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IDENTIFICATION OF MALARIA-INFECTED DRIED MOSQUITOES BY BIOTINYLATED DNA PROBE

LIU KE-YI,[1] CHEN XI-XIN, HUANG BING-CHENG AND CHENG YI-LIANG

Shandong Institute of Parasitic Diseases, Jining 272133, Shandong, People's Republic of China

ABSTRACT. Dot-blot hybridization with cloned genomic DNA labeled with photobiotin as a probe is used to detect *Plasmodium falciparum* sporozoites in dried mosquitoes. The assay is sensitive enough to detect 2 pg *Plasmodium* parasite DNA, or one infected mosquito in a pool of 40 insects using DNA extraction samples, or one infected mosquito in a pool of 25 insects without DNA extraction. A single mosquito squashed directly on a nitrocellulose filter for the determination of parasites provides a simple method for the detection of sporozoites. The mosquitoes triturated in reduction buffer were efficient for the screening of malaria infection of mosquitoes in a large number of samples. The species specificity, sensitivity, and ease of performance of this assay as well as the stability of the reagents may make it a useful epidemiological tool.

INTRODUCTION

Malaria transmission to humans occurs when an *Anopheles* mosquito infected with sporozoites of one of the 4 species of human *Plasmodium* (*P. vivax, P. falciparum, P. malariae,* and *P. ovale*) takes a blood meal from a human. Determination of sporozoites is an important component of epidemiological investigations of malaria. However, the traditional method of dissection of the salivary glands of individual *Anopheles* mosquitoes to search for sporozoites using a light microscope is labor intensive and time consuming because in many endemic areas, the vectors have very low rates of sporozoite infection. Thus, a great number of mosquitoes must be examined. Also, the dissection method cannot determine which species of malaria parasites is present in the mosquitoes because *Plasmodium* sporozoites of both human malaria species and other animal parasite species are difficult to morphologically distinguish from each other. Enzyme immunoassays have been developed to circumvent these problems (Wirtz et al. 1985, 1992; Beach et al. 1992; Somboon et al. 1993). However, these assays do have some limitations; for example, microtiter plate assays may require special equipment to perform and read the data, the samples need special treatment to reduce the background, and false negatives are difficult to avoid due to the variabilities of the antigens of *Plasmodium* parasites.

Because the genetic material DNA has the characteristic of species specificity, and DNA hybridization is not affected by whether the nucleotide state is active or inactive, DNA hybridization technology has been used extensively for the detection of malarial parasites from human blood (Franzen et al. 1984, Relf et al. 1990). We report here a method using dot-blot hybridization with cloned genomic DNA labeled with photobiotin as a probe to detect *P. falciparum* sporozoites from dried mosquitoes. The results indicate that this method may be of value in monitoring malaria control programs and in investigating transmission of *Plasmodium.*

MATERIALS AND METHODS

Preparation of DNA probe: Plasmodium falciparum (Fcc_1/HN strain) parasites were obtained through *in vitro* culture and purified by lysis with saponin. Genomic DNA of the parasites was isolated, subjected to quantitative digestion with the restriction endonuclease *Hin*dIII, and ligated to *Hin*dIII-digested plasmid pBR322 to construct a genomic DNA library. Chimeric plasmids were used to transform *Escherichia coli* strain HB101 (Maniatis et al. 1982, Liu et al. 1993). The library was screened for colonies containing highly repeated sequences of *P. falciparum* DNA. This was performed by *in situ* colony hybridization using a probe of total genomic DNA of *P. falciparum* radiolabeled with $\langle\alpha\text{-}^{32}P\rangle$dATP by nick translation as described by Rigby et al. (1977). Colonies with strong hybridization signals were selected. The recombinant DNA was isolated from selected clones by the alkaline miniprep method (Maniatis et al. 1982), and dissolved in distilled water.

Recombinant plasmid DNA solution (10 μg DNA) was mixed in subdued light with 2 volumes of photobiotin acetate (Yap et al. 1988) (product of Company of Chinese Academy of Military Medicine and Science, Beijing), and irradiated for 20 min on ice (tungsten filament reflector lamp LYQ12-100, at a distance of 10 cm). The labeled DNA was extracted twice with equal volumes of butanol after adding TE buffer (10 mM Tris-HCl, pH 9.0 and 1 mM EDTA) to

[1] Present address: Instituto de Biotecnología, Universidad Nacional Autonoma de Mexico, Apdo. Postal 510-3, Cuernavaca 62271, Morelos, Mexico.

500 μl, and recovered by precipitation with 2.5 volumes of ethanol and resuspended in sterile distilled water. The sensitivity of the labeled DNA was evaluated by direct incubation of the probe with avidin–alkaline phosphatase conjugate, and substrates of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

Preparation of mosquito samples: Plasmodium falciparum sporozoite-infected mosquitoes were obtained by membrane feeding (Collins et al. 1986) *Anopheles dirus* Peyton and Harrison mosquitoes with blood from gametocyte-carrying humans in Hainan Province of China. Levels of *P. falciparum* infection of mosquitoes were assessed by examining the midguts of representative samples of mosquitoes from different lots for oocysts. Then, lots of infected mosquitoes were kept for a time sufficient to allow the parasites to complete sporogonic development (assessed by looking for sporozoites in the salivary glands). Subsequently, the mosquitoes were collected and kept in bottles without any reagents, and stored at room temperature for use. For the control, some of mosquitoes were kept in 75% ethanol and washed with water before use.

A single dried mosquito was soaked in a solution of 10% sodium dodecyl sulfate (SDS) for 10 min, then put on a nitrocellulose filter, squashed with a heat-sealed micropipette tip. For the portion test, the mosquito was cut transversely between the thorax and the abdomen and the head portions (head plus thorax) and body portions (abdomen) were placed onto the filter. The filters were placed onto puddles of lysis buffer (0.5 M EDTA, pH 8.0, 0.5% SDS, 100 μg/ml proteinase K), incubated at 50°C for 1 h, and then air dried.

Detection of hybrids: The filters with mosquito samples were placed onto puddles of 0.5 M NaOH for 5 min to denature the DNA, then neutralized by 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl for 5 min after picking off the mosquito debris. The filters were then baked under vacuum for 30 min at 75°C after air drying. The prehybridization was carried out for 2 h at room temperature in prehybridization buffer (5× Denhardt's solution, 5× SSC [20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0], 100 μg/ml salmon sperm DNA, 50% formamide). The filters were hybridized overnight with photobiotinylated pBF4 DNA probe (100 ng/ml) at room temperature. The filters were washed twice in 2× SSC, 0.1% SDS, then twice in 0.1× SSC, 0.1% SDS for 10 min at room temperature with vigorous agitation.

The washed filters were blocked for 20 min at room temperature in blocking buffer (100 mM Tris-HCl, pH 7.5, 1.0 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100, 3% BSA). The blocking solution was replaced by 1 μg/ml avidin–alkaline phosphatase in the same buffer and incubated for 10 min at room temperature. The filters were washed twice in blocking buffer for 10 min, then in developing buffer (100 mM Tris-HCl, pH 9.5, 1.0 M NaCl, 5 mM $MgCl_2$) for 10 min. The results were revealed by adding fresh prepared substrates of BCIP (final concentration 0.2 mg/ml) and NBT (final concentration 0.3 mg/ml) after incubation for 20 min in the dark (Yap et al. 1988).

Specificity identification: To examine the specificity of the probe, various genomic DNAs were extracted by phenol/chloroform extraction and ethanol precipitation (Maniatis et al. 1982) from *P. falciparum* cultured *in vitro*, a *Plasmodium cynomolgi*-infected monkey, and a *Plasmodium berghei*-infected mouse. An oligonucleotide specific to *P. vivax* (Relf et al. 1990) (5'-CGGCCACCCTCCAGGACAGAAGACAGGTGTCCACC-3') was synthesized automatically. These DNAs were mixed with genomic DNA of *Anopheles* mosquitoes, spotted onto nitrocellulose filters, and hybridized to ^{32}P-nick-translated pBF4. The results were demonstrated by autoradiography.

Examination of sensitivity: Plasmodium falciparum genomic DNA was extracted from cultured parasites *in vitro* and diluted serially with distilled water, then dot-blotted onto nitrocellulose filters for hybridization. One *P. falciparum* sporozoite-infected mosquito was mixed with 9, 19, or 24 uninfected insects. Then the pooled mosquitoes were soaked in 100 μl, 200 μl, or 250 μl, respectively, of lysis buffer (2% SDS, 20% β-mercaptoethanol, 100 μg/ml proteinase K), and subsequently triturated completely with a pestle. Two microliters of each group were spotted onto the nitrocellulose filter for detection. Genomic DNA was extracted from pools of 50 mosquitoes with an infective rate of 20%, and resuspended with 400 μl distilled water. Two microliters of the DNA solution were spotted onto the nitrocellulose filter for analysis.

Comparison with dissection method: The same batch of infected mosquitoes was divided into 2 parts; one was used for dissection of the salivary glands for examining sporozoites, and the other part was used for DNA hybridization. These were done by different persons without communication with one another.

RESULTS

Clone containing repeated sequences: ^{32}P-labeled *P. falciparum* total genomic DNA was used as a hybridization probe to screen a *P. falciparum* genomic library under conditions that allowed detection of repeated sequences (Meinkoth and

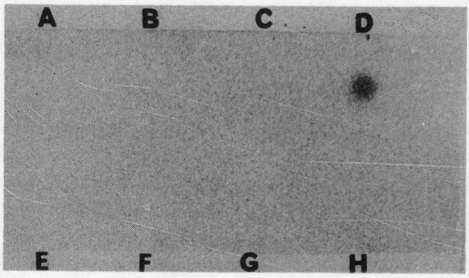

Fig. 1. Specificity of radiolabeled pBF4 DNA. A. *Plasmodium vivax* oligonucleotide fragment/*Anopheles dirus* mosquito DNA; B. *Plasmodium cynomolgi* genomic DNA/*Anopheles dirus* mosquito DNA; C. *Plasmodium berghei* genomic DNA/*Anopheles dirus* mosquito DNA; D. *Plasmodium falciparum* genomic DNA/*Anopheles dirus* mosquito DNA; E. Human blood cell DNA; F. Monkey blood cell DNA; G. Mouse blood cell DNA; H. *Anopheles dirus* mosquito DNA. (Amount of DNA per dot is 2 ng).

Wahl 1984). Approximately 0.26% of the clones contained repeated sequences (results not shown). After colony purification, the 5 plasmids producing the strongest signals were further analyzed. One of them, pBF4, contained an insert of 3 kb, determined by restriction enzyme analysis, and it hybridized to the 21mer oligonucleotide containing *P. falciparum* repeated sequence (5′-AGGTCTTAAC TTGACTAACA T-3′) described by Franzen et al. (1984).

Specificity and sensitivity: When samples containing different sources of parasite DNAs were hybridized with the ^{32}P-nick-translated pBF4, only the sample containing *P. falciparum* DNA gave a positive signal, whereas the others did not cross-hybridize to labeled pBF4 (Fig. 1).

When *P. falciparum* genomic DNA extracted from cultured parasites *in vitro* was dot-blotted onto nitrocellulose filters, 2 pg DNA could be

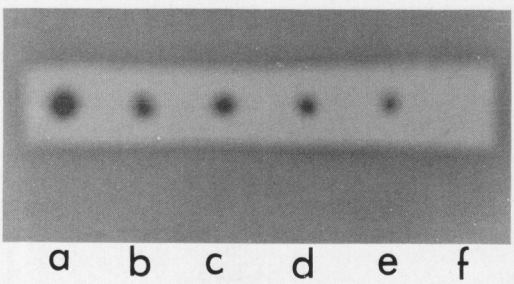

Fig. 2. Sensitivity of biotinylated DNA. Hybridization of the biotinylated pBF4 DNA to *Plasmodium falciparum* genomic DNA. a, b, c, d, e, f: represent 1,000 pg, 100 pg, 50 pg, 20 pg, 2 pg, 1 pg of DNA, respectively.

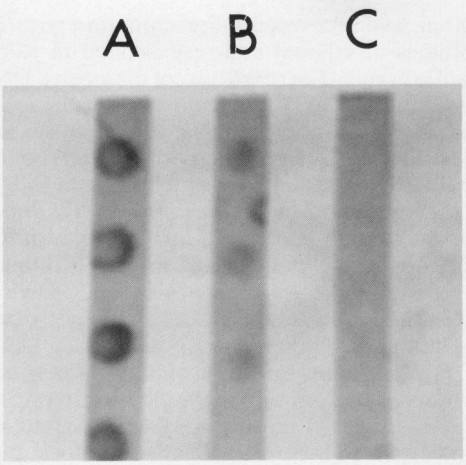

Fig. 3. Hybridization of biotinylated pBF4 DNA to triturated mosquitoes. A. Pellet of triturated infected mosquitoes; B. Supernatant of triturated infected mosquitoes; C. Triturated uninfected mosquitoes.

detected by the biotinylated pBF4 probe (Fig. 2). When 2 μl of the triturated sample with different mixture contents was spotted onto the nitrocellulose filter and hybridized with the biotinylated pBF4 probe, the positive blue spot could be observed in the samples of 10, 20, and 25 mosquitoes, which indicated that one infected from 24 uninfected mosquitoes could be detected (data not shown). When 2 μl of the DNA solution out of the 400 μl derived from 50 pooled mosquitoes with an infection rate of 20% was hybridized with the pBF4 biotinylated probe, a positive blue spot was easily observed, which corresponded to one infected mosquito from 40 uninfected mosquitoes.

Effect of sample preparation on the hybridization: After the dried mosquito samples were triturated, the supernatant and pellet (sediment) were spotted onto the nitrocellulose filter and subsequently hybridized with biotinylated pBF4 DNA. A distinctive purplish blue dot appeared in both pellet and supernatant of the sample, with the darker color in the pellet (Fig. 3). An individual dried infected mosquito squashed directly onto a nitrocellulose filter gave an easily detectable deep blue dot (Fig. 4A). Uninfected mosquitoes did not show this blue reaction background; only stains from mosquito debris were found (Fig. 4B).

Dissection vs. DNA probe detection: Two hundred eleven mosquitoes were examined by DNA hybridization, in which 114 showed a positive dot, with the positive rate of 54.0%. However, in the 189 mosquitoes that were dissected, 68 were positive, giving a positive rate of 36.0%. The infection rate using the hybridization meth-

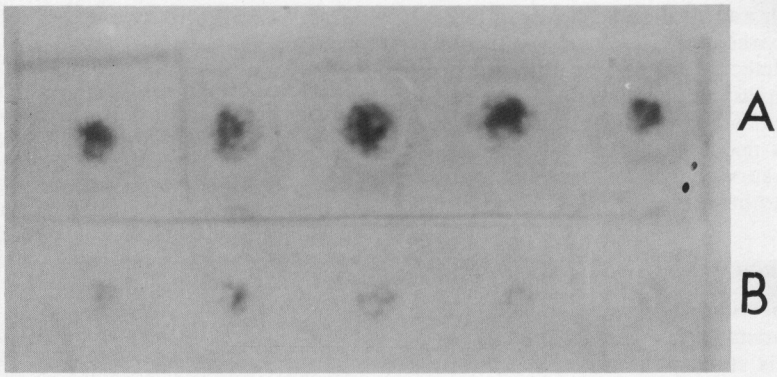

Fig. 4. Hybridization of biotinylated pBF4 DNA to single mosquito squashed on NC filter. A. Infected mosquito; B. Uninfected mosquito.

od was higher than that found in the dissection method ($\chi^2 = 13.1$, $P < 0.01$). The positive rate for the hybridization method represents the overall parasites present in the mosquitoes, whereas the positive rate in the dissection method represents only the sporozoites present in the mosquito salivary glands.

Mosquito portion test: The head portions (head plus thorax) and the body (abdomen) portions of the mosquitoes were examined with the biotinylated pBF4 DNA probe. In 32 mosquitoes tested, 10 gave positive spots for the head portions (31.3%), whereas 8 showed a positive reaction for the body portions (25%). This reflects the time difference of sporozoites entering the salivary glands of mosquitoes.

Comparison of biotinylation and radiolabels: The same batch of samples from different preparations was spotted onto nitrocellulose filters and hybridized in parallel either with the biotinylated or radiolabeled pBF4 DNA probes. As mentioned above, for the biotinylated probe hybridization there was no difficulty in distinguishing infected from uninfected mosquitoes using the different sample preparations. For radiolabeled probe hybridization, the infected mosquitoes produced a clear signal dot on X-ray film after exposure, but the uninfected mosquitoes did not. It was easier to distinguish uninfected mosquitoes from infected mosquitoes with the radiolabeled probe than with the biotinylated probe if the film was exposed for 72 h. However, the visibility of the biotin-labeled probes was even higher than that of radiolabeled probe if the exposure time for the radioprobe was limited to 4 h.

DISCUSSION

We have demonstrated here the successful application of a nonradioactive, biotinylated cloned genomic DNA probe for the identification of *P. falciparum* sporozoites in mosquitoes. The DNA probe used here is very specific, which is highly consistent with the oligonucleotide reported by Franzen et al. (1984) and has no cross hybridization with a specific *P. vivax* repetition (Relf et al. 1990). The cloned DNA in the plasmid is easy to prepare according to need and is more economical than synthesis of the oligonucleotide. The dry mosquito specimens used for hybridization have an important advantage because it allows mosquitoes captured in the field to be carried to the laboratory for batch detection. Using a nonradiolabeled probe in the assay will prevent harm to operators from a radiolabeled reagent, and provide convenience for storage and transportation to the field.

The traditional microscopic detection of sporozoite infection can neither distinguish the parasite species nor give high sensitivity. Lombardi et al. (1987) reported that light microscopy failed to reveal less than 500 parasites in salivary glands. The failure of microscopic detection of a proportion of infected mosquitoes could be particularly important in areas where the malaria vectors frequently carry a low parasite load. The enzyme immunobinding assay has a sensitivity of detecting 5–10 sporozoites, but it needs 100 μl per spot (Oprandy and Long 1990); such a large volume seems difficult to spot onto the membrane manually. In our study, using 2 μl of sample per spot, we can detect as little as 2 pg *P. falciparum* DNA, which corresponds to 100 sporozoites if one parasite contains 0.02 pg DNA (Gutteridge et al. 1971), indicating that the assay is very sensitive and may detect all infected mosquitoes because the average *P. falciparum* sporozoite load per infected mosquito is about 4,000 as reported by Burkot et al. (1987).

The results shown here demonstrate that when mosquito samples are soaked in sodium dodecyl

sulfate solution and then squashed on nitrocellulose filters, parasite DNA within the mosquitoes is easily detected, which simplifies the processing of samples, and enables the detection of individually infected mosquitoes. If a large number of samples needs to be screened in an epidemiological survey, the triturating method should prove to be very efficient and rapid.

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