Abstract

One line approach of the National Program to Eliminate Lymphatic Filariasis (PELF) in Thailand during fiscal years 2002-2006 that focuses on multiple-dose diethylcarbamazine (DEC) mass treatment is to large-scale control imported bancroftian filariasis to prevent introduced infection in vulnerable local population. The disease caused by the nocturnally periodic strain of \textit{Wuchereria bancrofti} is common among cross-border Myanmar migrant workers. This study was aimed at evaluating drug responses (i.e., parasitological and serological) in both microfilaremics and amicrofilaremics, and to investigate resistance mutation of the \textit{W. bancrofti} population in microfilaremic carriers. Of the 1,133 migrants whose blood samples were parasitologically and serologically determined, the 14 antigenemic cases including 7 microfilaremics and 7 matched amicrofilaremics were recruited. They were treated with a 300 mg single oral-dose DEC, and parasite loads (microfilaremia and/or antigenemia) were monitored at DEC-post treatment, 1, 2, and 3 months. Both groups had dramatic reduction of antigen loads in relation to time required to clear, but month 2 showed significant difference in antigen loads \((p = 0.013)\). In microfilaremic group, reduction of microfilariae and antigen loads at individual level was independent of time required to clear infection and intensity. By \textit{W. bancrofti} beta-tubulin gene-specific polymerase chain reaction, all the microfilariae isolates gave positive amplification of 607 bp amplicons which deduced amino acid sequences did not confer resistant mutations at position neither Phe 167 Tyr nor Phe 200 Tyr over a period of treatment. The principal outcomes would likely be beneficial for the PELF’s implementers at the provincial level to evaluate and monitor the PELF implementation effectiveness in drug response and resistance and in particular towards short-term migrants.

Key words: imported bancroftian filariasis, long-term migration, multiple-dose diethylcarbamazine, Myanmar migrants, National Program to Eliminate Lymphatic Filariasis, resistant mutations, short-term migration, \textit{Wuchereria bancrofti}
Introduction

The imported bancroftian filariasis, caused by the nocturnally periodic strain of *Wuchereria bancrofti*, is thought to acquire mosquito-borne transmission outside Thailand\(^{1-4}\). Asymptomatic microfilaremic carriers are common in adult males aged over 20 years\(^{1,4-6}\). Asymptomatic antigenemic persons outnumbering twenty-fold higher than microfilaremics are most vulnerable of diagnosis\(^{2,7,8}\). The emergence of the disease, common in cross-border Myanmar migrants, has been issued on social context and health consequences due to “Push and Pull effects” that resulted in movement of large numbers of those migrants into Thailand as a host country for occupational opportunity\(^{3,9,10}\).

One pillar of the National Program to Eliminate Lymphatic Filariasis (PELF) in Thailand, during fiscal years 2002-2006, is multiple-dose diethylcarbamazine (DEC) mass treatment\(^{3}\), aiming at preventing introduced infection of the nocturnally periodic *W. bancrofti* in at-risk local population in areas where breeding grounds of potent vector, *Culex quinquefasciatus*, are established\(^{11}\). This large-scale transmission control of imported bancroftian filariasis covers not only for those who have long-term migration and renew their work permit, but also for those who come for a short-term migration in risk areas. It is clear that the strategy (a combination of DEC provocation, biannual DEC mass treatment and supportive DEC mass treatment) has been shown to contain microfilaremics in those target migrant population\(^{3}\). Also, the antigenemic and/or microfilaremic burdens in those migrants with respect to a long-term migration are considered to be zero-ground prevalence after rounds of annual or biannual DEC mass treatment\(^{8,12}\).

A short-term migration (usually 3 to 6 months) is a vulnerable factor that favors treatment delay and levels of antigenemia or peripheral microfilaremia in those short-term migrants are independent of time and DEC treatment history\(^{3,4}\). Peripheral microfilaremia recurring or occurring in those vulnerable persons are believed to be below transmission threshold. The *W. bancrofti* populations in infected persons either naive or discontinuously-treated with DEC are, however, potential for transmission. Besides the PELF, most of registered migrants are once given a single-dose combination of 300 mg DEC and 400 mg albendazole\(^{8}\) in their lifetime during staying in Thailand and thereby selection of albendazole can evolve in the *W. bancrofti* populations\(^{13,14}\). These circumstances give rise to questioning about susceptibility to DEC selection pressure in those migrants targeted for the PELF.

Therefore, the study objectives and benefits were to evaluate drug responses (i.e., parasitological and serological) to a 300 mg single-dose DEC in those microfilaremics, and to characterize \(-\text{tubulin gene isotype 1 homologue, as known for resistance mutation under benzimidazole selection (13-15). Either two of point mutations of } W. bancrofti\) (\(-\text{tubulin at position Phe 167 Tyr or Phe 200 Tyr was investigated.}

Methodology

Case finding survey and recruitment

Both non- and registered Myanmar migrant workers (n = 1,133 persons) aged 20 years were selected during the case-finding surveys in Phang-nga Province, Southern Thailand. Seven microfilaremics subjects were recruited as the cumulative microfilaremic (Mf+)/antigenemic (Ag+) subjects during the night-time microfilaremia surveys of the non-registered migrants (n = 735) between August and October 2005. They were confirmed by Giemsa-stained blood film and immunochromatographic test (ICT) specific for *W. bancrofti* circulating filarial antigen (CFA) as described elsewhere\(^{2}\). Other seven antigenemic subjects were recruited and matched as the cumulative amicrofilaremic (Mf-)/antigenemic
(Ag+) subjects during the day-time antigenemia surveys of the registered migrants (n = 398) between August and October 2006. Laboratory-confirmed cases were also done by both methods. Information on age, gender, DEC treatment profiles and population migration patterns was recorded in assistance with Myanmar translators. Ethical clearance and approval for the study was obtained from the hospital and Institutional Review Board at Mahidol University.

**Drug Administration and Monitoring**

In prior to treatment, all the cumulative 14 antigenemic (Ag+) subjects including the same 7 microfilaremics (Mf+) and amicrofilaremics (Mf-) were individually requested to visit the hospital for blood examination and for treatment with a 300 mg single oral-dose DEC and monitoring. Also, the physical examination and interviewing were done in association with the Myanmar translators. All gave informed consents after they were informed about the purposes of the study. Then they were treated with the drug and, if the adverse drug reaction-ADR develop, they were requested to visit the hospital for ADR management(16). Subsequently, intravenous blood samples in Mf+/Ag+ individuals were collected at night time close to peak hour, as for the Mf-/Ag+ subjects day blood was used(4). The ethylenediamine tetraacetic acid (EDTA) was used as anticoagulant for plasma and whole blood preparation. All the samples were then transferred to the laboratory and they were refrigerated at 4°C or at -20°C until use. After DEC treatment, both groups were follow-up monitored at months, 1, 2 and 3, and the EDTA blood samples were examined according to the methods mentioned earlier. The night blood samples were examined for microfilaremia using the Giemsa-stained blood films. The plasma samples were examined for antigenemia using the Og4C3 enzyme-linked immunosorbent assay (ELISA). They were used to evaluate parasitological and serological responses, as described elsewhere(2,4).

**PCR Amplification of Beta-Tubulin Gene**

All the homologous sequences of *W. bancrofti* and other nematode parasites were retrieved from the genome databases and homology at DNA and protein levels was analyzed using the online available programs, BLASTN, BLASTx and BLASTP for sequence similarity algorithms (http://www.ncbi.nlm.gov/blast) and ClustalW for multiple sequence alignment (http://www.ebi.ac.uk/clustalw/). The exons 4 and 5 of β-tubulin gene of *W. bancrofti* were used as target sequences to design specific primers that can discriminate *B. malayi* β-tubulin gene homologue by flanking intron sequences.

Purified genomic DNA templates of the 7 microfilaremic subjects were extracted according to the method described by the manufacturer (QIAamp DNA Blood Mini Kit, QIAGEN GmbH, Germany). The quality of purified gDNA templates with an A260/A280 ratio of 1.7-1.9, as well as of Mf-/Ag+ subjects as internal control, was spectrophotometrically determined and electrophoresed. Then, the optimized PCR amplification of human and *W. bancrofti* β-tubulin gene homologues was performed on temperature gradient that, using individual DNA templates, specific primers differentiated authentic DNA fragments with specific annealing temperature. The amplicons (607 bp and 210 bp) were amplified using primers specific for *W. bancrofti* β-tubulin gene isotype 1 and human β-tubulin gene homologue, respectively. In a 25 μl separate reaction, the mixture contained 20 ng of purified gDNA templates (of *W. bancrofti* microfilariae isolates), primer set concentration (1.0 μM each of primers), and Go Taq® Green Master Mix (Promega, USA) containing Go Taq® DNA Polymerase, Green Go Taq® Reaction Buffer (pH 8.5), 200 (M each of deoxyribonucleotide triphosphates (dATP, dGTP,
dCTP and dTTP) and 1.5 mM MgCl2. The predenaturation was done at 95°C for 5 minutes, and followed by 40 thermocycles of denaturation at 95°C for 1 minute, annealing at 54°C for 40 seconds and extension at 72°C for 1 minute. The last extension was done at 72°C for 5 minutes. In a reaction containing a primer set of BT13 and BT14, annealing temperature was at 50°C for 1 minute. The human gDNA templates either isolated from the Mf+/Ag+ subjects or Mf-/Ag+ subjects were used as internal control for quality control of the PCR amplification throughout the study. Also, a negative control (with no DNA) was run always using the same amplification conditions. The amplification fidelity of all the gDNA samples of *W. bancrofti* isolates was performed triplicate.

**Data Analysis and statistical methods**

To test drug sensitivity response to 300 mg single-dose DEC, changes in the *W. bancrofti* CFA levels in both groups (Mf+/Ag+ vs. Mf-/Ag+), or geometric mean (GM) of arbitrary antigen titers (AU/ml) of both groups in the response DEC treatment in relation to time (months), were compared. Identical treatment effects on reduction in GM antigen loads were analyzed by one-way analysis of variance (ANOVA) at significant level, ( = 0.05). In individual microfilaremics (Mf+/Ag+), reduction in microfilarial loads in relation to time required to clear microfilaremia was also analyzed.

For post-PCR analysis of all the PCR products, one-fifth of the amplicons was subjected to 1.2-1.5% agarose gel electrophoresis at a constant voltage of 10 V-cm⁻¹. The DNA gel was stained with an ethidium bromide at a working concentration of 0.5 μg/ml for 15 minutes, and then destained with electrophoresis buffer at room temperature for 10-20 minutes. It was visualized under the UV wavelength by using the Gel Documentation. To test drug resistance of the *W. bancrofti* microfilariae isolates in all the DEC-treated Myanmar microfilaremic subjects, nucleotide sequences of the exons 4 and 5 were sequenced using direct PCR sequencing through both 5 end directions. Unanimous homology against the sequences deposited in the protein databases as described above was analyzed for point mutations at positions Phe167Tyr (or TTT/TAT) and Phe200Tyr (or TTC/TAC).

**Results**

**Drug Sensitivity Response**

In order to evaluate drug response between the Mf+/Ag+ and Mf-/Ag+ groups, parasite loads both microfilaria and antigenemia were monitored prior to and DEC-post treatment. The Mf+/Ag+ groups had geometric mean (GM) antigen load (SD) of 137089.5 (40082.4) AU/ml prior to treatment (Fig.1 and Table 1). The GM microfilarial load (SD) was 333.4 (341.6) Mf/ml. Over a period of 3 month post-treatment, GM antigen load (SD) was 113345.0 (33383.4) AU/ml. The GM microfilarial load (SD) was 376.8 (321.7) Mf/ml. Reduction of antigen loads was more likely to be dramatically declined in relation to time required to clear parasite intensity in the group. Moreover, in prior to treatment, there was no relationship between microfilarial loads and antigen loads at individual level in the group (p > 0.05) (data not shown). There was more likely to have effects on reduction of parasite loads in the group, as reduction of microfilarial and antigen loads were independent of time required to clear infection and intensity (Fig.1).

All the 14 antigenemics had antigen load of 107630 AU/ml before treatment and there was no significant difference in antigen loads between the Mf+/Ag+ and Mf-/Ag- subjects (F = 3.982, p = 0.063) (Table 1). Both groups tolerated well over a 3 month period of treatment and, in general, reduction of antigen loads was remarkably shown in relation to time...
required to clear infection in both groups. Month 2 showed significant difference in antigen loads between the groups (F = 8.375, p = 0.013). On the other hand, reduction of antigen loads between the groups was significantly different (F = 6.147, p = 0.029).

**Table 1** Antigenemia between amicrofilaremic (Mf-) and microfilaremic (Mf+) antigenemic (Ag+) groups, before and after DEC treatment (months).

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
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<tbody>
<tr>
<td>Mf+/Ag+ (n=7)</td>
<td>137,090</td>
<td>121,663</td>
<td>116,049*</td>
<td>113,345</td>
</tr>
<tr>
<td>Mf-/Ag+ (n=7)</td>
<td>84,500</td>
<td>71,958</td>
<td>57,895*</td>
<td>58,799</td>
</tr>
<tr>
<td>Total antigenemics (n=14)</td>
<td>107,630</td>
<td>93,567</td>
<td>81,968</td>
<td>81,637</td>
</tr>
</tbody>
</table>

Ag = antigen, AU/ml = antigen units per milliliters, GM = geometric mean.
*Significant for two-independent samples.

**Fig. 1** Drug sensitivity response of the microfilaremic (Mf+) and antigenemic (Ag+) subjects before and after DEC treatment. Reduction in parasite loads was monitored over a period of 3 months. Geometric means (GM) of parasite intensity are presented in terms of microfilarial load (Mf/ml) and antigen load (AU/ml).

**PCR Amplification and Sequence Analysis of Beta-Tubulin Gene Homologues**

The optimized PCR amplification of β-tubulin gene homologues was performed on temperature gradient as shown in Fig. 2. All the 7 Mf+/Ag+ subjects gave positive amplification using the specific primers BT9/12 that amplified 607 bp amplicons. Meanwhile they also gave positive amplification of 210 bp amplicons authentically derived from human β-tubulin gene homologue. At 3 month DEC-post treatment, sequence analysis of the authentic fragments of *W. bancrofti* revealed that they did not retain point muta-
Management by objectives approach:

1. Establishing overarching objectives.
2. Developing and issuing assignments, plans, procedures and protocols.
3. Establishing specific, measurable objectives and directing support of defined strategic objectives.
4. Documenting results to measure performance and facilitate corrective action.

A four-step process for achieving the incident goals

Fig. 2 Amplification of β-tubulin gene homologues of human and *W. bancrofti*. The optimization of PCR amplification using a combination of primer sets (BT9/12 and BT13/14) and purified gDNA template of the M7 as representative was performed on temperature gradient with 10°C decrement of 58.0°C, 57.2°C, 56.0°C, 54.1°C, 51.7°C, 49.9°C, 48.7°C, and 48.0°C (lanes 1-8, respectively). The primers BT9/12 gave 607-bp amplicons as indicated by the arrow that retained exons 4 and 5 as shown in Fig. 1. The primers BT13/14 gave 210-bp amplicons as indicated by the arrow. The DNA gel was electrophoresed using 1.2% agarose. The 100-bp DNA ladder as molecular weight marker (lane M) is shown.

tion neither Phe167Tyr (or TTT/TA T) nor Phe200Tyr (or TTC/TAC) (Fig. 3).

Discussion

Population migration is the most complex and volatile demographic variable. Chain migration (both international and internal migration) of the cross-border migrants is thought to play an important role in an increased risk of communicable diseases. Also, it can jeopardize lymphatic filariasis elimination program in Thailand. In the present study, the majority of Myanmar migrants with long-term migration in the study area had DEC treatment history, as most of short-term migrants were not treated with the drug before. These findings agreed well with previous findings that not only short-term migrants can favor increased risk of treatment delay, but they have also less access to health services in the residence areas. They often move from one area to another and resulting chain migration also plays an important role in rural-to-urban migration. This may be reasons why DEC treatment profile was insufficient for reliable data collection and surveillance of drug response and resistance among the Myanmar target population in permitted provinces. Another major challenge is necessary for the PELF’s implementers at the provincial level to evaluate and monitor the PELF’s implementation effectiveness, which relies on surveillance systems of drug response and resistance and in particular towards short-term migrants.

Single oral-dose DEC 6 mg/kg has been reported to immediately decrease *W. bancrofti* microfilarial den-
Fig. 3 Multiple sequence alignment of deduced amino acid sequences of β-tubulin gene homologues of *W. bancrofti* in comparison with other related and unrelated taxa including human. Consensus amino acids (*) are shown. The *W. bancrofti* β-tubulin sequences of the M1 to M7 do not confer resistance mutation at position neither Phe167Tyr nor Phe200Tyr, denoted as the asterisk. The selected taxa whose sequences with accession nos deposited in the protein databases were used: Ac, *Ancylostoma caninum*; Ad, *A. duodenale*; Bm, *Brugia malayi*; Bp, *B. pahangi*; Ch, *C. elegans*; Cn, *Cylindocyclus nassatus*; Co, *Cooperia oncophora*; Cr, *Cylindocyclus radiatus*; Di, *Dirofilaria immitis*; Fh, *Fasciola hepatica*; Hc, *Haemonchus contortus*; Na, *Necator americanus*; Ov, *Onchocerca volvulus*; Ovi, *Opisthorchis viverrini*; Sh, *Schistosoma haematobium*; Sj, *S. japonicum*; Sr, *Strongyloides ratti*; Ss, *S. stercoralis*; Ta, *Taenia asiatica*; Tr, *Trichostrongylus tenuis*; Tt, *Trichuris trichiura*; Wb, *Wuchereria bancrofti*. 
sity in the Myanmar patients\cite{4,7,18}. This has been shown that DEC 300 mg single oral-dose used as the DEC provocative day test provides simultaneous treatment in Myanmar migrants with a short period of stay in the permitted province\cite{7}. In general, a sharp decrease in microfilarial density was observed after DEC intake with respect to its pharmacokinetics\cite{19-21}. Prior study demonstrated that all the microfilaremics had a decrease in their mean microfilarial densities near peak hour (0100 h) soon after the DEC intake, but an increase in the mean microfilarial densities was observed afterwards\cite{4}. A linear model of \textit{W. bancrofti} microfilarial density reduction is predicted for the 2 weeks after drug intake. The efficacy of microfilaremia reduction by 300 mg oral-dose DEC occurs for as long as 2 to 4 weeks after ingestion. However, the microfilaria tend to be recur thereafter. There are no differences in the microfilarial densities between pretreatment levels and those at 4 to 12 weeks post-treatment. A non-linear model for \textit{W. bancrofti} microfilarial density can happen over time unless there is a DEC treatment repeat. Our study agreed well with these findings that, over a period of 3 month, the microfilaremia (Mf+/Ag+) group did not respond strongly to 300 mg single-dose DEC by showing a sharp decrease in microfilaria in relation to time required to clear the infection and intensity. Meanwhile, the group responded well to the dose, as shown by a dramatic decrease in antigenemia. Similar to previous findings\cite{2,8}, it was however clear that this dose reduced antigen loads in relation to time required to clear parasite loads in the group. In addition, there was no relationship between the decreases in microfilaria and antigenemia in susceptible recipients (data not shown). It was unwise to suggest that levels of microfilaria and antigenemia in the subjects with a history of DEC treatment reflect resistance. Detectable microfilaria levels at 3 month DEC-post treatment were equivalent at pre-treatment level. Our findings suggest that all the Mf+/Ag+ subjects had well responses to 300 mg single-dose DEC but fluctuations in parasite loads in individual recipients may result from immediate effects on active \textit{W. bancrofti} infection in the group. On the other hand, such dramatic declines in antigen loads between the microfilaremics (Mf+/Ag+) and amicrofilaremics (Mf-/Ag+) were proxy measures of drug response, regardless of the initial worm loads.

In order to understand about what resistance mutation of the \textit{W. bancrofti} population is associated with, the exons 4 and 5 of \(\beta\)-tubulin gene of \textit{W. bancrofti} microfilarialia isolates were analyzed after administration with 300 mg single-dose DEC. The \(\beta\)-tubulin gene isotype 1 responsible for a molecular mechanism of drug resistance is genetically inherent in the nematode parasites under selection of benzimidazole compounds\cite{22-24}. Resistance to albendazole in veterinary nematodes is known to be caused by either two of single amino acid substitutions from phenylalanine to tyrosine in parasite \(\beta\)-tubulin at position 167 or 200. This genetic marker of drug resistance in the \textit{W. bancrofti} population has been also shown for genotypic frequencies responsible for drug pressure in Ghana and Burkina Faso\cite{13}. The \textit{W. bancrofti} population isolated from the patients treated with a combination of albendazole and ivermectin had significantly higher genotypic frequencies associated with resistance at position 200. The allelic frequency of this mutation of the microfilariae isolates from the treated patients was two-fold higher than that of the untreated patients. A resistance mutation was not detected at position 167. Similar to findings of Hoti et al\cite{15}, the \textit{W. bancrofti} populations in the Myanmar Mf+/Ag+ subjects had no resistance mutation against 300 mg single-dose DEC in this study. A resistance mutation at position neither 167 nor 200 was not detected. The result of undetected resistance mutation in the \textit{W. bancrofti} microfilarialia isolates may be caused by three
major factors that involved in power estimates of resistance, if allele frequency is present in the population. Firstly, sequencing of individual microfilariae isolates may select predominant allele of the β-tubulin gene\(^{(15)}\) and thereby neither two of resistance mutations at position 167 nor 200 was not sensitive enough for analyzing resistant genotypes under selection of 300 mg single-dose DEC. Secondly, numbers of microfilariae isolates referred to as population sample unit tested in the study were limited and the result of selection pressure of the DEC had no longer effects on molecular mechanism of resistance in the \(W. \text{bancrofti}\) population. Lastly, even the subjects may or may not have experience with albendazole before the study, perhaps treatment with DEC alone does not have effects on morphological changes of adult worms\(^{(25)}\), and/or discrete microfilariae, in treated patients. Given their population migration patterns and epidemiologically-linked backgrounds, short-term migrants exposed to the microfilaremic infection are sentinel. Such phenomenon would be taken into account for long-term investigation in index areas. However, in this study, it was clear to state that both parasitological and serological responses of the \(W. \text{bancrofti}\) in DEC-treated Myanmar subjects were not associated with resistance mutation.

In conclusion, as to possibility of relationship between population migration patterns and DEC treatment profiles in the Myanmar subjects, those with long-term migration had DEC treatment history rather than those with short-term migration. Parasite loads (i.e., microfilaremia and antigenemia) were proven useful as surrogate measurements of active \(W. \text{bancrofti}\) infection prevalence and intensity in the target Myanmar migrant population. Reduction of parasite loads in relation to time required to clear parasite infections and intensities over a 3-month period of 300 mg single-dose DEC treatment was truly caused by antifilaricidal activity. However, even no selection pressure of the DEC, genetic analysis of the \(W. \text{bancrofti}\) population in the studied subjects would be critical for surveillance and monitoring of drug resistance in bancroftian filariases both imported and border forms in Thailand. Taken altogether, the principal outcomes would likely be beneficial for the PELF’s implementers at the provincial level to evaluate and monitor the PELF implementation effectiveness, and improve elimination strategy and rational approach to the target Myanmar population and in particular towards short-term migrants.

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