Recent Diagnostic Methods of Malaria and Filariasis in the Department of Parasitology, Faculty of Medicine University of Indonesia, Jakarta, Indonesia

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Abstrak

*Penyakit parasitik masih merupakan masalah kesehatan masyarakat di Indonesia. Selain penyakit kecacingan yang ditularkan melalui tanah, masih terdapat penyakit **malaria** dan **filariasis** yang tersebar luas di berbagai daerah di Indonesia. Pada makalah ini akan dibicarakan terutama mengenai aspek diagnostik kedua penyakit tersebut. Diagnostik **malaria** terutama didasarkan atas hasil pemeriksaan mikroskopik darah tepi, baik sediaan darah hapus atau sediaan darah tebal yang dipulas dengan Giemsa dengan menemukan parasitnya. Selain cara pemeriksaan konvensional ini, terakhir terdapat tes serologi yang dipakai untuk penelitian sero-epidemiologi malaria seperti IFA (Indirect Fluorescent Antibody) dan RESA-IFA (Ring Erythrocyte Surface Antigen - IFA) yang sudah dipakai di Bagian Parasitologi, FKUI, dan telah diterapkan di beberapa daerah di Indonesia dengan hasil yang cukup baik. Selain itu pemeriksaan dengan teknik QBC (Quantitative Buffy Coat) menunjukkan hasil positif yang lebih tinggi dibanding dengan cara konvensional. Teknik Kawamoto yang menggunakan mikroskop biasa ditambah dengan beberapa macam filter ternyata merupakan alat yang cukup murah dan canggih untuk menemukan parasit mallaria pada sediaan darah malaria yang konvensional. Teknik ini akan dicoba di Bagian Parasitologi, FKUI. Untuk diagnosis **filariasis**, selain cara konvensional telah dikembangkan beberapa teknik diagnostik yaitu : 1. digunakannya antibodi monoklonal yang spesies dan stadium spesifik untuk menemukan larva Brugia malayi dalam tubuh nyamuk 2. penggunaan prob DNA radioaktif dan non-radioaktif untuk menentukan diagnosis spesies mikrofilaria B. malayi pada manusia dan kucing, merupakan alat penting dalam rangka pengendalian pemberantasan filariasis. Selain itu penggunaan teknik PCR (Polymerase Chain Reaction) sangat bermanfaat demi peningkatan sensitivitas penemuan parasit pada tes "Assay" berdasarkan prob DNA.*

Abstract

Parasitic disease are still considered as a public health problem in Indonesia. Besides the soil-transmitted helminth infections, there are still some parasitic diseases such as malaria and filariasis which are widely distributed in many areas in Indonesia. In this paper, a description mainly on the diagnostic aspects of these two diseases will be given. The diagnosis of malaria is mainly based on the microscopic blood examinations, whether thin blood smear as well as thick blood smear stained with Giemsa, with the aim to find the parasites. Besides these conventional methods, there are serological tests which are used for sero-epidemiological study for malaria such as IFA (Indirect Fluorescent Antibody) and RESA-IFA (Ring Erythrocyte Surface Antigen - IFA) and have been used in the Department of Parasitology, Faculty of Medicine, University of Indonesia; these tests have been applied for field studies in several areas in Indonesia with good results. On the other hand, a QBC (Quantitative Buffy Coat) technique showed higher number of parasites detection in comparison with the conventional method. Besides those methods, there are still the Kawamoto technique which utilized an ordinary microscope equipped with additional filters seems to be a cheap and sophisticated instrument which could be used for malarial parasites detection in ordinary conventional blood smears. This technique will be applied in the Department of Parasitology, Faculty of Medicine, University of Indonesia. For the diagnosis of filariasis, besides the conventional method, some other techniques have been used such as: 1. the use of monoclonal antibody which are stage and species specific for finding the Brugia malayi larvae in mosquitoes 2. the use of radioactive and non-radioactive DNA probe to make the diagnosis of B. malayi microfilariae in human and cat, seems to be an important tool for monitoring the control of filariasis. In addition, the use of PCR (Polymerase Chain Reaction) technique is very useful in increasing the sensitivity of parasite detection in Assay tests based on DNA probe.

Keywords *: Serologic test, Malaria; Monoclonal antibody - DNA probe, Filariasis*

INTRODUCTION

Recently some parasitic diseases are still considered as one of the major public health problems in Indonesia. Besides the "soil- transmitted helminthiasis", we still have problems with **malaria** and **filariasis.** This paper will discuss mainly the diagnostic aspects of the two diseases.

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MALARIA

Malaria is still a public health problem in Indonesia. Before the use of DDT in 1959, no single area in Indonesia was free of malaria, with the exception of the high mountainous areas. The Malaria Control Program which was named Malaria Eradication Program ("Komando Pembasmian Malaria" or KOPEM) was started in 1959 by utilizing DDT spraying in the areas of Java, Bali and Madura. This program had succeeded in reducing the number of malaria cases in Indonesia up till its cessation in 1968. After that period, malaria cases increased gradually. In 1968, the Malaria Control Program was integrated into the National Health System. So the control activities were mainly focussed in Java, Bali and other islands which consisted of transmigration, production and development areas as well as the border areas in Indonesia. Although many achievements have been established during the fourth Five Years Development Plan, where the situation of malaria in Java and Bali was under control, but outside that area, especially in the Eastern part of Indonesia, the morbidity as well as the mortality of malaria were still high, and the main causative agent was *Plasmodium falciparum.*

Diagnosis

The specific diagnosis is mainly based on microscopic examination by using the conventional **Giemsa stained thick and thin smear technique.** These techniques provide us to identify the species as well as giving us the viability picture of the parasites in the blood. The drawback of this technique is that it required an experienced technician to do the job; in addition a longer time is needed to identify the parasite. Besides that, it is not efficient for surveying malaria in areas with low transmission. So, based on the following facts new diagnostic techniques were developed, namely the serological tests (the IFA test and RESA-IFA test) and the **QBC (Quantitative Buffy Coat)** technique; in addition a modified fluorescent microscope technique of Kawamoto will be applied.

The serological tests

The **IFA test** was used for sero-epidemiological study of malaria in a hypo-endemic area in Central Java, Indonesia.[1] As antigen, the *Plasmodium falciparum* Flores strain was used in this study, which were collected from the in vitro continuous culture of *P. falciparum* in our laboratory.

The test sera were collected from the population living in a hypoendemic area in Jelamprang and Besani villages, Central Java. As a negative control, thirty sera collected from people who had never visited malaria endemic areas were used. The conjugate used was the goat anti-human IgG-fluorescein Isothiocyanate produced by Sigma. The IFA technique used in this study was the modification method of Collins et alin 1964.[2] All the slides were examined by means of a fluorescent microscope. A total of 284 sera were examined by this technique, which consisted of 177 females and 107 males with ages varying from 15 months up to 90 years. The results showed that the positive titres varied between 1:200 to 1:3200, and the largest number found showed the 1:200 titre (57 sera), whereas there were only 10 sera which showed the highest titer of 1:3200; among them 2 were from the under - 15 years age-group, and the other 8 were from the above-15 years age-group. The total number of positive sera was 127 (44.7 %) from the 284 sera examined, among which 24 sera (21.6 %) were from the under 15 years age-group, and 103 sera (58.9 %) were from the above-15 years age-group. All the people who showed splenomegaly had positive IFA test, whereas the people who showed parasitemia, only 7 (87.5 %) had positive IFA.

The **RESA-IFA test** was used for the sero-epidemiological study of malaria in three different areas in Indonesia, namely the hypoendemic, mesoendemic and hyperendemic malaria areas.[3] For hypoendemic area the same villages namely the Jelamprang and Besani in Central Java were chosen, and for the mesoendemic and hyperendemic malaria areas, the villages in Flores were taken as the study area. For the mesoendemic, the Sante Piso and Sante Lemarang villages in Flores, and for hyperendemic, the Jengkalang village in Flores was chosen. For the antigen, the ring-stage (young trophozoit) ot *P. falciparum* was used in this test, which came from the same in vitro culture in the laboratory, with a parasite concentration of 3 - 5%. The sera were collected from the above mentioned endemic areas, and kept in the freezer with a temperature of minus 20°C. The negative control sera used were similar to the IFA test. The RESA-IFA test used in this study was the modified method of Perlmann et al.[4] A special object-glass was used in this technic; the antigen which was dropped on it was then reacted with the sera from the population diluted with PBS pH 7.2 which contained 0.05 % BSA. The initial dilution used was 1 : 5, and followed by 5 times dilution. The conjugate used was similar with the previous test.

The results showed that all the 30 negative control did not show positive results. From a total of 301 sera from the hypoendemic area examined, one hundred

and one sera (36.5 %) were positive. The geometric mean of the seropositive titres was 12.38. In general the seropositive value increased in the above 26 years age-group. The highest seropositive value was found in the above 40 years age group (53.5 %). The highest titre was 1 : 125, and was found in the above 40 years age group. From the 162 sera of the mesoendemic area examined, ninety nine sera (61.1 %) were positive. The mean geometric seropositive titre was 97.95. In general, the seropositive rate increased by age, starting from the above 11 years age-group. The highest seropositive rate was found in the 36-40 years age group. The highest titre was 1 : 3125 and was found in the above 40 years age-group.

From the hyperendemic area, there were 201 people examined, and one hundred forty six sera (72.6 %) were positive. The mean geometric seropositive titre was 133.55. In general the seropositive rate increased, starting from age 0, and the highest number was found in the above 40 years age group (96.3 %). The highest titre was 1 : 3125, and was found in the 40 years age group.

The QBC (Quantitative Buffy Coat) technique

The QBC technique was introduced by the Becton Dickinson and Co (Franklin Lakes, NJ, USA). This diagnostic test was based on the centrifugal stratification of haematoparasites; in this way parasites were concentrated in the erythrocyte layer of the buffy coat, and were easily detected after staining with acridine orange. This technique for detecting malaria is based on a high- precision glass capillary tube, containing an equally precise plastic float. When the blood-filled tube is centrifuged for five minutes, the float is buoyed by the packed red cells, automatically positioning it within the buffy coat layer between the packed red cells and the plasma. Under centrifugation, blood cells in the buffy coat separate according to their densities, forming visibly discrete bands. Because of their density, malaria and other haematoparasites separate and concentrate in a predictable area of the tube. A reagent coated in the QBC tube promote the separation of these cells and provide staining to enhance their visibility under blue violet light. Examination of the centrifuged blood under a fluorescence microscope readily permits the detection of malaria parasite in infected red cells. In this way, malaria parasites area easily seen and it takes a few seconds to detect, and this can be carried out by untrained personnel who have undergone a few hours of training. Besides its superiority, this technique is very expensive, as it needs highly sophisticated instruments to work with.

In the Department of Parasitology, Faculty of Medicine, University of Indonesia, **QBC** technique was initiated for the diagnosis of malaria and compared with the conventional method.[5] This technique was done to screen blood samples collected from malaria endemic area, namely the Berakit village, North Bintan Regency, Riau Province. A total of 495 blood samples were examined. The results showed that 104 samples were positive by QBC as well as thick blood smear. Nine samples were found negative by QBC, but positive by thick blood smear. One hundred and sixty samples were positive by QBC and only 82 samples were positive by thick blood smears.

In contrast, the **Kawamoto technique** is very cheap, as this technique utilized an ordinary light microscope additionally equipped with an interference filter in the diafragma and a barrier filter in the eyepiece. By this technique, an ordinary thin or thick blood film stained with acridine orange directly by adding one drop of the AO solution onto the fixed blood film. In this way examination of the blood smear could be done instantly by using direct sun-light or halogen light as light source. The parasite were also easily detectable by observing the yellow green nuclei and orange stained cytoplasma among the dark background.

FILARIASIS

Filariasis is still a public health problem in Indonesia, with many endemic foci spread over the whole country. More than 20 million people are at risk of acquiring the infection, and 3-4 million people were estimated to be infected. So far 3 species consisting of at least 5 epidemiologically different types have been confirmed infecting man. Both urban and rural types are confirmed in small areas only, with *Culex guinguefaciatus*, while the rural type is widely distributed, with Anopheles and Aedes as vectors. *Brugia malayi* has been found strictly rural. There are two types, namely the zoophylic type which may infect animals has Mansonia mosquitoes as vectors, while the anthrophophylic type has mostly *Anopheles barbirostris* as vector. *Brugia timori* so far has been found endemic in the southeastern part of the Archipelago and involves the provinces of Nusa Tenggara Timur, East Timor and the southern part of Maluku.

Filariasis is mostly a problem in rural areas, effecting the people of the low socio-economic group. It is also a lowland diseases but may be found in some of the hilly areas.

The impact of the infection to human health is mainly concerned with the lymphatic system. Acute

signs consist of fever, lymphadenitis, lymphangitis which recurred every month, usually several years elapsed after the acute infection. The chronic signs are lymphedema, hydrocele, chyluria, and elephantiasis. The disease is not fatal, but will cause suffering and disability to patients resulting in a decrease of total production.

Diagnosis

The diagnosis of filariasis was based on the discovery of microfilaria in **thick blood smears** with Giemsa stain. The time of collection of blood samples was done in relation to the periodicity of the parasite. Similar to malaria, this conventional method is time consuming, and needs experienced health personnel to do the job. The recent development of monoclonal antibodies and recombinant technology of DNA have made it possible to apply these new tools in the diagnosis of filariasis especially in the study of the epidemiology.

Field testing of a stage and species specific monoclonal antibody

A stage and species specific monoclonal antibody to detect a unique surface epitope of *B. malayi* infective larvae has been developed by the New England Biolabs.[6] An ELISA field assay was developed to identify infective larvae of *B. malayi* in vector mosquitoes. The sensitivity of detection of this assay was in the range of 70 to 80 % if the monoclonal antibody was incubated with the parasites for 1 hour in the laboratory in Jakarta, which was slightly lower than that obtained at the base laboratory. By increasing the incubation time to two hours the sensitivity was increased to more than 90 %. The monoclonal antibody did not cross-react with the infective larvae of *B. pahangi,* but did so with the non-sympatric L3 of *B. timori.* The reagent was field tested in South Kalimantan in an area endemic with *B. malayi* and enzootic with *B. pahangi,* where DEC control measures against brugian parasites in humans have been repeatedly applied. More than 3000 potential vectors were dissected for filarial larvae. Only 131 infective larvae were recovered and these small fraction of them were identified as *B. malayi.* This is a good example of how the use of modern technology can help endemic countries to cost-effectively control brugian filariasis.

Field testing of filarial specific DNA probes

A DNA probe to detect Brugian parasites has been developed by the New England Biolabs.[7] Based on the region of divergence of the *Hha*I repeat of the *B. malayi* (Bm) and *B. pahangi* (Bp), a 29-mer *Bm* specific and a 21-mer *Bp* specific oligonucleotide probes were developed.[8] These probes were radiolabeled with P32 and used in a dot blot assay to detect brugian microfilariae from donors in South Kalimantan, where *Bp* had been reported to occur in humans.[9] The tests were blindly performed using *Bm* and *Bp* from experimentally infected animals as control, and the results compared with those obtained by parasitological examination of microfilariae. Two hundred human blood samples were tested by both methods, and the agreement was 97 %.

An additional 64 cat blood samples were also blindly tested by both methods and the agreement was 95 %.

Preliminary field testing to nonradioactive DNA probes

A variety of non-radioactive DNA probe have been constructed and tested under laboratory conditions. Most of the effort was concentrated on the development of biothinylated oligonucleotide probes. The initial radio-labeled *Bm* specific 29-mer oligonucleotide and the *Bp* specific 21-mer oligonucleotide DNA probes were able to detect as little as 0.1 ng of *Brugia* DNA (approximately equal to 1 mf) and are species specific.

Recently two new oligonucleotide probes were synthesized based upon the *Hha*I repeat region of divergence : a 45-mer *Bm*-specific and a 44-mer *Bp*-specific DNA probe. They were labeled with 45 biotinylated uridine residues added to the 5' end. The new probes were field tested on 43 human and 14 cat blood samples, collected from microfilaraemic donors on the island of Rampang near Tanjung Pinang.[10] This study used the polymerase chain reaction (PCR) to compare the sensitivity of radioactive and biotinylated species specific oligonucleotide probes. The cloning characterization of the *Hha* I repeat DNA family found in filarial parasites of the genus Brugia, and the development of species-specific probes for *B. malayi* and *B. pahangi* based on these repeats. The PCR amplified samples were accurately identified by both the radioactive and biotinylated *B. malayi* and *B. pahangi* probes. Even samples with as few as two microfilariae per 100 ul of blood were easily detected. The samples that were not PCR amplified were accurately identified after only long exposures (longer than one week) to the radioactive probes. The biotinylated probes were not sensitive enough for accurately identification of the non-PCR amplified samples. The

polymerase chain reaction is therefore a promising new tool for enhancing the sensitivity of parasite detection assays based on DNA probes. This will be especially important in designing an assay based on non-radioactive DNA probes.

DISCUSSION

From the results of IFA test carried out in the hypoendemic malaria areas, it shows that quite high positives were found (44.7 %) from a total of 284 samples examined. This might be due to the fact that the people in these areas were infected for quite a long time. The IFA test is very sensitive and can detect malaria antibody for up to 2 years.[11]

Concerning the sero-epidemiology tests of malaria, it was evident from the results that in RESA-IFA test the seropositive rate in hypoendemic areas was much lower in comparison with that of the hyperendemic areas, and the difference was significant. In hypoendemic areas where the malaria transmission was low, the seropositivity rate increased, starting from age 26 or older, whereas in hyperendemic areas, the seropositive rate increased starting from younger age groups.

It seems that the result of QBC is much higher in comparison with the conventional method; this is due to the higher sensitivity of this test. On the contrary, the specificity was found to be lower in comparison with the conventional method.

The use of monoclonal antibodies and recombinant technology of DNA seems to be a new tool in the diagnostic aspect of filariasis. The field testing of a stage and species specific monoclonal antibody to detect *B. malayi* infective larvae in mosquitoes appears to be an excellent tool for the exact determination of the infective rates in vector mosquitoes, in comparison with the conventional method of dissection which revealed higher result more than ten fold, and those larvae found might consist of larvae from other species of Brugia.

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