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A SENSITIVE AND SPECIFIC DNA PROBE FOR PLASMODIUM YOELII

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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We have produced a DNA probe, designated pJC1Ø4, for the sensitive and specific identification of Plasmodium yoelii. The probe incorporates the major DNA repeat of the parasite and has a sensitivity that exceeds that of all conventional methods of malaria diagnosis. The probe represents a major breakthrough for the Navy's malaria vaccine development program, as it provides a means for quantifying the protective effects of experimental immune interventions in the mouse -- P. yoelii system. Specifically, protection against challenge infection by passive transfer of antibody, adoptive transfer of immune cells, infusion of bio-active substances and immunization with subunit vaccines can now be reliably measured.

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Plasmodium yoelii, a parasite causing murine malaria, has found many applications as a model system for studying human malaria. Currently, malaria vaccine development efforts are focused on interrupting the development of the parasite in the host's liver. Various biochemical and immunological interventions have been developed to kill or slow the development of the parasite at this stage, however, quantification of the effects of these treatments has been problematic. Current methods for quantifying the inhibitory effects of immunization protocols on liver stage development have been largely inferential, based on reduction in blood parasitemias, or have relied on examination of stained tissue sections.

The objective of the studies reported in this paper was to produce a DNA probe that could be used for the identification and quantification of *P. yoelii* infections in infected blood, in liver tissue sections *in situ*, and in total DNA extracts of infected liver.

MATERIALS AND METHODS

Production and purification of *Plasmodium yoelii*-infected erythrocytes

Plasmodium yoelii (17X NL strain) was used to infect 40 Balb/c mice. When parasitemias reached 10-20%, mice were bled retro-orbitally into Na-heparin. The pooled blood was layered over an equal volume of acid washed glass beads (Sigma, St. Louis, Missouri) in 50 cc syringe barrels. Glass beads were pre-wetted with IX SSC (150 mM NaCl, 15 nM Na citrate, pH 7.0), and the blood was washed through the column with additional IX SSC. The blood eluate was then layered over a 2X volume of CF-II cellulose (Whatman, Hillsboro, Oregon) lightly packed into 50 cc syringe barrels. The CF-II was thoroughly pre-wetted with IX SSC, and additional IX SSC was used to elute the blood until the cellulose cleared to pale pink.

The diluted blood was centrifuged 10 min at 1500 rpm and resuspended to its original volume in IX SSC. The blood was then layered over lymphocyte separation medium (LSM - Organon, Durham, North Carolina) and spun at 2500 rpm for 20 min. The majority of infected RBCs banded at the LSM/plasma interface, and this band was harvested, washed twice in IX SSC and used for further processing.

Isolation and purification of parasite DNA

The LSM band, containing infected RBC and essentially free of contaminating mouse WBC, was resuspended in 30 ml of IX SSC containing 1% saponin and placed on ice for 15 min. The preparation was spun at 8000 rpm for 10 min, the supernate was discarded, and the saponin lysis was repeated twice, until the supernate was clear. The pellet, containing free parasites and erythrocyte ghosts, was suspended in 4 ml of a 4% solution of sarkosyl containing 1 mg/ml proteinase K. The preparation was incubated at 37 C for 1 hr and solid CsCl was added to a final concentration of 1 gm/ml. Hoescht dye was added to a final concentration of 1 ug/ml, and the preparation was spun at 35, 000 rpm for 48 hr in a Beckman 50Ti rotor.

After centrifugation the dense, uppermost band was recovered, extracted with isopropanol and dialyzed 1: 10^6 against IX TE (10 mM Tris, 1 mM EDTA, pH7.5).

Production of plasmid library of *P. yoelii* DNA

Purified *P. yoelii* DNA was cut with Hind III, ligated into Hind III cut plasmid pBR322 according to standard methods (1), and plated onto Luria broth (LB) agar containing 50 ug/ml ampicillin (amp). Ampicillin resistant colonies were transferred to duplicate LB plates containing amp or tetracycline (tet - 28 ug/ml). Ampicillin resistant, tet sensitive colonies were then transferred to duplicate LB amp plates. The following day, colonies were lifted onto nitrocellulose filters, processed according to Davis et al. (2_{32}) (hybridized with either nick-translated ^{32}P-*P. yoelii* DNA or ^{32}P-mouse DNA. Colonies that hybridized strongly with *P. yoelii* DNA but failed to hybridize with mouse DNA were selected for further analysis.

Selection and characterization of *P. yoelii* DNA clones

From the pBR322 library of *P. yoelii* DNA fragments 5 clones were selected for further analysis. Cross-hybridization of these clones with each other suggested that they shared a common sequence, and the strength of the hybridization signal to *P. yoelii* genomic DNA suggested that this sequence encoded a major repeated element. One clone, pJClO4, gave an exceptionally strong signal and was selected as a candidate probe. Restriction enzyme analysis of pJClO4 revealed an insert of 3.16 kb, containing sites for the restriction enzymes Cla I, Sca I and Ssp I (Fig. 1).

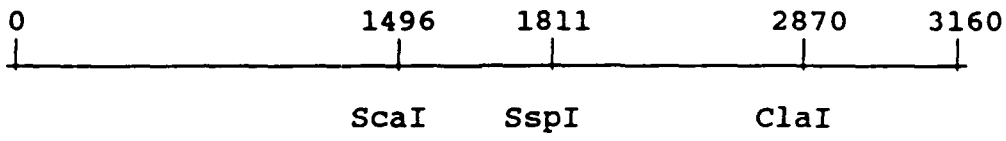
Applications of the probe for detecting *P. yoelii* DNA

To determine the absolute sensitivity of the probe, dilutions of *P. yoelii* DNA (1 pg - 1 ng) were applied to nitrocellulose in 50 ul volumes, using a slot blot apparatus (Minifold II, Schleicher & Schuell, Keene, New Hampshire). Nitrocellulose blots were processed by placing them over filter papers saturated with reagents as follows: 0.5 N NaOH, 10 min; IM Tris, pH 7.0, 2 min (twice) ; IM Tris, pH 7.0, 1.5 M NaCl, 10 min. The blots were then dried and baked at 80 C for 2 hr. Processed blots were hybridized against ^{32}P-pJClO4. The probe was also hybridized against Southern blots of Hind III digested *P. yoelii* genomic DNA to determine the banding pattern.

To determine the sensitivity of the probe for detecting *P. yoelii* DNA in the presence of host DNA, dilutions of *P. yoelii* DNA were made into a solution of mouse DNA to produce samples containing *P. yoelii*:mouse DNA in ratios of $1:10^7$ to $1:10^3$. Total DNA concentration of the samples was held constant and 1 ug of each was applied to an agarose gel. After electrophoresis, the samples were transferred to nitrocellulose and hybridized against ^{32}P-pJClO4. Alternatively, *P. yoelii* DNA was diluted into 20 ug of mouse DNA, in the same ratios as above, and applied in 100 ul volumes to 1 inch nitrocellulose squares. Nitrocellulose was processed as for slot blots, and then hybridized against the probe.

To determine the sensitivity of the probe for detecting erythrocytic stages of *P. yoelii*, mouse blood infected with *P. yoelii* was diluted with uninfected mouse blood in eppendorf tubes to produce parasitemias ranging from 1.0% - .0001%. Twenty ul of each dilution was spotted onto nitrocellulose and air dried. Spots were then processed according to Mucenski et al., (3) and hybridized against ^{32}P-pJClO4.

RESTRICTION MAP OF PJC104 INSERT



insert = 3160 bp

FIGURE 1

To determine if pJCIO4 could be used for detecting liver stages of *P. yoelii*, Wistar rat hepatocytes were grown in microculture on Lab Tek chamber slides (Nunc, Naperville, Illinois), and infected with sporozoites of *P. yoelii* (17X NL strain). Duplicate cultures containing IO^5-hepatocytes were prepared and stained with monoclonal antibody NYSI (1/100 dilution) against the circumsporozoite protein of *P. yoelii* to confirm and quantitate liver cell infections. Uninfected liver cell cultures were maintained as negative controls.

Cell cultures were transferred and lysed by inverting the slides onto nitrocellulose in petri dishes, over filter paper saturated with IM Tris, pH 7.0, containing 10% sarkosyl and 1 mg/ml proteinase K. Petri dishes were covered, sealed with parafilm and held for 2-24 hr at 37°C. The nitrocellulose was then processed as for slot blot preparations and baked at 80°C in vacuo for 2 hr to fix the DNA to the paper. Duplicate samples were prepared as described above and hybridized to ^{32}P-pJCIO4. One set of the hybridized cell transfers was used for autoradiography, while the duplicate set was counted in 5 ml of scintillation fluid in a Beckman LS 9000 scintillation counter.

Additionally, detection of liver stage parasites was studied by *in situ* hybridization. Ten micron-thick cryosections of *P. yoelii* infected mouse liver on glass microscope slides were processed according to the method of Lum (4) and hybridized with ^{32}P-pJCIO4. Tissue sections on the slides were washed, dipped in photographic emulsion (NTB2, Kodak, Rochester, New York), and held at 4 C in the dark for periods ranging from 12 hr to 1 wk. Slides were developed, counterstained with hematoxylin and eosin and examined at 400 or IOOOX in a compound microscope for exposure of the emulsion at the locations of parasites.

To determine the specificity of pJCIO4 for *P. yoelii* DNA, dilutions of *P. falciparum, P. vivax, P. yoelii, P. berghei*, human, chimpanzee and mouse DNA were slot-blotted to nitrocellulose and hybridized with the probe.

RESULTS

Production/purification of *P. yoelii* DNA

The purification procedure described above resulted in very high yields of exceptionally pure *P. yoelii* genomic DNA. As mentioned in Materials and Methods, the infected mouse RBCs were localized primarily in the LSM band. Interestingly, the same protocol applied to the purification of human malaria parasites (*P. falciparum, P. vivax, P. malariae* and *P. ovale*) from the blood of experimentally infected chimpanzees resulted in the localization of infected RBC in the erythrocyte pellet rather than the LSM band after centrifugation (J. Campbell, unpublished results - see NMRI technical report, June 1989).

Ultracentrifugation through CsCl resulted in two bands - an upper band of *P. yoelii* DNA and a lower, minor band of host (mouse) DNA.

Detection of *P. yoelii* DNA using pJCIO4

Using the nitrocellulose slot blot, pJCIO4 detected a minimum of 1 pg of *P. yoelii* genomic DNA, equivalent to the DNA of 10 parasites. Southern blot analysis using the probe revealed a pattern of

multiple bands (Fig. 2) on Hind III digested *P. yoelii* genomic DNA. After agarose gel electrophoresis and Southern blotting, dilutions of *P. yoelii* DNA in mouse DNA were detected at a ratio of 1 ng *P. yoelii*:1 ug mouse, ie, in the presence of a thousand-fold excess of host DNA. However, autoradiography of the diluted DNA applied directly to nitrocellulose allowed detection of parasite DNA in the presence of a 10^5-fold excess of host DNA, after a 48 hr exposure.

Spots of mouse blood (20 ul) were detected by the probe with parasitemias as low as .001%, equivalent to 10^4 parasites. In liver cell cultures transferred to nitrocellulose the probe successfully detected 29 schizonts/10^5 cells and produced a visible signal after 18 hr exposure on the autoradiograph. These results were corroborated by scintillation counter results which revealed counts in the infected samples more than 4 SD above negative (uninfected) control samples. Tissue sections probed *in situ* with pJCIO4 revealed specific, focal hybridization of the probe to liver stage parasites, with a distinct autoradiographic signal by 16 hr, however background was high. Experiments to reduce background and increase the signal:noise ratio are continuing.

With the specificity of pJCIO4 for purified *P. yoelii* DNA taken to be 100%, cross-reactivity of less than 1% to *P. berghei* DNA was observed. The probe failed completely to hybridize to purified DNA from *P. falciparum, P. vivax*, human, chimpanzee or mouse.

DISCUSSION

Data presented in this paper indicate that pJCIO4 contains a sequence that is homologous to a major repeated element in *P. yoelii* DNA. The sensitivity of this probe for detecting blood infections equals or exceeds that available by examination of Giemsa stained smears. This probe provides the first sensitive and reproducible method for diagnosing and quantifying liver stage infections in the mouse - *P. yoelii* model system.

Development of probe pJCIO4 has taken two years, and represents a major breakthrough for the Navy's malaria vaccine development program. Up to this time the program has been handicapped by the lack of a sensitive, reproducible method for quantifying the effects of experimental immune interventions in its *P. yoelii* animal model system. The specificity and the high sensitivity of this probe will allow the effects on protection of such treatments as passive transfer of hyper-immune sera or monoclonal antibodies, adoptive transfer of immune cells or infusions of bio-active substances (eg., ganMa interferon) to be quantified. Additionally, the effects of ablating portions of the immune system (T-cell subsets, Ig-producing cells) on protection against infection can be studied, to clarify host-parasite relationships. Last, but not least, experimental vaccines (whole natural antigens, recombinant proteins, subunit peptides and anti-idiotype antibodies) can be tested in the murine system and their effects on subsequent challenge infection can be measured with this probe.

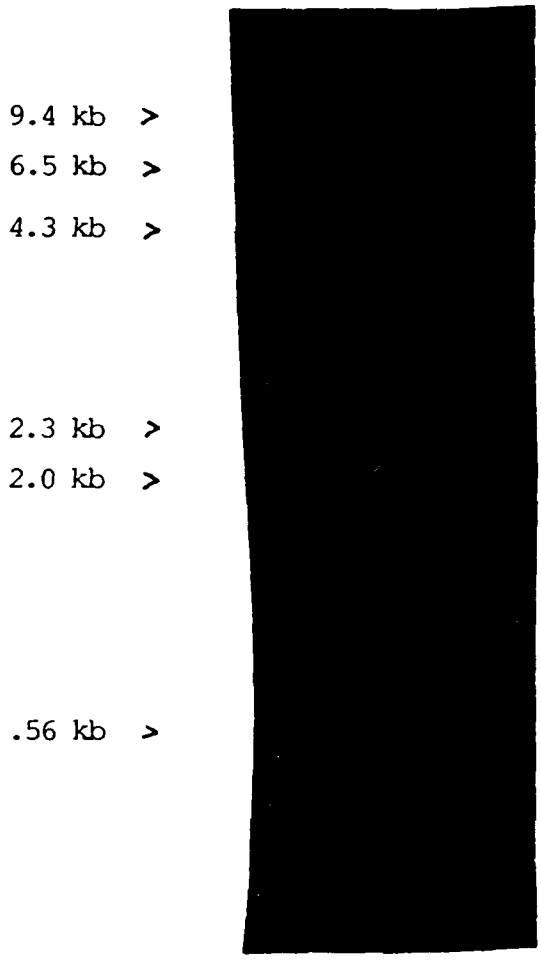


FIGURE 2

Southern blot of Hind III digested P. yoelii DNA hybridized to pJC104

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