DETECTION OF BRUGIA MALAYI INFECTED MOSQUITOES WITH SPECIES SPECIFIC DNA PROBE pBm 15, IN RIAU, INDONESIA

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ABSTRACT

A species specific DNA probe (pBm15) was used in a field area where 2 filarial infections coexist: *B.malayi* in man and *B.pahangi* in cats.

In our laboratory in Jakarta, this DNA probe proved to be sensitive enough to detect 500 ng DNA. One to two infective larvae of *B.malayi* could be detected with ease. This DNA probe did not react with infective larvae of *wuchereria bancrofti, B.pahangi,* and *Dirofilaria* spp.

Non specific binding caused by undefined mosquito components was overcome with proteinase K and chitinase treatment. This additional step, made it possible for whole body mosquitoes to be squashed directly onto nitrocellulose paper.

A comparative study of experimental infections of laboratory bred mosquitoes infected with *B.malayi,* showed no difference in infection rate between the group examined by dissection or by DNA probing.

Mosquitoes which are vectors in Riau were collected and fed on microfilaremic patients of Riau. The set of mosquitoes were tested in parallel with mosquitoes infected with *B.pahangi* from cats. All fed mosquitoes were tested after 10-12 days. Only mosquitoes infected with *B.malayi* reacted in the assay.

This study shows a success in applying the DNA probe technique in Jakarta. Further application in the field should be encouraged, with some modification of the DNA probing technique, for cheaper and easier implementation.

INTRODUCTION

Assessment of transmission of lymphatic filariasis depends on measuring the annual biting rate of the vector and the number of infective larvae found in the vector. It is obvious that identification of the infective larvae is very important. Failure of identifying the infective larvae will result in the wrong mosquito being incriminated as vectors, which further resulted in transmission studies and control programs being based on false premises[1].

Infective larvae of a different genus can be differentiated by an experienced scientist. Distinguishing infective larvae within a different genus by morphological criteria is dif-

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ficult or even impossible, which then necessitates infecting laboratory animals for identification of the characteristic adult worm of the species[1].

In areas where *B.malayi* and *B.pahangi*, which are trasmitted by the same mosquito vector, coexist, differentiating the infective larvae becomes a very important issue.

A DNA clone, pBm1 5 produced from microfilaria of the subperiodic *B.malayi* (maintained in laboratory animal), was shown to be sensitive enough to detect two infective larvae or 300 pg purified *B.malayi* microfilarial DNA[2]. Labelled clone pBm15 hybridized with infective larvae of *B.malayi*, but not to that of *B.pahangi*. It cross hybridized with microfilaria *B.timori* at a lower level than to microfilaria of *B.malayi*[3]. Cross hybridization of this clone to *B.pahangi* was at least 1000 times less sensitive than to DNA of *B.malayi*[2]. All the above mentioned hybridization was done with infective larvae revealed from individually dissected mosquitoes and blotted onto nitrocellulose paper.

For further use of this DNA probe in the field it is necessary to get rid of the non specific binding of the undefined components of mosquitoes squashed onto the nitrocellulose paper[3].

This species specific *DNA* probe pBm15 was used for examining the presence of *B.malayi* larvae in mosquitoes caught in the field and compared the results with the mosquito dissection method.

MATERIAL AND METHOD

Study location

This study was carried out in the village of Teluk Sejua and Dusun Tuo, in Riau province, where microfilarial rate (mf rate) of *B.malayi* in human was 21 % and mf rate in cats was 16%.

Test samples

Extract of microfilarial and infective larva DNA of *B.malayi*, infective larvae of *W.bancrofti* and *D.imitis*, non infected *Mansonia uniformis*, *Mansonia dives/bonnae* and *Aedes togoi*, were tested for hybridization with clone pBm15 to measure the sensitivity and specificity of the test carried out in our laboratory and to ensure that non specific binding did not occur.

Aedes togoi was fed with blood containing microfilaria of *B.malayi* (30 mf/20 mmc blood) by membrane feeding.

Wild caught *Mansonia dives/bonnae* and *M. uniformis* were fed to individuals harbouring microfilaria of *B.malayi* from Teluk Sejuo and Dusun Tuo.

These mosquitoes mentioned above were then kept for 10-12 days before examination by mosquito dissection or by DNA hybridization. Mosquitoes were divided randomly into two groups. The first group was dissected, infective larvae counted, followed by blotting the recovered infective larvae onto nitrocellulose paper. In the second group, mosquitoes were squashed directly onto nitrocellulose paper.

Spotting infective larvae and squashing mosquitoes onto nitrocellullose filters.

Infective larvae recovered from mosquito dissections were spotted onto nitrocellulose paper. (prewetted with 6 X SSC buffer: 0.9 M NaCl, 0.09 M Na citrate) using a minifold vacuum filtration apparatus (Biorad). Mosquitoes were squashed on prewetted nitrocellulose paper as well. These filters were air dried. DNA extracted from microfilaria of B. malayi and non infected mosquitoes served as positive and negative control in every test.

Samples were denaturated with 0.5 M NaOH and neutralized with 1 M Tris HCl pH 7.4 and 0.5 M Tris HCl pH 7.4 plus 1.5 M Na Cl. These filters were baked for 90 minutes at 70 degree Celcius.

Enzymatic treatment

Prepared filters were treated with 150 µg/ml proteinase K (Boehringer Mannheim 161 519), for 2 hours at 37 degree Celcius, washed with SSC 2X, dried and further treated with 100 µg/ml chitinase (Sigma, G1525) in 0.1 M Na citrate pH 6 for 24 hours at room temperature, washed with SSC 2X and dried before hybridization.

DNA hybridization

Samples were prehybridized for 2 hours at 42 degree Celcius in Denhart's (0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrolidone), 2.5X SSC, 100 µg/ml herring sperm DNA, 50 % formamide and 0.1 % SDS. The filters were then hybridized for 16 hours with nick translated P 32 labelled pBm15. After washing with 0.1X SSC, 0.5 % SDS for 3X 30 minutes at 50 degree Celcius and then air dried. Filters were then left in minus 20 degree Celcius exposed onto XAR-5 Kodak for 10-12 hours using an intensifying screen. Results seen on the X ray film, were evaluated.

RESULTS

Figure 1 shows the results of DNA probing of infected and non infected mosquitoes with clone pBm15. Non specific binding was shown by the uninfected mosquitoes.

Enzymatic treatment with proteinase K and chitinase avoided non specific binding caused by uninfected mosquitoes (figure 2).

Hybridization to extract DNA of microfilariae and infected larvae of *B.malayi*, infective larvae of *W.bancrofti* and *D.immitis*, non infected *Mansonia uniformis, Mansonia Dives* and *Aedes togoi* mosquitoes showed that hybridization occured only on *B.malayi* samples and had a sensitivity of 500 pg purified *B.malayi* microfilarial DNA and 1-2 infective larvae of *B.malayi*. These samples were treated with protein-K and chitinase and no specific binding to non infected mosquitoes was detected.

Two hundred ninety two alive *Aedes togoi*, 10-12 days after being fed with *B. malayi* microfilariae, were divided into two groups. One hundred forty six were examined by mosquito dissection method followed by DNA hybridization of the recovered infective larvae. The other 146 mosquitoes were squashed on nitrocellulose paper for DNA hybridization only. Sixty out of the 146 (41.1%) mosquitoes dissected showed the presence of infective larvae. Labelled clone pBm15 hybridized with all dot blotted infective larvae found by dissection. In the test performed one infective larvae was detected by the DNA probe as shown in table 1. No clear cut difference was seen in the autoradiograph that could show relationship of density of infective larvae and the spots on autoradiograph. Fifty three out of 146 (36.3 %) mosquitoes squashed directly on nitrocellulose paper showed hybridization with clone pBm15 (Table 1). No significant differences were observed between these two approaches ($p > 0.05$).

Eighty one of 162 wild caught mosquitoes fed to individuals harbouring microfilaria of *B.malayi* were dissected individually. Nineteen out of those 81 (23.5%) showed the presence of infective larvae. All 19 specimen showed hybridization with clone pBm15. Fifteen out of 81 (18.5 %) mosquitoes squashed hybridized with clone pBm15. No significant difference between

these two observations was seen ($p < 0.05$).

Six mosquitoes out of 22 (27.3 %) fed on cats infected with *B.pahangi* were found to harbour infective larvae, but none of them show any hybridization with clone pBm15. Mosquitoes fed on cats infected with *B.pahangi* which were squashed on nitrocellulose paper did not show any hybridization with this clone.

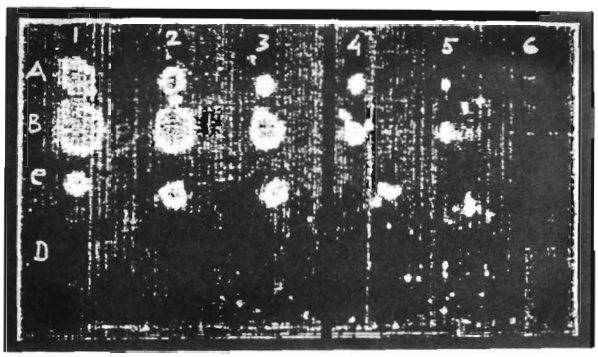

Figure 1. **Infected *B.malayi* mosquitoes (A,B and C) and uninfected mosquitoes were squashed onto nitrocellulose paper and hybridized with radiolabeled pBm15. Non specific binding of the pBm15 probe with uninfected mosquitoes was shown (D).**

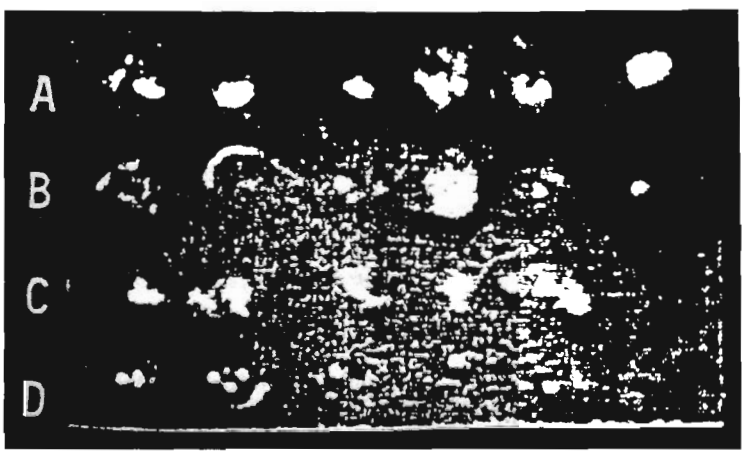

Figure 2. **Infected *B.malayi* mosquitoes (A and B) and uninfected mosquitoes were squashed onto nitrocellulose paper. Samples were treated with proteinase K (150 μg/ml, 2 hours, 37^0C) and chitinase (100 μg/ml, 24 hours, room temperature) and hybridized with radiolabeled pBm15. The enzymatic treatment avoided non specific binding (C).**

Table 1. Results of the presence of L3 of *B.malayi* by mosquito dissection method and hybridization to clone DNA pBm15 in mosquitoes infected with Brugia spp.

Number of L3/mosq.	Number of mosquitoes	Positive for the presence of L3	
		Disection method and hybridization	Squashed mosq. hybridization
0	86	0	
1	24	24	
2	12	12	
3	10	10	
4	12	12	
11	2	2	
	146	60/41.1%	53/36.3%

DISCUSSION

Enzymatic treatment with 150 µg/ml of proteinase K and 100 µg/ml of chitinase showed to be sufficient to avoid non specific binding. This treatment is very important when this technique would be used for examination of large numbers of mosquitoes in the field. Without this treatment, infective larvae should be released from individual dissected mosquitoes before the dissection fluid is transfered to nitrocellulose paper, which is quite cumbersome and ill suited for field conditions[3]. With enzymatic treatment mosquitoes could be squashed directly onto nitrocellulose paper in the field and carried back to the laboratory with ease.

Sensitivity and specificity of clone pBm15 were established in our laboratory and 500 pg of microfilarial DNA or 1-2 infective larvae DNA of *B.malayi* were detectable. Sim et al[2] could detect 300 pg of the microfilarial DNA or 2 infective larvae. With this level of sensitivity this test could be used for epidemiological studies as it is known that the vector of *B.malayi* mostly harbours 1-10 infective larvae[4]. The test was shown to be specific, it did not hybridize to filariid parasites other than *B.malayi* and *B.timori*[3].

The results of this study, where several hundreds of mosquitoes had been examined, confirmed the report of Sim et al[3]. The DNA probe was 100 % sensitive (table 1) and 100 % specific and all infective larvae revealed from mosquitoes fed to cats harbouring *B.pahangi* did not react. *B.pahangi* cross hybridize in such a low level to this probe, that no mosquito harbouring infective larvae of *B.pahangi* could be detected by this DNA probe, since hardly ever more than 40 infective larvae of *B.pahangi* would be found in nature. This is a very important breakthrough, since by using this probe it is possible to differentiate *B.malayi* from *B.pahangi*, where in some areas in Indonesia these two species coexist. The cross hybridization of this probe

to *B.timori* would not be of a problem in Indonesia, since until now there is no report that *B.malayi* and *B.timori* coexist in an area.

The percentage of mosquitoes showing hybridization in the group of mosquitoes that was squashed directly onto nitrocellulose paper was lower than the group that was dissected, followed by dot blotting the infective larvae onto nitrocellullose paper, though the difference was not of any significance. This difference might be caused by the hinderence of the infective larvae to stick onto the nitrocellulose paper by the mosquito components.

Infective larvae of Brugia spp. could be found anywhere in the body of the mosquito, while first stage larvae (L1) and second stage larvae (L2) would be found in the thorax[5]. In the dissection method to find L3 the whole mosquito should be dissected.

Clone pBm15 is not stage specific, thereby it does not differentiate infective larvae from L1 and L2. Thus, DNA probing using clone pBm15 alone would not give accurate information in discovering the vector responsible for transmission of malayan filariasis in man in a particular area. For the purpose of discovering the vector, mosquito dissection is needed, infective larvae looked for and then test it with clone pBm15. Once the vector has been identified, for further monitoring of transmission: the use of clone pBm15 would be of great advantage compared to the conventional mosquito dissection method.

With the fact that clone pBm15 differentiate *B.malayi* from *B.pahangi*, it is imperative to apply the DNA probing with the mosquito dissection method in areas where both parasites are present in an area in discovering the vector as well as in monitoring transmission. It would be of preference if DNA clone which is specific to *B.pahangi*[6] could be used simultaneously in the transmission studies in such areas.

This DNA probe would be a valuable tool for studying transmission and monitoring a control programme. Special effort should be given to modify the technique so that it could be easily applied in the field and in a modestly equipped laboratory: e.g. the use of P 32 replaced by enzym.

CONCLUSION

1. Enzymatic treatment using 150 µg/ml of proteinase K and 100 µg/ml of chitinase for retrieving infective larvae in mosquitoes is a very important treatment to avoid non specific binding. Mosquitoes could be squashed directly to the nitrocellulose paper in the field without any effort of dissecting the mosquitoes.
2. The high sensitivity and specificity of clone pBm15 enabled the detection of one infective larva of *Brugia malayi* and the differentiation from *Brugia pahangi* in areas where both species coexist.
3. Clone pBm15 can be used for monitoring a control programme in *B.malayi* areas. In discovering the vector in an endemic area, mosquitoes should be dissected, infective larvae revealed and the probe used to identify the larvae found. This approach enables the entomologist to identify the species of the infective larvae,thus no further effort to develop adult worm for characterization is needed.
4. This hybridization method using clone pBm15 needs further modification to achieve a method which is cheap and easy to implement.

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QUESTIONS AND ANSWERS :

1. Question: If the probe is not stage specific, how can the probe monitor control activities ?

   Answer : We modified this DNA probe with dissection method.
   Cut the upper part of mosquitoes, to eliminate the L_1 - L_2 stage, and collect the thorax part that contain the L3 stage larvae.
   Blotted it on to NCP and hybridized with this clone.

2. Question: When you applied enzyme treatment for eliminating nonspecific reaction, at the same time you might face the problem of decreasing or loosing some important determinants. What is your comment on this ?

   Answer : I don't think that we are going to loose the determinant since DNA only hybridized with their complementory.