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PROGRESS REPORT NO. 2

DNA PROBE FOR THE DIAGNOSIS OF VIVAX MALARIA

A RESEARCH PROJECT

U.S. - ISRAEL COOPERATIVE DEVELOPMENT

RESEARCH PROGRAM

GRANT NO. 936-5544-G-00-8047-00

SUBMITTED BY

PRAMUAN TAPCHAISRI

SAVANAT THARAVANIJ

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

FACULTY OF TROPICAL MEDICINE, MAHIDOL UNIVERSITY

420/6 RAJVITHI ROAD, BANGKOK 10400

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PROJECT PROFILE

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Project Title : DNA Probe for the Diagnosis of Vivax Malaria

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CONTENTS

1. Background/Introduction

Malaria is still an important public health problem plaquing the economic development of countries of the third world (1). Vivax malaria, which is caused by the parasitic protozoan *Plasmodium vivax*, is second in occurrence to falciparum malaria which is the most frequency worldwide disease caused by *P. falciparum*. Although vivax malaria does not cause as much mortality as falciparum malaria, nevertheless, it causes incapacitation by preventing people from work and thus undermines productivity of the nation. In addition, *P. vivax* is the major species responsible for relapsing malaria (2).

For more than a century, microscopic examination of blood smears has been used for diagnosis of malaria. However, this technique is laborious, requiring a well-trained personnels, difficult to reproduce quantitatively and not suitable for surveillance or for a large scale epidemiological studies. Very recently, DNA probes have been developed and used for rapid diagnosis of infections caused by many infectious agents including viruses, bacteria and protozoa (3,4,5,6,7,8). The DNA probes specific for *P. falciparum* have been reported but none to date for *P. vivax* (9,10,11,12,13).

2. Objectives

: The objectives of the present study are 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.

3. Methods and Results

3.1 Purchase of equipment

Equipment purchased

3.1.1 Microwave oven

3.1.2 Vaccuum pump and accessory

3.1.3 Agarose electrophoresis system

3.1.4 Microcentrifuge

3.1.5 UV Transluminator

3.1.6 Polaroid camera and accessories

3.1.7 Shaking water bath

3.1.8 Refrigerator

3.1.9 Air conditioner

3.1.10 Power supply and electrophoresis chamber

Equipment being ordered

3.1.11 Isotope survey meter

3.2 Collection of *P. vivax* infected cells

P. vivax infected cells were collected from patients with vivax malaria admitted to the Bangkok Hospital for Tropical Diseases of the Faculty of Tropical Medicine by passing through the sulfoethyl cellulose-sephadex G25 column and Percoll

gradient centrifugation (14) as described in the previous report. Blood samples from 87 patients with parasitemia ranged from 0.1-2.4% were obtained and processed during January to June 1989. The yield from each patient was between $2x10^6$ to $5x10^7$ infected cells. All the samples were lyophilized and kept at -70°C for DNA extraction and antigen analysis work.

3.3 Collection of antisera

Ninety-five plasma samples were obtained from patients with vivax malaria admitted to the Bangkok Hospital for Tropical Diseases. The plasma were kept in small aliquots at -20°C.

3.4 Indirect fluorescent antibody test for specific antibodies against P. vivax

One hundred and seventy-six plasma samples obtained during July 1988 - June 1989 from the patients with vivax malaria were tested for the presence of specific antibodies against erythrocytic stages of P. vivax by indirect fluorescent antibody (IFA) test similar to those described by Tharavanij et al. (15). The P. vivax infected erythrocytes were obtained from a vivax malaria patient with parasitemia of 2.4%. The infected erythrocytes were washed 5 times in phosphate buffered saline pH 7.4 (PBS) by centrifugation at 500xg at 4°C for 10 minutes. The pellet was resuspended to approximately 20% hematocrit. Thin smears of the blood were made on clean glass slides, quickly dried with an air blower, then kept at -20°C until use. The antigen slides were fixed in cold acetone at -20°C for 18 hours

then air-dried. Ten microliters of two-fold serially diluted plasma samples in PBS containing 0.5% gelatin (PBSG) starting from 1:60 was placed on a marked area on the slide and the reaction was allowed to occur at 37°C for 30 minutes. The slides were then rinsed, soaked three times in PBSG and air-dried. Ten microliters of a 1:80 dilution of fluorescein-isothiocyanate (FITC)-labelled goat anti-human immunoglobulins was placed on each marked area of the slides. The slides were incubated, washed and dried as above. The slides were mounted with buffered glycerol and covered with cover slips before observed under a Zeiss fluorescence microscope. the antibody titer of the plasma samples was the highest dilution of the samples which gave a definite positive IFA reaction (2 + on the 0 to 4 + scale). A positive IFA reaction for the antibodies against the parasites is shown in Figure 1.

The distribution of the antibody titers in 176 plasma samples obtained from the vivax malaria patients is shown in Figure 2. It was found that approximately 85% of the patients had positive IFA antibodies against P. vivax and that more than 60% of them had the titer equal to or more than 1:320.

3.5 Antigenic analaysis of erythrocytic stages of P. vivax

The plasma samples with high IFA titers were selected for further analysis of the antigens of erythrocytic stages of P. vivax by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

The electrophoresis was carried out in a vertical

slab gel apparatus using the method of Laemmli and Farve (16). A 4% acrylamide stacking gel and a 10% acrylamide separating gel were used. Samples containing $2x10^6$ P. vivax infected erythrocytes were heated at 100°C for 3 minutes in the presence of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol before loading into the gel. After electrophoresis the gel was electroblotted onto a 0.45 um nitrocellulose membrane according to the method of Towbin et al. (17). After blotting the unreacted sites on the membrane were blocked by soaking the strips in PBS containing 3% gelatin and 0.5% bovine serum albumin (BSA) and 0.04% sodium azide (PBSG-BSA) at 45°C for 1 hour. The paper strips were treated with 5 ml of a 1:100 plasma sample in PBS containing 1% BSA and 1% gelatin at 26°C for 45 minutes with gentle rocking, washed 5 times with PBSG with 0.05% Tween 20, and then treated with ^{125}I-labelled protein A ($1-5x10^5$ cpm/ml in PBS-BSA) at 26°C for 45 minutes. The strips were washed as above, dried and autoradiographed on Kodak X-Omat K film with intensifying screen at -70°C for 24-48 hours.

Four plasma samples were initially selected in the SDS-PAGE and Western blot analysis against P. vivax erythrocytic stages derived from a pool of 11 samples of the enriched parasite's preparations. It was found that the antigens were highly complex composing of more than 22 bands as revealed in the autoradiograms (Figure 3). The major antigenic bands are those with molecular weights (MW) higher than 200, 120, 100, 86, 84, 70, 65, 50, 38, 32, 27 and 14 kilodaltons. Normal red blood cell group AB was used as the control and it was found that none of the sera from the patients showed any reactivity.

3.6 Screening of P. vivax genomic DNA library for antigen-producing clone by using immune serum.

As mentioned in the previous report that a genomic DNA library of P. vivax was constructed by ligation of Sau3A partially cleaved purified P. vivax DNA to the BamH1 site of pUC13 plasmid vector through the assistance of Dr. Chev Kidson at the Queensland Institute of Medical Research, Australia. After transformation of the recombinant DNA to Escherichia coli JM 109, approximately, 1,500 colonies were obtained. These bacterial colonies were screened for the presence of P. vivax antigen producing clone by replicating the bacterial colonies from the culture plates directly onto the nitrocellulose membranes. The membranes were placed onto LB agar plates containing 50 ug/ml ampicillin and previously spreaded with 0.1 ml of 20 mM isopropylthiogalactoside (IPTG). The bacteria were allowed to grow at 37°C for 18 hours. The membranes were peeled off the plates and the bacteria were lysed by SDS/lysosyme and chloroform vapour. After washing with PBSG with 0.05% Tween 20 and the unreacted sites were blocked with PBSG-BSA, the membranes were treated with a 1:200 dilution of E. coli absorbed immune serum (Pv430 with the IFA titer of more than 1:5,120) at 26°C for 45 minutes. The membranes were washed, treated with ^{125}I-labelled protein A, washed, dried and autoradiographed as mentioned in section 3.5.

It was found that none of the bacterial colonies tested gave any positive signal indicating that the appropriate bacterial clond has not been obtained in this first

transformation.

3.7 Characterization of an *E. coli* clone positive for *P. vivax* DNA insert.

As mentioned in the previous report that an *E. coli* clone (VPL101) obtained from the genomic DNA library was positive for *P. vivax* DNA insert by screening of the transformants with nick-translated P^{32}-labelled purified *P. vivax* DNA. Further characterization are being done in our laboratory at the Faculty of Tropical Medicine, Mahidol University and at the Queensland Institute of Medical Research. A DNA minipreparation of the clone was made by growing the bacteria in LB broth, the bacteria were lysed by lysozyme and alkali treatment (18). The cell lysate was extracted two times with phenol/iso-amyl alcohol and chloroform solution and the plasmids were alcohol precipitated. Agarose electrophoresis and ethidium bromide staining revealed that the size of the *P. vivax* DNA insert was approximately 3.2 kb (Figure 4).

The purified VP101 plasmid DNA was biotin-labelled by nick-translation using biotin-7- ATP according to the manufacturer's instruction (Nick Translation Reagent Kit, Bethesda Research Laboratory, U.S.A.). The species specificity of the plasmid DNA was tested by using the biotinylated DNA as a probe to hybridize with its own DNA, the pUC19 vector and the human and *P. falciparum* DNA. DNA hybridization was performed by blotting various amounts of the denatured DNA in 0.4 N NaOH/10 mM EDTA onto the Zeta-Probe membrane (Bio-Rad Laboratories, U.S.A.) using a Bio-Dot apparatus (Bio-Rad Laboratories). The membrane

was rinsed with 2xSSC (1xSSC = 0.15 M NaCl, 0.015 M trisodium citrate), soaked for 2-3 minutes in prehybridization solution (2xSSPE, 1% SDS, 0.5% skimmed milk, 0.05% denatured salmon sperm DNA, 10% destran sulfate), then hybridized with 40 ng/ml of the heat-denatured biotinylated DNA in the prehybridization solution at 65°C for 18 hours. SSPE is 0.15 M NaCl, 0.1 M sodium dihydrogen phosphate and 0.001 M EDTA pH 7.4. The membranes was rinsed and washed with 2xSSC/0.1% SDS at 26°C for 30 minutes, 0.5xSSC/0.1% SDS at 65°C for 15 minutes and finally rinsed in the latter solution, dried on a filter paper. The hybridization reaction wsa detected by the streptavidin-alkali phosphatase detection system using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as the substrate (Bethesda Research Laboratory, U.S.A.). The result is shown in Figure 5. It was found that the plasmid hybridize only with P. vivax DNA and the vector but not with human or P. falciparum DNA even at much higher concentration.

3.8 Work collaboration with the Kuvin Centre for the Study of Infectious and Tropical Diseases, Hebrew Univeristy, Israel.

The principle investigator has made a visit to the Kuvin Centre for the Study of Infectious and Tropical Diseases, Hebrew University, Israel, during February 4-27, 1989. During the visit, DNA extracted from the P. vivax enriched samples was cut with 10 different restriction enzymes and separated by agarose gel electrophoresis in order to see whether repetitive sequences were present in the parasites genome. The enzymes used

were Eco R1, Bam H1, Hae III, Xho I, Pst I, Alu I, Kpn I, Bgl II, Hind III and Taq I. However, no detectable discrete band of the repetitive DNA sequence was detected in this first experiment by either ethidium bromide staining or Southern blotting. It was also found that contamination of human DNA in the P. vivax DNA preparation was minimal.

4. Conclusion/Remark

5. Workplan for the Next Period

5.1 Construction of P. vivax genomic DNA library using lambda phage vector EMBL and Escherichia coli (to be done at the Hebrew University of Jerusalem, Israel).

5.2 Screening of the phage vector containing P. vivax DNA inserts (to be done at the Hebrew University of Jerusalem, Israel and Mahidol University).

5.3 Screening of P. vivax genomic DNA library constructed in Australia for antigen producing clones and characterizing the existing clone positive for P. vivax DNA insert (to be done at Mahidol University).

5.4 Further characterization of P. vivax antigens using immune plasma serum from patients with vivax malaria (to be done at Mahidol University).

5.5 Field blood collection. Arrangement will be made with either with the Malaria Division, Ministry of Public Health or other institution to collect blood specimens from individuals living in malaria endemic areas in Thailand.

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7. Figures

Figure 1. A positive IFA reactivity of serum antibodies against erythrocytic stages of *P. vivax*.

Figure 2. Distribution of serum IFA titers against erythrocytic stages of P. vivax in 176 patients with vivax malaria.

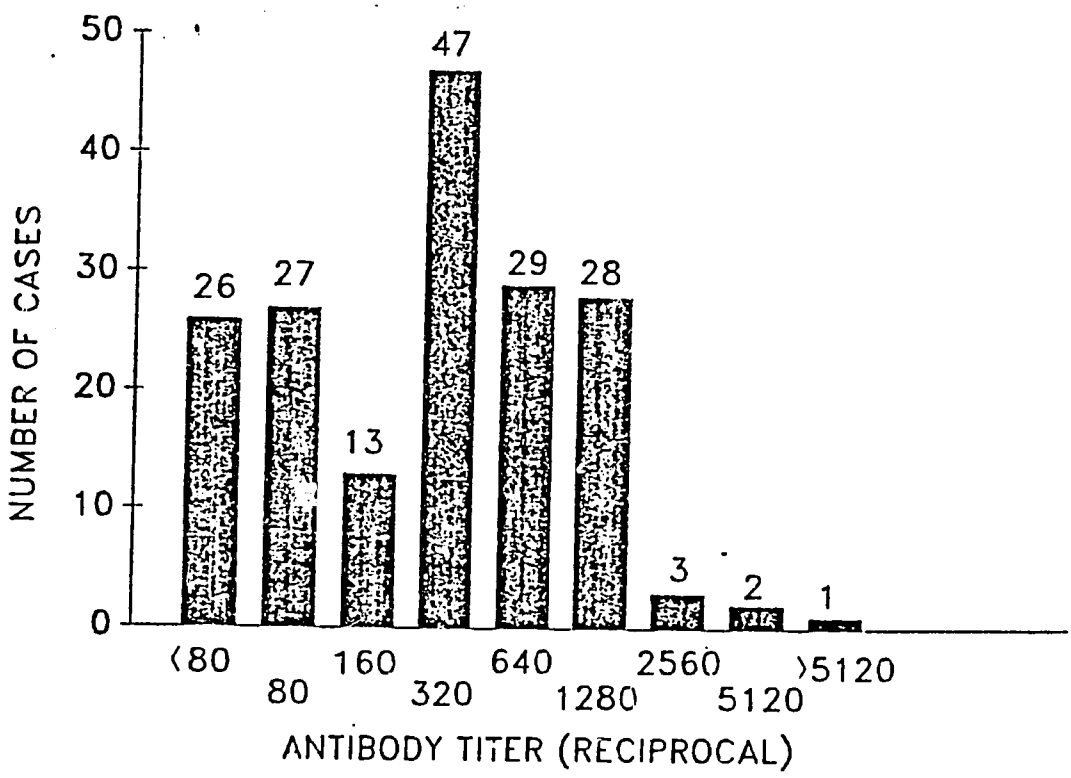

Figure 3. Reactivities of serum antibodies against P. vivax erythrocytic stages antigens in 4 patients with vivax malaria.

Antigens in Lanes 1-4 are P. vivax and Lanes 5-8 are human AB red blood cell controls. Sera and the IFA titers are: A, 1:5,120; B, 1:2,560; C, 1:2,560; and D, 1:1,280. Numbers at left indicate molecular weight markers in kilodaltons

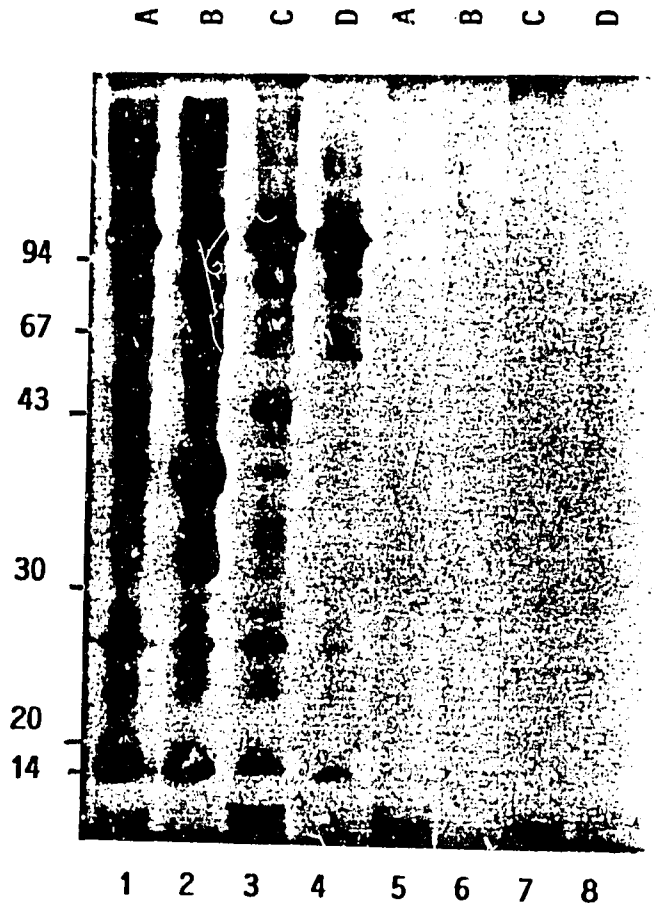

Figure 4. Plasmid VPL101 DNA before and after cutting by Bam H1 as shown by agarose gel electrophoresis and ethidium bromide staining. Lane 1 = linear pUC 19 vector cut with Bam H1, one band of 2.66 kilobases (kb); Lane 2 = pUC 19 vector uncut, upper band is relax form and lower band is supercoiled. Lane 3 = VPL101 cut with Bam H1, upper band is linear VPL101 of 5.9 kb, middle band is P. vivax DNA insert of 3.2 kb and lower band is the linear pUC vector; and Lane 4 = VPL101 uncut, upper band is relax form and lower band is supercoiled. Numbers at left indicate DNA markers in kb.

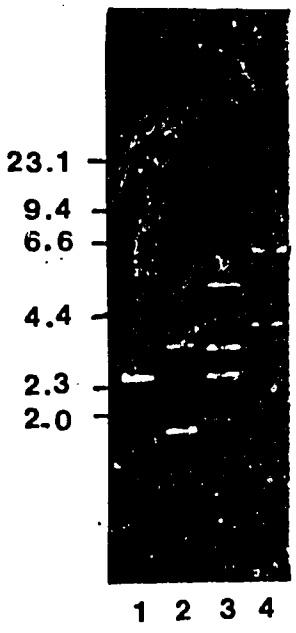
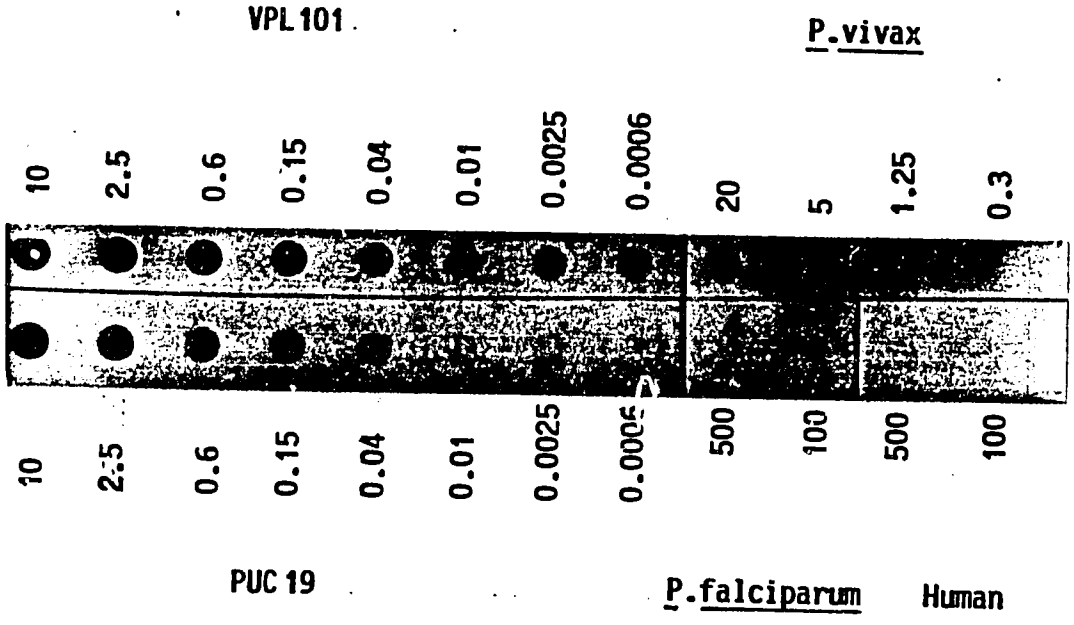
Figure 5. Hybridization of nick-translated biotin-labelled VPL101 to its own DNA, pUC19 plasmid vector, P. vivax DNA but not to P. falciparum or human DNA. The reactivities were detected by alkaline phosphatase-streptavidin and the and substrates nitroblue tetrazolium and 5-bromo-4 -chloro-3-indolylphosphate. The numbers adjacent to the samples indicate the amounts of various DNA in ng.