identical to those of pVM-1. Hybridization of the pVM-9 probe against the Sau3AI partially cut *P. vivax* genomic DNA revealed at least 5 bands with the sizes of approximately 0.6, 1.0, 1.2, 1.7 and 1.9 kb (Figure 18). Probing of these DNA fragments with $^{32}$P-labelled *P. vivax* genomic
Figure 17  The autoradiogram of hybridization of $^{32}$P-labelled pVM-8 DNA insert against the Sau3AI partially digested *P. vivax* genomic DNA after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 5 for sample descriptions.
Figure 18  The autoradiogram of hybridization of $^{32}$P-labelled pVM-9 DNA insert against the Sau3AI partially digested \textit{P. vivax} genomic DNA after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 5 for sample descriptions.
DNA revealed that only the 0.6 kb DNA fragment gave strong hybridization signal. Presently, the 0.6 kb fragment of pVM-9 was isolated, subcloned into the pUC19 plasmid and DNA sequenced.

5 Construction of *P. vivax* genomic DNA library using pUN121 plasmid vector at Mahidol University

Construction of *P. vivax* genomic DNA library was also carried out using pUN121 plasmid vectors (kindly provided by Dr. Sakol Panyim, Department of Biochemistry, Faculty of Science, Mahidol University). Approximately 500 ng of pooled purified *P. vivax* genomic DNA was completely digested with EcoRI and the DNA fragments generated were purified before ligating to the EcoRI site of pUN121. The ligated recombinant plasmids were transformed into the *E. coli* JM107 host cells as described in the Materials and Methods section. Approximately 50 ng of the EcoRI digested genomic DNA was ligated to the equal amount of pUN121 vector previously treated with EcoRI and bacterial alkaline phosphatase. The recombinant plasmids were used to transform the *E. coli* host cells and only the transformants harboring the DNA inserts were selected on LB agar containing the antibiotic tetracycline. Approximately few hundreds of the transformants were selected and screened for any clones containing the *P. vivax* specific DNA inserts by hybridizing with $^{32}$P-labelled *P. vivax* genomic DNA by the procedures described above. Four clones, namely, pVM-101, pVM-102, pVM-103 and pVM-104, which gave relatively strong hybridizing signals with only the *P. vivax* genomic DNA probe but not human genomic DNA probe were selected.
and used for further studies. The recombinant plasmids were purified and the insert sizes determined by agarose gel electrophoresis. It was found that the plasmids and the approximate sizes of the DNA inserts are as the followings: pVM-101, 2.2 and 7 kb; pVM-102, 0.6, 1.3, 2.2 and 6.0 kb; pVM-103, 20 kb; and pVM-104, 8.0 kb (Figure 19). Southern blotting and hybridization of these DNA fragments with $^{32}$P-labelled P. vivax genomic DNA revealed that the probe gave relatively high signals against all of these DNA bands (Figure 20). Currently, these DNA inserts are being studied for their sequences and the possibility for uses as the diagnostic probe for vivax malaria.
The EtBr staining of the 4 selected pUN121 recombinant plasmids containing *P. vivax* specific DNA insert cutting with EcoRI after agarose gel electrophoresis. Lane 1 is molecular weight size standards. Lanes 2, 3, 4, 5 and 6 are pVM-101; Lanes 7, 8, 9, 10 and 11 are pVM-102; Lanes 12, 13, 14, 15 and 16 are pVM-103; and lanes 17, 18, 19, 20 and 21 are pVM-104 at the amounts of 400, 100, 25, 6 and 1.5 ng, respectively. Lane 22 is the pUN121 plasmid vector. Numbers at left indicate the molecular sizes in kb.
Figure 20  The autoradiogram of hybridization of $^{32}$P-labelled *P. vivax* genomic DNA against the 4 selected pUN121 recombinant plasmids containing *P. vivax* specific DNA inserts after agarose gel electrophoresis and Southern blotting. Please see legend to Figure 19 for sample descriptions.
6. Work collaboration with the Kuvin Centre for the Study of Infectious and Tropical Diseases, Hebrew University, Israel.

The work carried out at the Kuvin Centre for the Study of Infectious and Tropical Diseases, Hebrew University, Israel is summarized from the three progress reports attached below since the final report is not available.

The work was initiated during the visit of the principal investigator to the Kuvin Centre for the Study of Infectious and Tropical Diseases in February 1989. A pool of *P. vivax* infected erythrocyte samples obtained from vivax malaria patients in Thailand were used for extraction of genomic DNA which showed minimal contamination of the human DNA. The genomic DNA was cut with 10 different restriction enzymes, namely, Eco RI, Bam HI, Hae III, Xho I, Pst I, Alu I, Kpn I, Bgl II, Hind III and Taq I. The DNA fragments generated were analysed by agarose gel electrophoresis in order to examine whether any repetitive DNA sequence was present in the parasite's genome. However, no discrete band of the repetitive DNA sequence was detected either by ethidium bromide staining or Southern blotting and hybridization using $^{32}$P-labelled *P. vivax* genomic DNA as the probe. Therefore, it was felt that the simple strategy of using the existing library of *P. vivax* or developing similar libraries (using EMBL-3 instead of pUC13) will not work. Therefore, another strategy was tried. The telomeric region from various species of *Plasmodium* could be hybridized with the telometric DNA fragment that has been cloned from *P. berghei* (34, 35). This fragment consists of a sequence of 4 base pairs which are
repeated about 70 times in *P. berghei* (36).

However, the subtelomeric region of *P. falciparum* was found to consist of four different tandem repeats of various sizes (37). These tandem repeat sequences are conserved within *P. falciparum* while there was no hybridization to DNA from other *Plasmodium* species. Therefore, the repeat sequences in the subtelomeric region of *P. vivax* might be useful as a specific *vivax* DNA probe.

In order to clone telomeric sequences, DNA was prepared from 20 different blood samples. These samples were tested for the presence of human DNA by dot blot hybridization, using human DNA as a probe. Samples that contained least amounts of human DNA were pooled and used for hybridization with *P. berghei* telomeric probe (kindly provided by Dr. M. Ponzi from Italy).

The results showed that the telomeric probe hybridized specifically with *P. vivax*. The lowest amount of DNA that was used for hybridization was 10 ng. No hybridization was found using human DNA, even using as much as 200 ng DNA. The DNA was digested with Bal 31, ligated to the pUC8 plasmid and transformed into *Escherichia coli* host cells (37). The DNA library was screen by hybridization using the telomeric probe from *P. berghei*. A few bacterial colonies were positive. However, subsequent isolation of the clones showed that they were not *P. vivax*-specific.
Further studies on the work done at the Kuvin Centre for the Study of Infectious and Tropical Diseases were not available due to the poor communication during the Middle East War. Professor Dan T. Spira informed us later that there was no additional result of the project during his visit to Bangkok in late August 1992.
CONCLUSION

Successful work on DNA cloning of *P. vivax* is presently very limited. To the best of our knowledge, only three antigen genes, one Duffy receptor, one small subunit ribosomal RNA gene and one DNA probe of the malaria parasite were studied in details (24, 33, 38, 39, 40, 41, 42). The main reason for the slow progress is probably because the parasite can not be cultivated *in vitro*. Therefore, the amounts of the parasite materials which were derived from the infected man or animals were frequently insufficient and usually contaminated with the host's cells which complicated the purification of the DNA and the cloning work. The main objective of the present study was to construct the *P. vivax* genomic DNA library with the ultimate aim at isolating the clone(s) containing the parasite's repetitive DNA sequence for use as the specific and sensitive diagnostic DNA probe suitable for large scale screenings of blood samples from the suspected malaria patients. This study took the advantage of the recent successful work by Tharavanij *et al.* (26) who were able to purify and enrich *P. vivax* infected cells from blood of vivax malaria patients with relatively free from the human leucocytes. Purified DNA prepared from such enriched samples was partially restricted with the restriction enzyme Sau3A1 separated by agarose gel electrophoresis, Southern blotted and probed with the $^{32}$P-labelled *P. vivax* genomic DNA to look for any repetitive DNA bands. This study was the first to reveal at least 6 repetitive DNA fragments with 1.7, 1.6, 1.3, 1.2, 0.7 and 0.6 kb in sizes (Figure 5). However, two of these bands, the
1.6 kb and the 1.2 kb were probably human-specific since these bands were also found after reprobing of the membrane with the $^{32}$P-labelled human genomic DNA (Figure 6).

Construction of the *P. vivax* genomic DNA library was done by ligation of the purified DNA fragments at the BamHI site of the pUC19 plasmid vector, a similar strategy which was successfully used in the cloning of *P. falciparum* repetitive DNA sequences (14, 15, 19, 43) and a *P. vivax* DNA probe (33). By screening of the genomic DNA library with the $^{32}$P-labelled *P. vivax* genomic DNA in comparison of the results with those of the $^{32}$P-labelled human genomic DNA, a total of 11 *P. vivax* specific clones which gave the relatively high hybridization signals were identified and isolated. The sizes of the *P. vivax*-specific DNA inserts ranged from approximately 0.6 kb to 2.8 kb. Cross hybridization studies revealed that they could be divided into 4 main groups in which some of them, i.e., pVM1, pVM3 and pVM6 or pVM-5 and pVM-7 may derived from a single original clone since they contained a similar sizes of the inserts with the same restriction enzyme digestion patterns. The findings of differences in the hybridization reaction which reflected the differences in DNA sequences among these clones groups were very interesting and suggested that more that one repetitive DNA sequences may be present in *P. vivax* genome, a phenomenon which has already been found in *P. falciparum* (22). Further complete Sau3AI digestion revealed that the groups 1, 2, 3 and 4 clones contained the common *P. vivax*-specific DNA fragments of 0.6, 1.2, 1.0 and 0.5 kb, respectively. Currently these DNA fragments are
being analysed for their sequences and limited data showed that they contained high (approximately 70%) AT base contents.

Purified DNA inserts from the representatives of the 4 groups of plasmids, namely, pVM-1, pVM-2, pVM-8 and pVM-9 were selected for used further as the probe for hybridization against DNA extracted from the vivax malaria patients. The findings that all of the $^{32}$P-labelled DNA inserts hybridized with the similar patterns to all of the DNA samples extracted from vivax malaria patients without any cross hybridization against relatively high amounts of human genomic DNA or *P. falciparum* DNA were very exciting and confirmed that the probe was indeed *P. vivax* specific and contained a common repetitive DNA sequence among the parasite's isolates from several endemic areas of Thailand. Comparative studies between the $^{32}$P-labelled DNA inserts and another *P. vivax* DNA probe, i.e., VPL101 and its subclone VPL101/5 (33), revealed that pVM-1 gave much higher signals and percent of positivity against these DNA samples than both VPL101 and VPL101/5 probe.

Although the lowest limit of the sensitivities of the probes in the detection of the *P. vivax* DNA has never been accurately determined in the present study because purified *P. vivax* was not available since significant amount of the human DNA was consistently found even in highly enriched *P. vivax* DNA samples, nevertheless, the probes could detect as low as 80 pg of a pooled purified *P. vivax* DNA sample (e.g., pVM-1 in Figure 14). This amount of the DNA detected by the probe was comparable to several DNA probes reported for *P. falciparum*, e.g., 30 pg of DNA.
by PFR1-AP (12), 12 pg of DNA by pPF14 (14), 25 pg of DNA by clone 26 (19), 100 pg of DNA by pUNK1-45 (20), etc. Considering that one ring stage parasite nucleus contained approximately 0.02 pg of DNA (44, 45) and that the sample contained no human DNA contamination, then the smallest number of the parasites which could be detectable by the probe was approximately 4,000 parasites. Assuming that a red blood cell count of $5 \times 10^6/\mu l$, this probe would be able to detect 80 parasites/ul in a single 50 ul volume of blood or a corresponding parasitemia of 0.0016%. Although this sensitivity was slightly lower than the limit of those detected by microscopy which generally detected 20-40 parasites/ul of blood (22), detection of the parasites by the DNA probe technique probably had more advantage than the microscopic technique in that it could be automated, thereby reducing the time and labor in processing a large number of the samples.

Beside their uses for diagnosis, the DNA probes based on the repetitive DNA sequences may also be used for detection of differences within the malaria species using the method of Southern blot hybridization and chromosome analysis (22, 46). The findings that the probes gave multiple banding patterns after hybridization against the Sau3AI restricted $P. \text{vivax}$ DNA samples suggested that it may be capable of differentiating different $P. \text{vivax}$ isolates provided that appropriate restriction enzymes were used. The ability to differentiate among strains of the parasites would be very useful for epidemiological study as well as discriminating the organisms causing relapses which occurred very frequently in vivax malaria.
The current requirement to use radioactively labelled DNA is another major obstacle in the field application of the probe. Since it was found in the present study that these DNA inserts had high potentials to be developed as specific and sensitive probes for vivax malaria, the current non-isotopic labelling systems which have been claimed to be as sensitive, e.g., chemiluminescent techniques (47, 48) should be further investigated for substitution of the isotopic technique.

In conclusion, more studies on the DNA sequences of these probes are needed in order to discover the repetitive sequences of the malaria parasites. Further applications, e.g., combinations of the oligonucleotides probes or the use of polymerase chain reaction using the primers based on the repetitive DNA sequences may provide a highly sensitive and specific tool for the diagnosis of vivax malaria.
REFERENCES


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Diagnosis of *Plasmodium vivax* malaria using a specific deoxyribonucleic acid probe

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Abstract

A deoxyribonucleic acid (DNA) probe which specifically distinguishes *Plasmodium vivax* from *P. falciparum* malaria has been derived from a *P. vivax* genomic DNA library. This probe, VPL101, consists of 3-2 kilobase pairs and does not hybridize with up to 6 µg of human or *P. falciparum* DNA. VPL101 contains at least two copies of a 70S base pair repeat sequence. The subcloned repeat probe, VPL101/S, reacted with 73 of 76 microscopically diagnosed *P. vivax* samples but not with any of 17 human DNA samples or any of 8 *P. falciparum* DNA samples from cultured parasites. It was possible to detect *P. vivax* in mixed infections in which only *P. falciparum* parasites were identifiable by microscopy. This *P. vivax* DNA probe provides a useful epidemiological tool for malaria control programmes.

Introduction

Control of malaria represents a major challenge, as one-third of the world's population inhabits endemic areas, with more than 100 million cases per year (Bruce-Chwatt, 1987). The 2 most prevalent human malaria parasite species are *Plasmodium falciparum* and *P. vivax*. With its higher worldwide frequency, its greater fatality rate and the appearance of drug-resistant strains, *P. falciparum* has attracted the most attention. The ability to culture *P. falciparum in vitro* has enabled more detailed studies and hence greater advances at the molecular level. But *P. vivax* is also widespread, causing more than 90% of malaria in regions as diverse as the Middle East, Central America and China, and is the main species responsible for relapsing malaria (Wernsdorfer, 1980).

Microscopists can generally detect 20-40 malaria parasites per µl of blood (WHO, 1986) but in thick films, typically, 10µl may be detectable. Control programmes require specific, sensitive, and cheap diagnostic methods to supplement and perhaps eventually to replace microscopy. This applies particularly in endemic areas where low parasitaemias and mixed infections may result in substantial underestimates of the malaria prevalence (Boyd & Kitchen, 1937; Bruce-Chwatt, 1980; Cattani et al., 1986). Deoxyribonucleic acid (DNA) probes have been developed for *P. falciparum* which can detect 40 parasites/µl under optimal conditions (Franzen et al., 1984; Pollack et al., 1985; Banker et al., 1986; Guntaka et al., 1986). However, in some corresponding field studies (Holmberg et al., 1987; McLaughlin et al., 1987a) fewer than 500-800 parasites/µl could not be reliably detected. DNA probes for *P. vivax* that could detect low parasitaemias would be a great asset for epidemiological and clinical investigations. Recent reports (Lal et al., 1989; Waters & McCutchan, 1989) demonstrated that short oligonucleotides, labeled with 32P, and derived from unique regions of *Plasmodium spp.* ribosomal RNA gene sequences can detect very low numbers of parasites and are species-specific. These have yet to be tested comprehensively with clinical samples, and hence shown to be suitable diagnostic tools for field analysis in situ. We report the development of a specific and sensitive DNA probe which can routinely distinguish *P. vivax* from *P. falciparum* in clinical specimens obtained from all of the world's major endemic regions. Furthermore, its size (3-2 kilobases [kb]) renders it useful for both radioactive and non-radioactive labelling techniques, an advantage which may well be exploited in field applications.

Materials and Methods

Preparation of probe

A *P. vivax* genomic DNA library was constructed using partially purified parasitized erythrocytes (Tharavanij et al., 1987) from a pool of 4 Thai blood samples. Following saponin lysis and digestion with proteinase K (Boehringer Mannheim), DNA was extracted (Maniatis et al., 1982) and purified (Hatori & Sakaki, 1986). Partially Sal3A-cleaved (Promega) DNA was ligated into the BssH1 site of pUC13 (Upcroft & Healey, 1987) then transformed into *Escherichia coli* JM109 host cells (Hanahan, 1983). Transformed colonies were transferred to nitrocellulose filters (Hanahan & Meselson, 1980) and screened with 32P-labelled nick-translated human *P. falciparum* or *P. vivax* DNA.

Hybridization of *P. vivax* clone to parasite DNA

Clone VPL101 reacting with *P. vivax* DNA and containing a 3-2 kb insert was hybridized to parasite DNA as follows. The test DNA samples were sheared using a 26-gauge needle, and heated in 0-5 ml 0-4 N sodium hydroxide (NaOH), 10 mm ethylene-diaminetetraacetic acid (EDTA) at 100°C for 10 min and applied to Zeta-Probe® membrane (Bio-Rad) under vacuum (Reed, 1987). The membrane was rinsed in 2×SSC (standard saline citrate) and stored at -20°C until required. VPL101 DNA insert was labelled with 32P by nick-translation and denatured with NaOH (Meinkoth & Wahl, 1984). The membrane was hybridized in 2×SSPE (0-18 M sodium
chlordane, 0.01 M sodium dihydrogen orthophosphate, 0.01 M EDTA), 1% sodium dodecyl sulphate (SDS), 0.05% non-fat milk powder, 0.3 mg/ml salmon sperm DNA and 10% dextran sulphate at 65°C. Washes were performed at 25°C in 2X SSC + 0.1% SDS for 30 min followed by 0.1X SSC + 0.1% SDS for 15 min at 65°C. Filters were exposed for one hour and overnight on Kodak XAR-5 film.

Subcloning of VPL101 insert
BamH1-cleaved VPL101 insert was purified (BURNS & BEECHAM, 1983), cleaved with HpaII, and the resulting DNA fragments were subcloned into the Accl site of pUC19. Subclones from VPL101 were prepared for dideoxy sequencing (SANGER et al., 1977; SANGER, 1981). The VPL101/5 clone was selected for hybridization studies using clinical samples.

Samples from patients
Malaria specimens were obtained with the cooperation of private and hospital pathology laboratories in Brisbane, Australia. Most specimens were collected from patients who had returned to Australia following a visit to malaria-endemic areas in the south Pacific, south-east Asia, south Asia, the Middle East and Africa, and from a small number of persons living in endemic areas and visiting Australia. One to 5 ml of blood were collected into EDTA, and overnight on Kodak XAR-5 film.

Preparation of the probe
The 32P-labelled VPL101 insert (100 ng DNA) was found to react with P. vivax DNA but not with up to 6 µg of pure human DNA or 6 µg of pure P. falciparum DNA obtained from cultured parasites (Fig. 1A). The insert of VPL101, although part of a Sau3A library, could be cleaved with BamHI. The excised insert was further cleaved with HpaII and subcloned. The sequences of the insert were determined from subclones VPL101/5 and VPL1/7 in Fig. 2. The 240 base pair (bp) sequence comprising VPL101/5 contains 205 bp of a repeat which occurs at least 3 times in the P. vivax genome, as judged from restriction endonuclease cleavage, hybridization and partial sequencing of VPL101 DNA insert together with sequence data from its subclones. Comparison of VPL101/5 and VPL1/7 shows that some base substitutions, insertions and deletions occur in the repeat sequences (Fig. 2).

Results
DNA of each sample was determined (MANIATIS et al., 1982), assuming one white cell nucleus to contain 6 pg DNA and one parasite ring nucleus to contain 0.02 pg DNA (WHITEFIELD, 1953; GOMAN et al., 1982). This calculation did not consider the proportions of the parasite stages present. Therefore total DNA refers to both white blood cell and parasite DNA. VPL101/5 DNA insert was radio-labelled and hybridized to DNA from the clinical specimens as described above.

Southern transfer and hybridization
5 µg total DNA were digested with HinfI, subjected to agarose gel electrophoresis and then transferred to Zeta probe nylon membrane (REED, 1987). VPL101/5 DNA insert was nick-translated as previously described and hybridized overnight.

Fig. 1. A. Specificity of clone VPL101 DNA for P. vivax by dot blot hybridization. Lane 1: dilutions of clone VPL101 DNA containing 40 ng, 8 ng, 4 ng, 500 pg, 50 pg, 5 pg and 0.5 pg DNA. Lane 2: dilutions of pools P. vivax genomic DNA isolated from white blood cell depleted samples (THARAVANTHI et al., 1987), the precise quantity of P. vivax DNA was indeterminable. Lane 3: human DNA, 6 µg and 1 µg. Lane 4: P. falciparum laboratory isolate HB3. Column 3: human DNA column 4: Patients P1, 1.1% parasitaemia. Column 5: P1, 0.025% parasitaemia. Column 6: Pn, 0.001% parasitaemia. Column 7: Pf, 0.14% parasitaemia. Column 8: Pf, 0.4% parasitaemia. Autoradiography was for 24 h.

B. Dot blot hybridization of VPL101/5 DNA insert labelled with 32P with samples from selected patients infected with P. vivax (Pv) or P. falciparum (Pf). Row a, 2 µg of total DNA; row b, 1.4 µg total DNA (except for column 1, in which row a represents 0.5 µg clone VPL101 DNA and row b is blank). Column 3: P. falciparum laboratory isolate HB3. Column 3: human DNA column 4: Patients P1, 1.1% parasitaemia. Column 5: P1, 0.025% parasitaemia. Column 6: Pn, 0.001% parasitaemia. Column 7: Pf, 0.14% parasitaemia. Column 8: Pf, 0.4% parasitaemia. Autoradiography was for 24 h.
Fig. 2. Nucleotide sequences of VPL101/5 and VPL101/7 DNA inserts. Row A: VPL101/5 DNA insert, and row B: VPL101/7 DNA insert. Vertical lines indicate matching bases; the gaps, nucleotide deletions, N= undetermined base; internal HpaII sites are underlined. The length of the repeat unit is 201 bp beginning at the first nucleotide as row A and continuing to the second arrow. In row B, the deletion of one base at position 3 creates an HpaII site.

Table. Reactivity of VPL101/5 DNA insert

<table>
<thead>
<tr>
<th>DNA source P. falciparum laboratory strains</th>
<th>Humana</th>
<th>Indeterminablec species</th>
</tr>
</thead>
<tbody>
<tr>
<td>76 patients</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>73 of samples</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

6 µg DNA samples from established laboratory P. falciparum isolates FCQ-27/PNG, FC1, V1, 7G8, FC64, NF7, K1, and HB3 were tested.

b9 µg of human white blood cell-derived DNA was tested.

cThese patients were infected with species which could not be identified by microscopy based on parasite morphology as judged by several independent microscopists.

clinical probe because it contained a single copy of the species-specific repeat sequence which may be highly conserved in the genomes of different geographical isolates.

DNA hybridization to patient samples

3P-labelled subclone VPL101/5 DNA insert did not recognize human DNA in dot-blot hybridization (Fig. 1B) but did react with 96% of patients' samples (Table) diagnosed as P. vivax by microscopy. Parasitaemias giving positive blots ranged from 0.0002% to 1% in 10-200 µl of blood. Patients came from Papua New Guinea, Solomon Islands, Vanuatu, Thailand, Malaysia, Indonesia, India, Sri Lanka, Pakistan, Afghanistan, Iran, Turkey and Zaire, so it can be concluded that a wide range of parasite strains was recognized by the probe. The 3 probe-negative P. vivax cases had parasitaemias which were theoretically in the detectable range (0.75%, 0.002%, 0.0005%). Perhaps the diagnosis by morphology was incorrect, but a technical basis for the non-reaction of these samples cannot be excluded (FRANZ et al., 1984; BARKER et al., 1986; MU et al., 1986; FLUSSING et al., 1987). P. vivax has long been thought to
Four of 10 cases in which the parasite species was indeterminable by microscopy were positive with the probe, thereby illustrating the uncertainties inherent in parasite identification by morphology alone (MAEG-RATH, 1948; PATTERSON et al., 1987). Possible cross-reactions with P. malariae infections were not available for follow-up, to mixed infections populations. Drug treatment of P. vivax by thick films (i.e., morphological examination) to provide important tools for epidemiological analysis. Both findings are consistent with the development of mixed infections in which the presence of different proportions of parasite developmental stages in each case, or to variation in the number of repeat sequences in different P. vivax isolates.

Four patients diagnosed as having P. falciparum malaria showed a positive result with VPL1015 DNA insert (Table). One patient (Pf 22) subsequently developed a P. vivax infection, confirmed by microscopy, 2 months after treatment with quinine and Fansidar® for the P. falciparum attack. A second patient (Pf 20) had recurrences of clinical malaria after apparently successful treatment for P. falciparum with quinine. Neither patient had returned to a malaria-endemic area. Both findings are consistent with initial mixed infections in which P. vivax parasites were undetectable by microscopy using thin and thick films (i.e., <10 parasites/μl) but were detectable by the DNA probe. Thus, other two P. falciparum cases were not available for follow-up, so mixed infections cannot be excluded in these cases. Since 6 μg of pure P. falciparum DNA samples derived from cultured parasites did not react with VPL1015 (Table), we conclude that the DNA probe does not recognize P. falciparum DNA.

Southern hybridization of patients’ samples

In Southern hybridization analysis of DNA cleaved with HindIII (Fig. 3), the VPL1015 DNA probe hybridized to a major 1.4 kb band in samples which had been diagnosed as positive for P. vivax by dot-blot analysis. Some minor bands of variable size were not available for follow-up. This was only true for the 2 P. falciparum cases with assumed concurrent P. vivax infections (Pf 22 and Pf 20), which gives confidence that the positive dot-blot did indeed reflect the presence of P. vivax. 5 μg of pure P. falciparum or human DNA failed to react.

Discussion

The VPL101 and VPL1015 DNA probes provide an objective means of distinguishing P. vivax from P. falciparum in many areas of the world where both species cause human disease. Like other DNA probes, they require adaptation using a non-radioactive label (McLAUGHLIN et al., 1987b). Preliminary non-isotopic labelling experiments with VPL101 have detected <-1 pg of target DNA. The capacity to use stable, non-radioactive labelling will be an advantage for field studies. If necessary, signal amplification could be achieved by the polymerase chain reaction (ERLICH et al., 1988). This would be feasible in large laboratory-based investigations, as would the use of short oligonucleotides, labelled with 32P, from ribosomal genes for detection of Plasmodium spp. (LAI et al., 1989; WATERS & MCCUTCHEON, 1989). Both these techniques are highly sensitive, but usage would be limited in field studies because of strict technological requirements.

The use of VPL101 in mixed infections could be of particular interest. Within 2 months of successful drug treatment of P. falciparum malaria, P. vivax appeared in 104 (33%) of 319 patients in Thailand (LOOARKEEWSAN et al., 1987). These data are consistent with acute suppression of P. vivax parasitemia by P. falciparum (BOYD & KITCHEN, 1937), with the concurrent P. vivax infection becoming apparent only after the elimination of P. falciparum. This illustrates the potential usefulness of the VPL101 DNA probes to identify a concurrent P. vivax infection. The P. vivax and P. falciparum DNA probes now available provide important tools for epidemiological analysis and control programme monitoring. They may also be useful in screening blood donors, especially in malaria-endemic countries. In addition, the development of P. falciparum vaccines and their application to human populations may result in an upsurge of P. vivax, which will require accurate monitoring by such means.

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References


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DNA PROBE FOR THE DIAGNOSIS OF VIVAX MALARIA

CDR Grant No. 936-5544-6-00-0047-00

I. Quarterly Progress Report


Presented by Dan T. Spira P.I.
The first stages of this project were devoted mainly to establish basic technologies needed for the successful performance of the various steps outlined in the grant proposal. Due to the lack of available \textit{P. vivax} DNA at this time we used \textit{P. berghei} from infected mice and \textit{P. falciparum} from cultures as "surrogate" DNA. Although we are aware, that DNA from different species of \textit{Plasmodium} differs both in composition and in structure, we considered it an appropriate model system.

Numerous extractions of DNA from Saponin treated \textit{P. berghei} infected mouse red blood cells yielded about 1-2 ug DNA per ml infected blood at 30% parasitemia. It was obvious that, \textit{P. vivax} DNA samples will never be of this magnitude, and therefore only the most frugal experiments could be performed in the future. \textit{P. berghei} DNA served also as the model for the preparation of a genomic library using \textit{EMBL3} vector. We concentrated on this vector to complement and expand the library already prepared by the Thai-Australian team, so that two different libraries obtained by different means could be compared in the future. To achieve maximal cloning efficiency the DNA had to be purified to a high degree. Various techniques of DNA preparation have been compared to achieve purity with minimal loss during this process, so that even tiny amounts of DNA extracted from \textit{P. vivax} samples will yield useful preparations.

Among the techniques examined, which included direct extraction from gel slices, electrolution and "Elutip D" columns and other accepted procedures, we chose Schleicher & Schull
Et'utip D as the most efficient and DNA conserving. For maximal efficiency the appropriate physical conditions had to be established. The standardization of this step took most of our efforts prior to Prof. Pramuang's visit and the delivery of *P. vivax* samples.

The first experiments using *P. vivax* from infected blood collected from patients in Thailand and concentrated using the method described in the grant proposal, were DNA extractions using the Maniatis method, as employed by the Thai-Australian team. This extraction procedure was later compared to the method used by Wallach et al. for *P. falciparum* and *P. berghei*.

At present we are attempting to find more probes that would hybridize to different isolates of *P. vivax* and not hybridize to DNA from other species of Plasmodia which could be used to analyze blood samples from patients suspected of being infected with the parasite. *P. vivax* shows a high level of karyotype polymorphism as has been shown by Langley (1988) on isolated parasites obtained from different patients. Our approach is to find a repetitive sequence that can be used as a highly sensitive DNA probe. For this purpose, we extracted DNA from *P. vivax* parasites isolated from four pools of infected patients, cut with different restriction enzymes in order to see whether they contain repetitive sequences. The enzymes used were HaIII, XhoI, PstI, AVUI, KpnI, BglII, HindIII and TaqI. The samples were run on 0.8% agarose gel, and then stained with ethidium bromide. No detectable discrete band proving the presence of repetitive
sequences have been detected. To improve on sensitivity, in addition to direct ethidium bromide Southern blotting was also used to detect discrete bands but this highly sensitive method also could not show repetitive sequences in our samples.

The results of our Southern blotting experiments thus far showed that no hybridization was obtained using 100-200 ng P. vivax DNA, 100 ng human DNA or clone VPL101 (20 ng) as probes on a blot containing 60-100 ng of P. vivax DNA cut with the restriction enzymes BamHI and HindIII and hybridized overnight. We then performed blotting experiments to determine the level of sensitivity of these probes. Human DNA used as a probe could detect as little as 1 ng of human DNA on a blot. In addition, we were able to detect 100 pg of clone VPL101 in an overnight exposure. Thus it would appear that P. vivax DNA contains either very low amounts of repetitive DNA, or extreme heterogeneity of repetitive sequences which do not cross-hybridize within a given isolate. These results also show that there is very little (less than 1%) human DNA present in the P. vivax samples so far analyzed.

In order to distinguish between the possibility of either little or no repetitive DNA in the P. vivax genome, or a very high level of heterogeneity between repetitive elements, Southern blotting experiments will be carried out under conditions of low stringency. Should hybridization be detected, then various restriction enzymes would be tested to produce a pattern most suitable for cloning. However, if under low stringency conditions
little or no hybridization is detected, we would conclude that finding a DNA probe with greater sensitivity and specificity than VFL101 is very unlike.
DNA PROBE FOR THE DIAGNOSIS OF VIVAX MALARIA

CDR Grant No. 926-5544-G-00-A027-00

II. Quarterly Progress Report

1 April 1987

31 July 1987

Presented by Dan T. Spira
During the visits of Profs. Pramuan in Jerusalem and Spira and Wallach in Bangkok it was felt that the simple strategy of using the existing library of \textit{P. vivax} or developing similar libraries (using EMBL-3 instead of PUC13) will not work. Testing for repetitive sequences using a variety of restriction enzymes also did not give useful results. This, in addition to the high level of DNA polymorphism in \textit{P. vivax}, led us to seek a different strategy. Therefore, after consultations and deliberations we decided to try the following strategy: The telomeric region from various species of \textit{Plasmodium} could be hybridized with the telomeric DNA fragment that has been cloned from \textit{P. berghei} \cite{1,2}. This fragment consists of a sequence of 4 base pairs which are repeated about 70 times in \textit{P. berghei} \cite{3}.

However, the subtelomeric region of \textit{P. falciparum} was found to consist of four different tandem repeats of various sizes \cite{4}. These tandem repeat sequences are conserved within \textit{P. falciparum} while there was no hybridization to DNA from other \textit{Plasmodium} species.

We have therefore decided to look for repeat sequences in the subtelomeric region of \textit{P. vivax}, which might lead us to a specific \textit{vivax} DNA probe.

In order to clone telomeric sequences, DNA was prepared from...
20 different blood samples, of those originally supplied by Prof. Pramuan. These samples were tested for the presence of human DNA by dot blot hybridization, using human DNA as a probe. Samples that contained least amounts of human DNA were pooled and used for hybridization with P. berghei telomeric probe that was kindly given to us by Dr. M. Ponzi from Italy.

The results showed that the telomeric probe hybridized specifically with P. vivax. The lowest amount of DNA that was used for the hybridization was 10 ng. No hybridization was found using human DNA, even using as much as 200 ng DNA.

The next steps in our study will be:

1) Cloning of telomeric sequences in PUC8.
   The cloning strategy for this process has been described by Van der Ploeg et al. (5). Briefly it consists of digesting the DNA with Bal 31 in order to create blunt ends and ligating the digested DNA to a linearized plasmid. The recombinant molecule would then be digested with an enzyme that does not cut the vector. This would enable to obtain smaller molecules. These will then be diluted and circularized. Finally, transformation into an appropriate host would be done.

2) The library will be screened with the telomeric probe from P. berghei to find clones containing some of the subtelomeric region. These cloned would then be used to continue screening the
3) The various clones would be sequenced. Those containing repeats would be checked for further use as probes with the various samples of P. vivax DNA. The sensitivity of different probes can then be performed by doing the hybridization at different levels of stringency. The most sensitive probes can then be field as described in the original proposal.

At the time of writing this report the first experiments concerning the telomere cloning have been done but more conclusive answers could be obtained only in the near future. Not to prolong the period between reports any longer, this one therefore expresses more our theoretical considerations and library work than laboratory progress. This, I am convinced will be different in the next period.
REFERENCE


DNA PROBE FOR THE DIAGNOSIS OF VIVAX MALARIA

CDR Grant No. 536-5544-8-00-8027

Progress Report

1 March 1990

Dan T. Spira
In our last report we proposed to concentrate on subtelomeric repeat sequences as an appropriate strategy to achieve sensitive and specific probes for \textit{P. vivax} diagnosis. The telomere was found to be common to all plasmodial species (1), while the subtelomeric region contains species specific repeat sequences promising both sensitivity and specificity. \textit{P. falciparum} subtelomeric region consists of four tandem repeats of various sizes (2).

The strategy we adopted in this work was to clone the telomeric sequence. At first we tried it on human DNA, since it is difficult to obtain \textit{P. vivax} DNA. The procedure used which has been described by Van der Ploeg and Ponzi M. (3, 4), consists of following steps:

\begin{enumerate}
\item Digestion of high molecular weight DNA with nuclease Bal 31 for 5 minutes which reduces molecular extremities by a limited number of base pairs and creates blunt ends. From previous experience we treated the DNA for 5 min reducing the end by about 60 nucleotides.
\item The vector used was pUC8 which have been double digested with SmaI which creates blunt ends and by BamHI which creates sticky end. After digestion with the restriction enzymes, DNA was extracted with phenol/chloform and precipitated by ethanol.
\end{enumerate}
c. Ligation of Bal 31 digested DNA to SmaI restricted pUC8 was performed overnight at room temperature in the presence of 3 WU T₄ DNA ligase.

d. The ligation mixture was then digested with Bgl II which has no restriction sites in pUC8, but generates in the plasmid DNA (or in human DNA) sticky ends complementary to those generated by BamHI in the plasmid.

e. The ligation mixture was digested for a second time with SmaI and BamHI to reduce the background caused by reconstituted plasmid, not containing plasmoidal DNA.

f. DNA was extracted by phenol/chloroform and precipitated by ethanol.

g. Dilution of the mixture to a plasmid concentration of 2µg/ml to favour circularization of the recombined molecules. Sticky end ligation was performed overnight at 40°C by adding 2 WU T₄ DNA ligase.

h. Transformation of XLI blue cells was performed using the CaCl₂ procedure described by Maniatis et al. (5). Selection of recombinant clones was by plating in the presence of IPTG and X-gal. Foreign DNA at the cloning site results in the loss of β-gal complementation.
We repeated this procedure 3 times without success. Therefore we did the following changes of the original procedure to ensure more efficient ligation of the blunt ends.

1) After treating the DNA with nuclease Bal 31 only a fraction of DNA molecules in the reaction mixture will have blunt ends that are suitable for ligation. DNA treated with Bal 31 can therefore be repaired by an end filling reaction using the Klenow fragment of *E. coli* DNA polymerase I. This reaction has been performed after step a), using reaction condition described by Maniatis et al. (5).

2) Step e) which contains double digestion with SmaI and BamHI had been omitted because to this reaction the plasmid DNA fragment could also be digested with SmaI and therefore will not have sticky ends available for the second ligation. Instead it has been digested with BamHI only or has not been digested. We found that both changes of the original method gave almost the same results improving yield of clones.

Using the method with the modifications that have been described above we got a *P. vivax* library of about 200 clones. 40 clones have been isolated and have been checked for the presence of an insert by double digestion with PstI-EcoRI (these two enzyme sites are located near the ends of the insert.)
Seven clones have been found to contain inserts ranging in size from 2.3Kb to 0.3Kb. Southern blot was performed for these clones with probe pTel, prepared from P. berghei (4). Two clones were found to hybridize to this probe.

The two clones have been used for the hybridization with ten different P. vivax DNA samples using dot blot system. All the 10 different samples have been found to hybridize to these clones.

The next steps in this program will be to sequence the two clones. If a subtelomeric region is indeed found, it will be submitted and checked for its ability to serve as a specific probe for detection of P. vivax. The two clones will be used as a probe to test the remaining 5 clones containing P. vivax DNA that did not hybridize with pTel but might contain fragments of the subtelomeric region.

REFERENCES


cloning. A Laboratory Manual published by Cold Spring Harbor
Laboratory Press, N.Y.