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Usefulness of a New DNA Diagnostic Method for Malaria; Microtiter Plate-Hybridization

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For malaria diagnosis, microscopic examination of Giemsa-stained blood films has been used. Although microscopic examination is simple and does not require highly equipped facilities, it is time consuming, laborious, and tend to mislead in identifying parasite species, especially in cases of low-grade parasitemia, mixed-parasite infection, or parasite-degeneration by drug treatment.

As an alternative approach to the diagnosis of malaria, various methods which detect parasite-specific antibody in sera have been reported. However, they are of limited value because a patient may not generate enough antibody titer in the acute febrile stage, nor can we discriminate between present and past infections, even if antibody titer is high.

Several researchers have introduced various DNA probe-based, RNA probe-based or PCR-based diagnostic methods for detection of malaria parasites. However, these methods require complicated manipulations or handling of radioisotopes, which make them impractical under clinical or field conditions.

We have developed a new PCR-based diagnostic method "microtiter plate-hybridization" (MPH) for detection of human malaria parasite. Its procedure is as follows. Ten microliter of blood was collected by a finger prick, and suspended in 150 μl of PBS containing nystatin and gentamicin in a microcentrifuge tube. Blood was hemolyzed by adding saponin solution. The mixture was centrifuged and the supernatant was removed. The above centrifugation was repeated after dissolving the pellet with 200 μl of PBS. Destruction and proteolysis of the parasites were carried out by adding the lysis solution containing proteinase K and detergents. A pair of oligonucleotide primers were designed for the amplification of the conserved region of the gene coding for the 18S small subunit ribosomal RNA. Species-specific oligonucleotide probes were also designed and immobilized on microtiter wells. After heat inactivation of proteinase K, the target sequence of malaria parasites in human blood was amplified by the polymerase chain reaction (PCR) and the PCR-amplified product was captured by the species-specific probe on the microplate well. The biotin-streptavidin system was used to detect the captured materials. Positive samples gave yellow color by the chromogenic reaction.

MPH has been evaluated in clinical conditions of imported malaria. We obtained blood samples by finger puncture from 201 donors. The patients enrolled in this study consulted the Department of Infectious Diseases and Applied Immunology, Institute of Medical Science, University of Tokyo, between January 1992 and March 1997. They were classified as malaria-positives or negatives by Giemsa-staining microscopy. And the results of microscopic examination were compared with those of MPH method. The results of our MPH method were highly sensitive and specific for the detection of the four species of human malaria parasites.

Among the 201 cases tested, 63 cases were positive for *P. falciparum*, 41 were positive for *P. vivax*, 16 were positive for *P. ovale*, 2 were positive for *P. malariae*, and 74 were negative by MPH. Four cases were diagnosed as *P. ovale*-variants. The diagnostic results obtained by MPH method completely accorded with those obtained by Giemsa-stained microscopy, except one case which was diagnosed as *P. vivax*-positive by microscopy, but as negative by MPH method. This discrepancy was probably due to the fragility of gametocytes, only which were present in the blood sample.

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We have also performed an epidemiological research using our MPH method in Vietnam. During July to August in 1994 and August in 1995, we collected a total of 229 malaria-positives from 780 symptomatic and asymptomatic patients by the acridine orange microscopy. It was demonstrated that our MPH method is useful for mass-diagnosis in an epidemiological research.

Furthermore, we can find a new type of human malaria parasites comparing with results by microscopy and MPH method. If malaria parasites are microscopically positive but MPH-negative with corresponding probes, they are potentially new variants of human malaria parasites. We show here the usefulness of this system, and also report the discovery of a variant of *P. ovale*, whose sequence of the 18S ribosomal RNA gene differs from that of the typical sequence. Using MPH method, we found 5 cases of *P. ovale*-variant, whose PCR-amplified DNA did not hybridize with the probe for typical *P. ovale*.

We conclude that microtiter plate-hybridization is a simple and sensitive detection system, which does not require the prior DNA extraction from the blood nor the use of radioisotopes either. It should also be emphasized that our DNA diagnosis method does not depend upon the skill of individual examiners, making it useful in malaria control programs. Our MPH system has the advantages of rapid diagnosis and detection of new human malaria parasites. MPH is useful not only for clinical management, but also for epidemiological research.