

Treatment Follow-up of *Brugia malayi* Microfilaraemic and Amicrofilaraemic Individuals with Serological Evidence of Active Infection

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ABSTRACT

Filariasis caused by *Brugia malayi* and *Brugia timori* affects ~13 million Asians. In order to ensure elimination of these infections in the context of the Global Programme for Elimination of Lymphatic Filariasis (GPELF), assays which are more sensitive than night blood examination must be employed. IgG4 assay using BmR1 recombinant antigen has been shown to be highly specific and sensitive for diagnosis of brugian filariasis. To provide further evidence of the diagnostic value of this assay, treatment follow-up study was performed on *B. malayi* microfilaraemic and amicrofilaraemic individuals who were positive by the BmR1-based IgG4-ELISA. Group 1 comprised 22 treated microfilaraemic individuals; group 2A comprised 13 treated amicrofilaraemic individuals and group 2B (control group) comprised 16 untreated amicrofilaraemic individuals. Group 1 individuals demonstrated decline in IgG4 levels with treatment and all participants were negative by the end of the 21 months study period. Group 2A also demonstrated IgG4 decline to negativity by 21 months, with re-treatment at 12 months performed on 3 individuals. In group 2B untreated individuals, at 21 months seven participants remained IgG4 positive while nine individuals were IgG4 negative, possibly through spontaneous death of adult worms. Significant difference ($p=0.008$) was observed when proportions between group 2A and group 2B were compared. This study showed decline of filaria-specific IgG4 post-treatment in both microfilaria positive and microfilaria negative individuals. In addition amicrofilaraemic IgG4 positive individuals were shown to be infected as evidenced by the significant difference between treated and untreated groups of individuals. Therefore, this study strengthened the reported findings that IgG4 assay based on BmR1 recombinant antigen is a good diagnostic tool for brugian filariasis.

Keywords: Treatment, follow up, IgG4-Elisa, BmR1, recombinant antigen

INTRODUCTION

Brugian filariasis, caused by *Brugia malayi* and *Brugia timori* affects ~ 13 million individuals in several Asian countries. It is characterized by a wide range of manifestations which can be divided into four main categories i.e. amicrofilaraemic with no clinical disease, amicrofilaraemic with clinical disease, microfilaraemic with no clinical disease and microfilaraemic with clinical disease.

Serodiagnosis is useful in diagnosis of lymphatic filariasis especially in microfilaria negative individuals. However, early serodiagnostic techniques using soluble worm extracts to detect IgG antibodies were neither specific (Maizels, *et al.*, 1985) nor able to differentiate previous infection, or exposure to the parasite (aborted infection), from current active infection (McCarthy, 2000). Problems arose as most residents of filariasis-endemic regions were found to be antibody positive (Ottesen, *et al.*, 1982). However, serological assays that detect filarial-specific IgG4 were found to possess better specificity as compared to those that detect total IgG (Turner *et al.*, 1992; Wamae *et al.*, 1992). In addition, elevated levels of specific IgG4 were shown to be suitable markers of active

filarial infection (Ottesen *et al.*, 1985; Kwan Lim, *et al.*, 1990; Kurniawan, *et al.*, 1993; Rahmah *et al.*, 1998; Haarbrink *et al.*, 1999) and non-detection of filarial-specific IgG4 antibodies effectively excludes infection (McCarthy, 2000).

Availability of excellent diagnostic and new treatment strategies were two important factors that contributed to the establishment of the Global Program to Eliminate Lymphatic Filariasis (GPELF) by 2020 (Ottesen, 2000). However in brugian filariasis, a good antigen detection tool is still unavailable, thus the aim to interrupt transmission of *B. malayi* infections could be hampered by the lack of a sensitive and specific diagnostic tool needed to monitor and evaluate the efficacy of mass treatment.

BmR1 recombinant antigen (expressed by *Bm17DIII* gene, GenBank accession no. AF225296), employed in ELISA and rapid test formats, has been shown to be highly sensitive and specific in detection of *B. malayi* (Lim *et al.*, 2001; Rahmah *et al.* 2001a; 2001b; 2003a; 2003b; Lammie *et al.*; 2004; Jamail *et al.*, 2005). However more data is needed to determine the period of IgG4 positivity post-treatment. It is also important to demonstrate that IgG4 positive individuals who are asymptomatic amicrofilaraemics are infected.

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Thus the aim of the present study is to further demonstrate the diagnostic value of BmR1 by employing the recombinant antigen in investigating the decline of antifilarial IgG4 in two groups of individuals. Group 1 comprised treated microfilaria (mf) positive individuals; while Group 2 comprised amicrofilaraemics who were subdivided into treated and untreated individuals.

MATERIALS AND METHODS

Study population

A total of 51 individuals from two low endemic areas in Peninsular Malaysia participated in this study. The endemic areas, located ~ 100 km apart, were Setiu in the state of Terengganu and Pasir Mas in the state of Kelantan. As shown by previous studies, there was no significant difference ($p=0.072$; Fischer exact test) between prevalence of filariasis by Brugia ELISA in the study areas in Setiu (28/1134) and the study areas in Pasir Mas (18/1283) (Lim *et al.*, 2001; Rahmah *et al.*, 2003b).

The individuals were all positive by an IgG4-ELISA which employed BmR1 recombinant antigen. The participants were asked questions to elicit information regarding history of lymphoedema, adenolymphangitis and/or recurrent fever. Group 1 comprised 22 mf positive individuals. They were identified by the filariasis teams of the respective states. Group 2 comprised 29 mf negative individuals, and were subdivided into groups A and B. They were identified during previous field studies (Lim *et al.*, 2001; Rahmah *et al.*, 2003b). Group 2A received treatment and comprised 13 individuals, 10 males and 3 females with an average age of 39.8 years, ranging from 12 to 67 years. Group 2B served as the control group, and comprised 16 individuals who were untreated during the study period. They were 11 males and 5 females, and the average age was 17.1 years, ranging from 7 to 70 years. In compliance with the University Research and Ethics Committee, informed consents were obtained from all subjects prior to enrollment in this study.

Treatment

Two different treatment regimens were employed to ensure treatment compliance. Group 1 individuals received six doses of diethylcarbamazine (DEC) treatment at 6 mg/kg body weight; this is the standard treatment regimen for filariasis recommended by the Malaysian Ministry of Health (MOH). One participant received an additional dose of DEC at 12 months post-treatment.

Group 2A individuals received one dose of DEC (6 mg/kg) and one dose of albendazole (400 mg) i.e. the treatment regimen recommended by WHO for GPELF. This treatment regimen was employed to avoid non-compliance since asymptomatic mf negative individuals are not indicated for treatment by MOH; thus the participants (and district health personnel) were not agreeable to the full course of DEC treatment. Three

participants from group 2A were given an additional dose of DEC at one year post-treatment. At the end of the study, recommendations were made to the district medical officer to treat individuals in group 2B who remained IgG4 positive.

Sample collection

A previous follow-up study of 15 treated microfilaraemics demonstrated that most individuals became IgG4 negative after about 21 months post-treatment (Rahmah *et al.*, 2001a), thus the same period was employed for the present study. For individuals in group 1 and group 2A, serum samples were monitored approximately once every two to four months until the end of the study period or until the IgG4-ELISA showed negative results. To encourage enrolment into group 2B, the individuals were only required to provide finger-pricked blood two times i.e. once at the beginning and another at the end of the study period.

Serum samples were collected during the daytime. The middle finger of the subject was wiped with 70% ethanol and about 6 drops of blood were collected in a microtainer and transported to the laboratory in cold condition.

PCR of blood clots

The microfilaria negative status of group 2 individuals were confirmed by performing PCR on blood clot samples from both first and second specimens; using tissue kit DNA extraction kit (Genispin™ Tissue DNA kit, Biosyntech, Malaysia), and according to published methods (Lizotte *et al.*, 1994; Rahmah *et al.*, 1998). Each PCR run was accompanied by the appropriate controls i.e. DNA from blood clots from normal and mf+ individuals and elution buffer.

ELISA

ELISA was performed as described previously (Rahmah *et al.*, 2001a). Follow-up serum samples were tested on the same microtiter plate. Briefly, microtiter wells (Nunc, USA) were coated with 100 μ l recombinant antigen at 20 μ g/ml in NaHCO₃ buffer (pH 9.6). After a blocking step, serum samples were incubated for 2 h, followed by 0.5 h incubation with monoclonal anti-human IgG4-HRP (CLB, Netherlands) diluted at 1:4500. Subsequently ABTS substrate (Roche Diagnostics, Germany) was added for 30 minutes before the optical densities (OD) were read at 410 nm with an ELISA spectrophotometer (Dynatech, USA). Serum samples that demonstrated average optical density (OD) readings of ≥ 0.300 (Rahmah *et al.*, 2001a) were considered to be IgG4 positive.

For participants in group 1, serum dilutions of 1:50 were performed on each sample. Follow-up samplings on the participants were discontinued when the serum samples showed average OD of < 0.300 . For samples from group 2, serial dilutions were performed for each sample and the highest dilution which gave average OD

value of ≥ 0.300 was considered as the IgG4 titer. Antibody titer was considered zero when the assayed serum sample at 1:50 dilution showed an OD of < 0.300 .

Statistical Analysis

Statistical analysis was performed in SPSS for Windows version 10.0. Proportions between participants of groups 2A and 2B at 21 months after the first sampling were compared by chi-square test; if indicated Fisher's exact test was used instead.

RESULTS

From the responses to the questions asked, none of the participants seemed to have had any history of adenolymphangitis or lymphoedema. Figure 1 illustrates the decline in OD of follow-up samples of 22 microfilaraemic individuals post-treatment. One participant was IgG4 negative by 3 months post-treatment, while eight, four, seven, one and another one participants were negative at 6, 9, 12, 15 and 21 months p.t. respectively. A total of 20 participants were ELISA negative by one year post-treatment. One participant whose OD did not show much decline from 6 to 12 months post-treatment, was given an additional dose of DEC 6 mg/kg; and three months later his serum sample demonstrated negative ELISA result. All 22 participants in group 1 were ELISA-negative by 21 months post-treatment.

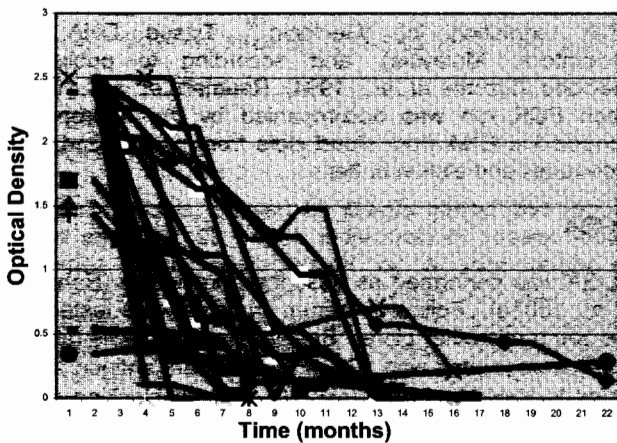


Figure 1: Decline in optical densities (OD) of follow-up samples of 22 microfilaraemic individuals post-DEC treatment. Cut off for positive result is OD value of ≥ 0.300

PCR performed on all blood clot samples from participants in group 2 reduced negative results, thus confirming the amicrofilaraemic status of these individuals. Figure 2 shows the \log_{10} geometric mean titres (GMT) of group 2A and group 2B. In group 2A, the average BmR1-specific IgG4 titer of pre-treatment serum samples was 877. At two months post-treatment, the GMT dropped to 715, in which one participant

demonstrated a decline in titer from 100 to zero. After five months post-treatment, seven more participants were found to demonstrate zero titer, and the GMT was 165. A one year post-treatment, 10 participants (77%) were found to be negative by *Brugia*-ELISA. Three participants who were still positive after a year post-treatment, was each given an additional dose of DEC, and they became ELISA negative by 5-7 months post-treatment. In group 2B (untreated amicrofilaraemic individuals), the initial serum samples showed GMT of 162.5. Twenty one months later, the value dropped to 109.4; however it was still above the cut-off titer of 50.

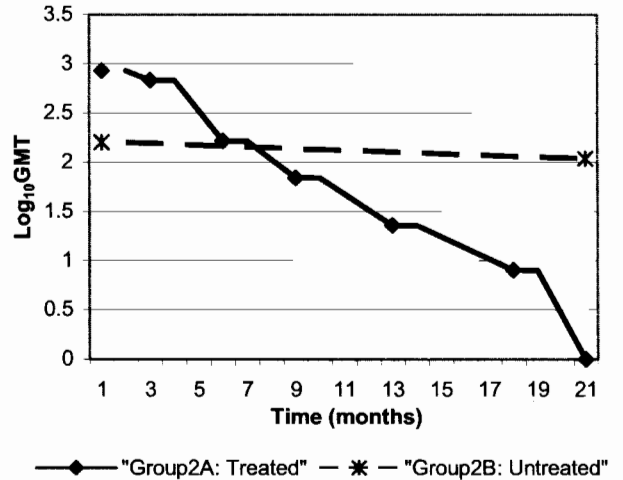


Figure 2: \log_{10} geometric mean titres (GMT) of IgG4-ELISA from follow-up samples of treated (group 2A) and untreated (group 2B) amicrofilaraemic individuals. Cut off value is 1.7 ($\log_{10} 50$).

The results of the individual participants of group 2A (Figure 3) shows that filaria-specific IgG4 antibody ceased to be detected at different sampling points. The results of the individual participants of group 2B are depicted in figure 4. At 21 months, nine participants were found to be IgG4 negative while seven individuals remained positive. Among the IgG4 positive individuals, antibody titers in three participants were found to increase i.e. titers of two participants had increased from 50 to 100 and the other increased from 100 to 200. Two participants maintained their high antibody titers throughout the 21 month period i.e. at titers of 400 and 800. However two participants demonstrated a drop in their antibody titers i.e. from 100 to 50 and from 2000 to 100. Comparison between group 2A (0/13) and Group 2B (7/16) at the end of the 21 month study period showed that, at 0.05 level of significance, two tailed p value was 0.008. Thus there is a significant difference in proportions between Group 2A and Group 2B.

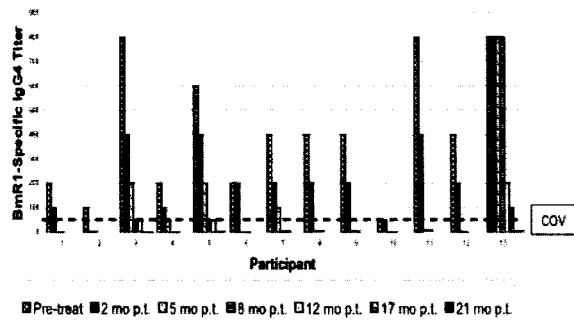


Figure 3: Follow-up IgG4-ELISA titres of 13 amicrofilaraemic participants treated with one dose of DEC and one dose of albendazole (group 2A)

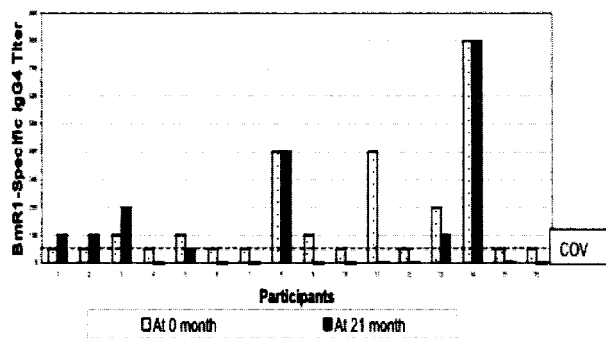


Figure 4: Follow-up IgG4-ELISA titres of 16 untreated amicrofilaraemic participants (group 2B)

DISCUSSION

Examination of thick blood smear is still being widely used for the routine diagnosis of *B. malayi* infection due to the unavailability of a good antigen detection test. Although this traditional method is cheap and specific, low sensitivity remains one of its major drawbacks in which persons with low microfilaraemia, single-sex infections and those at the amicrofilaraemic stages of the infection were often left undiagnosed (Turner *et al.*, 1992; McCarthy, 2000). Its application as a monitoring tool in post-treatment of infected individuals would produce erroneous results; thus an alternative serological test is needed for this purpose.

A previous study reported that the development and incidence of lymphangitis and lymphoedema was found to be significantly higher in amicrofilaraemic, antibody positive subjects as compared to microfilaraemic subjects (Dissayanake, 2001). It is therefore important to diagnose and treat individuals with cryptic infections and this is only feasible by a serological test. IgG4 assays based BmR1 recombinant antigen has been shown to be highly specific and sensitive for detection of brugian filariasis (Rahmah *et al.*, 2001a; 2001b; 2003a; 2003b; Lammie *et*

al.; 2004; Jamail *et al.*, 2005). In an animal experimental study, nine of 31 mf negative gerbils (29%) were found to demonstrate consistently high levels of BmR1-specific IgG antibody. At necropsy, all animals were found to harbour adult worms, although microfilaria was never detected throughout the study period (Lim *et al.*, 2004). Therefore BmR1 recombinant antigen detected cryptic infections in the gerbils. He present study was conducted to further demonstrate the diagnostic value of BmR1 recombinant antigen by follow-up studies of treated and untreated BmR1 positive individuals from endemic areas.

Group 1 comprised 22 treated mf positive individuals. The results showed that the filaria-specific IgG4 levels in these individuals declined post-treatment and a 100% negative ELISA results were achieved by 21 months p.t. However re-treatment of one individual was necessary to expedite the decline of the IgG4 antibody level. Previous studies had also documented decrease of IgG4 post-treatment. Using the same recombinant antigen a treatment follow-up of 15 microfilaraemic individuals showed progressive decline in IgG4, and all participants were negative by about 2 years (Rahmah *et al.*, 2001a). In a cross-sectional study on microfilaraemic subjects, IgG4 levels to *B. malayi* soluble worm extract antigen were found to decrease sharply (65%-78%) within 12 months post-DEC treatment (Kurniawan *et al.*, 1995). Significant decline in IgG4 responses were also reported among microfilaraemic bancroftian patients treated with DEC compared to their pre-treatment values at 180-720 days post-treatment. (Wamae *et al.*, 1992). In another study, patients treated with DEC who had clearance of circulating antigen (CFA) showed significantly greater decrease in antifilarial IgG4 than those treated individuals who did not clear their CFA (McCarthy *et al.*, 1995).

However a longitudinal study performed in a filariasis endemic area in Indonesia demonstrated that not all microfilaraemic patients who received intensive DEC treatment for at least 30 days (total dosage of at least 180 mg/kg) showed reduction in anti-filarial IgG4 levels (Terhell *et al.*, 2003). This could be due to the high endemicity in the Indonesian population as compared to the population in Malaysia; as reflected in the more aggressive treatment in the former. Thus, unlike the population in the present study, many individuals in the Indonesian study probably had high worm burden that were not all killed by the treatment. Furthermore, there is more likelihood for re-infection in the Indonesian endemic area due to the high transmission rate.

In Group 2A, the decline of average BmR1-specific IgG4 titer was rather gradual at two months post-treatment i.e. from 877 to 715. However, a sharp drop in the titer to 165 was observed between two to five months post-treatment, in which 8 (62%) participants were negative by the IgG4-ELISA. This shows the two-drug combination regimen took about half a year to clear the infection from about 60% of the participants. Upon receiving an additional dose of DEC, the persistent antibody titers observed at one year post-treatment in three participants were found to decline to an

undetectable level after 5 to 7 months. Since DEC is known to be an inefficient macrofilaricidal agent, it is not surprising that re-treatment is necessary in some individuals. At the end of the 21-month period, all 13 individuals became IgG4 negative.

In group 2B, seven of sixteen (44%) participants were found to be still IgG4 positive after the 21 month period. The negative ELISA results observed in 9 participants was probably due to the spontaneous death of adult worms in these individuals. The low-endemicity of the study area also implies that most infected individuals harbour low numbers of adult worms, thus spontaneous death of some worms can rapidly clear the infection. In addition the younger participants in Group 2B (average age of 17.1) probably harbour lower numbers of worms than participants in Group 2A (average age of 39.8) due to physiological factors; as alluded in a previous report (Terhell *et al.*, 2001). Despite the above limitations a significant difference in proportions between groups 2A and 2B could still be demonstrated; therefore providing evidence that BmR1-based ELISA detects actively infected individuals.

Therefore in this study, BmR1 recombinant antigen-based ELISA showed decline of IgG4 antibody in microfilaraemic individuals within less than 2 years post-treatment. It also showed evidence that amicrofilaraemic individuals who were positive by this assay were most probably infected. In addition, this study also demonstrated the usefulness of re-treatment of individuals who do not demonstrate a satisfactory decline in IgG4 levels. Therefore assay based on BmR1 recombinant antigen is useful in diagnosis and treatment follow-up of individuals infected with *B. malayi*.

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