RESEARCH NOTE

DIAGNOSIS OF MALARIA BY ENZYMATIC DETECTION OF POLYMERASE CHAIN REACTION PRODUCTS

Y Normaznah[1], T Furuta[2], K Saniah[1], A Noor Rain[1], S Kojima[2] and JW Mak[1]

[1]Biotechnology Center, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia;
[2]Department of Parasitology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan

Conventional laboratory diagnosis of malaria is by microscopic examination of stained blood smears. Although the method is simple and cheap, it requires a skilled microscopist, is subjective, and not suitable for the screening of large numbers of blood samples. New methods are continuously being developed for epidemiological studies and for monitoring the effectiveness of control programs. Detection of parasite DNA with DNA probes as an alternative method for malaria diagnosis has been developed (Boonsaeng *et al,* 1989). Recently, amplification of plasmodial DNA by polymerase chain reaction (PCR) using specific primers for sequences of the small subunit ribosomal RNA (ssRNA) gene from *Plasmodium falciparum* and *Plasmodium vivax* was found to be a highly sensitive technique for detection of parasite DNA (Snounou *et al,* 1993). Demonstration and identification of PCR products are usually be gel electrophoresis and ethidium bromide staining, Southern hybridization and dot blot hybridization. Although these techniques are reliable, they are not suitable for routine diagnosis and large scale epidemiological study. The enzymatic colorimetric method (ED-PCR) for detecting PCR products using microplate has recently been developed (Ubukata *et al,* 1992).

In this study, a rapid and simple method based on PCR using specific primers of ssRNA gene from *P. falciparum* and *P. vivax* sequences, and ED-PCR was evaluated. One *P. falciparum* isolate cultured *in vitro* in the Institute for Medical Research using the standard technique (Trager and Jensen, 1976) and 9 specimens from malaria infected patients from Kuala Lumpur Hospital and Gombak Hospital, Selangor, were used to examine the specificity and sensitivity of the method. The patients were diagnosed microscopically by hospital laboratory technicians using thick and thin blood films stained with Giemsa. The parasite density was determined by counting the number of parasites present in relation to the number of white blood cells. The number of parasites was counted against a total of 500 white blood cells, and the parasite density was calculated based on the white blood cell count. The confirmation of the parasite species was based on morphological characteristics of the parasites in thin blood films. Blood films were considered negative for malaria when no parasite was detected in 200 microscopic fields. Negative control samples were obtained from 8 healthy individuals who had no malaria parasites. Out of 9 positive samples, two were infected with *P. falciparum* with parasite counts between 800-182,400 (asexual stage)/0 (sexual stage) per µl of blood, and 7 were positive for *P. vivax* with parasite counts 800-6, 880/0-480 per µl of blood. Ten µl of blood was taken from each sample for DNA extraction following the method published previously (Wataya *et al,* 1993). Briefly, the blood was treated with 0.15% saponin, and centrifuged at 10,000 rpm for 10 minutes. The pellet was subsequently resuspended with lysis solution containing 200 µg proteinase K per ml. Ten µl of PCR mixture containing 1 unit of Tth DNA polymerase (Toyobo Co Ltd, Japan) and 0.76 µM each of 2 biotinylated primers (5'-CAGATACCGTCGTAATCTTA-3' and 5'-CCAAAGACTTTGATTTCTCAT-3') was then added, giving the final volume of 50 µl. This set of primers amplifies a 138 base pair fragment of small subunit ribosomal RNA (ssrRNA) common to both *P. falciparum* and *P. vivax* (McCutchan *et al,* 1988; Waters and McCutchan, 1989) and the labeling of primers was performed using Aminolink II (Applied Biosystem, USA) for synthesis; this was followed by purification and a reaction with biotin N-hydroxy-succinimide ester or dinitrofluorobenzene (Coull *et al,* 1986). PCR was run with the amplification cycle of 15 seconds at 94°C, 15 seconds at 50°C and 15 seconds at 72°C for a

total of 30 cycles by a DNA thermal cycler (Perkin-Elmer Cetus, Model 9600, USA). The PCR products were then added into each well of streptavidin-immobilized microplate containing a solution of anti-dinitrophenol antibody conjugated with alkaline phosphatase. After incubation, the plate was washed and the substrate solution (p-nitrophenol phosphate solution) was added into each well. The microplate was incubated for 30 minutes and then measured for absorbance at 405 nm.

The distribution of optical density values (OD) for the specimens tested is shown in Fig 1. The mean optical density (OD) of 8 negative samples was 0.079 ± 0.007. The cut-off point for sensitivity was taken as 0.100 (mean OD $\pm$ 3 standard deviation). The ten positive samples including cultured *P. falciparum,* all showed higher OD values than the cut-off point. The mean OD for all microscopically positive samples was 0.354 ± 0.162. From the results of this limited study, the sensitivity and specificity of the method was 100%, when compared with the results of microscopic examination. The usual diagnosis of malaria is by microscopic examination of stained blood films, and its detection level is 20-40 parasites per μl of blood (Bruce-Chwatt, 1984). The lowest detection level of our method was 10 parasites/μl of blood (data not shown), and therefore it was thought that this detection level is sufficiently sensitive enough for routine diagnosis of malaria. Recently, new methods using specific probes for malaria parasites were developed to diagnose malaria, and generally, their sensitivity and specificity are high. However, these methods require complicated manipulations and handling of radioactive isotopes, which are not suitable for clinical or field conditions in endemic areas. In our method, detection of the PCR products was performed by standard enzyme-linked immunosorbent assay, this permits the examination of a lot of specimens at the same time, thus saving time required for routine clinical diagnosis.

In conclusion, these preliminary results show that this method is quite sensitive, specific and useful for routine diagnosis of malaria.

ACKNOWLEDGEMENTS

We would like to thank the Director, Institute for Medical Research, Kuala Lumpur for permission to publish and the Director, Gombak Hospital for permission to collect the samples. Thanks are also due to Mr Y Osada and Dr K Kita, Institute of Medical Science, the University of Tokyo, Dr Akio Yamane, Institute for Biotechnology Research, Wakunaga Pharmaceutical Co Ltd, and Dr Hiroshi Tanaka, Chief Advisor for IMR-JICA projects for their useful discussion, advice and support. The study was performed as part of the Institute for Medical Research and Japan International Cooperation Agency Research Project on Tropical Diseases.

REFERENCES

Boonsaeng V, Chansiri K, Vilasineekul P, *et al.* Detection of *Plasmodium falciparum* using a cloned DNA probe: A simple procedure suitable for field application. *Southeast Asian J Trop Med Public Health* 1989; 20 : 519-22.

Bruce-Chwatt LJ. DNA probes for malaria diagnosis. *Lancet* 1984; 1 : 795.

Coull JM, Weith HL, Bischoff R. A novel method for the introduction of an aliphatic primary amino group at the 5'-terminus of synthetic oligonucleotides. *Tetrahedron Lett* 1986; 27 : 3991-4.

McCutchan TF, de la Cruz VF, Lal AA, *et al.* Primary sequences of two small ribosomal RNA genes from *Plasmodium falciparum. Mol Biochem Parasitol* 1988; 28 : 63-8.

Snounou G, Viriyakosol S, Jarra W, *et al.* Indentification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of high prevalence of mixed infection. *Mol Biochem Parasitol* 1993; 58 : 283-92.

Trager P, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; 193 : 673-5.

Ubukata K, Nakagami S, Nitta A, *et al.* Rapid detection of the mecA gene in Methicillin-resistant Staphylococci by enzymatic detection of polymerase chain reaction products. *J Clin Microbiol* 1992; 30 : 1728-33.

Wataya Y, Arai M, Kubochi F, *et al.* DNA diagnosis of falciparum malaria using a double PCR technique; a field trial in the Solomon Islands. *Mol Biochem Parasitol* 1993; 58 : 165-7.

Waters AO, McCutchan TF. Partial sequence of asexually expressed SU rRNA gene of *Plasmodium vivax. Nucleic Acids Res* 1989; 17 : 2135-6.